UNIVERSITAT DE VALÈNCIA

FACULTAD DE FARMACIA

T E S I S D O C T O R A L I N T E R N A C I O N A L

PROGRAMA DE DOCTORADO EN PARASITOLOGÍA HUMANA Y ANIMAL
DEPARTAMENTO DE FARMACIA Y TECNOLOGÍA FARMACÉUTICA Y PARASITOLOGÍA
FACULTAD DE FARMACIA, UNIVERSITAT DE VALÈNCIA

HUMAN AND ANIMAL FASCIOLIASIS IN ANDEAN REGIONS OF ARGENTINA: LYMNAEID VECTORS AND LIVESTOCK RESERVOIRS

by

ROBERTO MERA Y SIERRA

Directors

Prof. Dra. María Dolores Bargues Castelló
Catedrática de Parasitología
Departamento de Farmacia y Tecnología Farmacéutica y Parasitología
Facultad de Farmacia, Universitat de València

Dr. Patricio Artigas Bascur
Investigador Doctor
Departamento de Farmacia y Tecnología Farmacéutica y Parasitología
Facultad de Farmacia, Universitat de València
y

Prof. Dr. Dr.h.c. Santiago Mas-Coma
Catedrático de Parasitología
Departamento de Farmacia y Tecnología Farmacéutica y Parasitología
Facultad de Farmacia, Universitat de València

Valencia, September 14, 2018

AREA DE PARASITOLOGÍA
DEPARTAMENTO DE FARMACIA Y TECNOLOGÍA FARMACÉUTICA Y PARASITOLOGÍA, FACULTAD DE FARMACIA, UNIVERSITAT DE VALÈNCIA
Av. Vicent Andrés Estellés s/n
46100 Burjassot, Valencia
Tel.: 963544298
Fax: 963544769

Los abajo firmantes, Prof. Dra. MARIA DOLORES BARGUES CASTELLÓ, Catedrática de Parasitología, Dr. PATRICIO ARTIGAS BASCUR, Investigador Doctor, y Prof. Dr. Dr.h.c. SANTIAGO MAS-COMA, Catedrático de Parasitología, del Departamento de Farmacia y Tecnología Farmacéutica y Parasitología, por la presente:

CERTIFICAN:

Que Don ROBERTO MERA Y SIERRA ha realizado íntegramente el trabajo experimental titulado “Human and animal fascioliasis in Andean regions of Argentina: lymnaeid vectors and livestock reservoirs” (Fascioliasis humana y animal en regiones andinas de Argentina: lymnaeidos vectores y ganado reservorio) en el laboratorio del Departamento antedicho en la Facultad de Farmacia de la Universitat de València bajo su dirección y con el fin de optar al Grado de Doctor.

Y para que así conste a los efectos oportunos, firman la presente en Valencia, a 14 de Septiembre de 2018

Fdo.:	Fdo.:	Fdo.:
Prof. Dra. M. D.Bargues Castelló	Dr. P. Artigas Bascur	Prof. Dr. Dr.h.c. S. Mas-Coma

MERA Y SIERRA (ROBERTO), 2018.- FASCIOLIASIS HUMANA Y ANIMAL EN REGIONES ANDINAS DE ARGENTINA: LYMNAEIDOS VECTORES Y GANADO RESERVORIO: (DIRECTORES: M.D. BARGUES, P. ARTIGAS & S. MAS-COMA).

La fascioliasis es una enfermedad parasitaria zoonótica causada en América por *Fasciola hepatica.* Produce serias pérdidas económicas a la ganadería y es un importante problema en salud pública a nivel mundial. Las más altas prevalencias en humanos se hallan en regiones andinas. La especie de lymnaeido vector involucrada condiciona el escenario epidemiológico. En Argentina existía gran controversia acerca de las especies de lymnaeidos vectores presentes. Además de los bovinos, escasos estudios han considerado el rol que cumplirían otras especies domésticas y silvestres como reservorios. La fascioliasis humana ha sido considerada una enfermedad de escasa relevancia. El objetivo del presente trabajo fue identificar y caracterizar las especies de lymnaeidos vectores en zonas andinas, su relación con la fascioliasis animal y humana y el rol como reservorio del ganado y animales silvestres. Se caracterizaron genética y fenotípicamente los lymnaeidos; se realizaron estudios parasitológicos en diversas especies de ganado y animales silvestres; se llevó a cabo una exhaustiva revisión y análisis bibliográfico de fascioliasis humana. Los lymnaeidos se identificaron en base a marcadores moleculares; genes 18S y 16S, espaciadores del ADN nuclear ribosomal ITS-1 e ITS-2, y gen *cox*1 del ADN mitocondrial. La revisión elevó los casos humanos para Argentina de 85 a 629, la mayoría en zonas montañosas. Se demostró la existencia de *Galba truncatula* y *Lymnaea neotropica,* esta última naturalmente infectada por *F. hepatica,* además de *Lymnaea viator y* asociadas a altas prevalencias en animales. La fascioliasis en bovinos demostró un gradiente altitudinal y se halló una elevada prevalencia en caprinos y en animales silvestres, que hasta la fecha no eran considerados como reservorios de relevancia. La variedad de lymnaeidos hallada, su extrema adaptación a diversos ambientes, la prevalencia en ganado y animales silvestres además de la subestimación histórica de la fascioliasis humana, alerta sobre la real situación de la fascioliasis en regiones andinas de Argentina.

Palabras clave: Fascioliasis, *Fasciola hepatica*, humanos, ganado, Lymnaeidae, caracterización molecular, patrones epidemiológicos, Argentina, Cordillera de los Andes.

MERA Y SIERRA (ROBERTO), 2018.- HUMAN AND ANIMAL FASCIOLIASIS IN ANDEAN REGIONS OF ARGENTINA: LYMNAEID VECTORS AND LIVESTOCK RESERVOIRS: (DIRECTORS: M.D. BARGUES, P. ARTIGAS & S. MAS-COMA).

Fascioliasis is a zoonotic parasitic disease caused in the American continent by *Fasciola hepatica*. It is responsible for severe economic losses to the livestock industry and is an important public health problem worldwide. Andean regions have the highest prevalences in humans ever described. The epidemiological scenario differs according to the species of lymnaeid vector involved. In Argentina, there has been great confusion referred to the species of lymnaeid vectors present. Few studies have focused on the role as reservoirs of other species beside cattle and human fascioliasis has been considered a sporadic, infrequent disease. The aim of this study is to characterise the lymnaeid species involved in the transmission of fascioliasis in Andean regions of Argentina, their relation with human and animal fascioliasis and the role as reservoirs of livestock and wild species. Lymnaeid snails were genetically and phenotypically characterised and parasitological studies were performed in livestock and wild animals. A thorough bibliographical search and analysis of human fascioliasis was done. Molecular markers used in lymnaeids were the 18S and 16S genes, the ITS-1 and the ITS-2 spacers of the nuclear ribosomal DNA and the *cox*1 gene of the mitochondrial DNA. The bibliographical search elevated the cases described for Argentina in previous studies from 85 to 629, the majority in mountainous regions. Results demonstrated that in Andean regions, besides *Lymnaea viator*, there are two other species; *Galba truncatula* and *Lymnaea neotropica,* the latter naturally infected with *F. hepatica* and associated with a high prevalence in domestic animals. Cattle fascioliasis showed to increase with altitude and a high prevalence was found in goats and also in autochthonous as well as introduced wild species, which adds to the epidemiological complexity. The diverse species of lymnaeids found, their extreme adaptation to different environments, the high prevalence in domestic and wild animals and the historical underestimation of human fascioliasis alert us of the real situation of fascioliasis in Andean regions of Argentina.

Key Words: Fascioliasis, *Fasciola hepatica*, humans, livestock, Lymnaeidae, molecular characterisation, epidemiological patterns, Argentina, Andes Mountains.

INDEX

ACKNOWLEDGEMENTS 19

1.- FASCIOLIASIS BACKGROUND 25
1.1.-Fascioliasis: Current situation 25
1.2.-The causal agent: Systematics, phenotype and genotype 28
1.3.- Parasites life cycle and disease transmission 29
1.4.- The animal reservoirs 32
1.5.- The snail vectors 33
1.5.1.- Classification of lymnaeid snails 33
1.5.2.- Molecular characterization 34
1.5.2.1.- DNA nuclear ribosomal markers 35
1.5.2.2.- Mitochondrial DNA markers 37
1.5.3.- Lymnaeid snails described in South America 39
1.6.- Epidemiology 43
1.6.1.- Worldwide geographical distribution 43
1.6.2.- Transmission patterns of fascioliasis 45
1.6.3.- Epidemiological situations of human fascioliasis 46
1.6.4.- Human infection sources 47
1.7.- Diagnosis 48
1.8.- Treatment 50
1.8.1.- Antiparasitic drugs available 50
1.8.2.- Resistance of *F. hepatica* to antiparasitic drugs 52
1.9.- Prevention 53

2.- HUMAN FASCIOLIASIS IN ARGENTINA 57
2.1.- Retrospective overview, critical analysis and baseline for future research 57
2.2.- Materials and methods 57
2.2.1.- Information sources for case reports 57
2.2.2.- Information sources for climatic data 59
2.3.- Results 60
2.3.1.- First human cases in Argentina 60
2.3.2.- Characteristics of the infected population 63
2.3.3.- Biogeographical aspects 64

2.3.3.1.- Geographical distribution according to provinces....64
2.3.3.2.- Distribution according to altitude....67
2.3.3.3.- Links to livestock infection....68
2.3.3.4.- Seasonality....68
2.3.3.5.- Relationships with annual climate changes....72
2.3.4.- Sources of human infection....73
2.3.5.- Diagnostic aspects....74
2.3.5.1.- Methodological and technical problems....74
2.3.5.2.- Coprological analyses....77
2.3.5.3.- Serology....77
2.3.5.4.- Intradermal tests....84
2.3.5.5.- Diagnosis by non-invasive techniques....84
2.3.5.6.- Ectopic cases....85
2.3.5.7.- Clinical/epidemiological diagnosis....85
2.3.6.- Medical aspects....87
2.3.6.1.- Clinical findings....87
2.3.6.2.- Co-infection with other parasites....88
2.3.6.3.- Treatment....89
2.3.6.4.- Surgical cases....91
2.3.7.- Recent reports of human fascioliasis in Argentina....93
2.4.- Discussion....94
2.4.1.- Lessons from the thorough literature search....94
2.4.2.- A critical analysis of past and present human fascioliasis knowledge in Argentina....95
2.4.2.1.- Country situation....95
2.4.2.2.- Geographical distribution of human cases....96
2.4.2.3.- Epidemiological characteristics....97
2.4.2.4.- Diagnostic and treatment problems detected....98
2.4.2.5.- Present situation and future perspectives....100

3.- MOLECULAR AND MORPHO ANATOMICAL CHARACTERISATION OF LYMNAEIDS FROM ARGENTINA....103
3.1.- Background....103
3.2.- Objectives....104
3.3.- Molecular characterisation of *Galba truncatula*....105

3.3.1.- Geographical origin of the specimens studied 105
3.3.2.- Methodological procedures 107
3.3.3.- Results obtained in the study of rDNA markers 108
3.3.4.- Impact of *G. truncatula* finding in Argentina in the transmission of fascioliasis 111
3.4.- Genetic and phenetic description of *Lymnaea neotropica* n.sp. 114
3.4.1.- Geographical origin of the specimens studied 114
3.4.2.- Molecular study 115
3.4.2.1.- Molecular markers used 115
3.4.2.2.- DNA extraction 115
3.4.2.3.- Sequencing of rDNA and mtDNA markers 116
3.4.2.4.- Representation of the 18S rRNA secondary structure 117
3.4.2.5.- Phylogenetic inference 118
3.4.3.- Phenotypic study 118
3.4.3.1.- Shell measurements 118
3.4.3.2.- Anatomical studies 119
3.4.4.- Molecular results 119
3.4.4.1.- Results obtained in the study of rDNA markers 120
3.4.4.1.1.- 18S rRNA gene 120
3.4.4.1.2.- ITS-2 rDNA 121
3.4.4.1.3.-ITS-1 rDNA 128
3.4.4.2.- Results obtained in the study of mtDNA markers 130
3.4.4.2.1.-*cox*1 mtDNA 130
3.4.4.3.- Results obtained in the phylogenetic analyses 133
3.4.5.- Phenotypic results 133
3.4.5.1.- Systematic-taxonomic characteristics of *Lymnaea neotropica* n.sp. 133
3.4.5.2.- Description of the shell 135
3.4.5.3.- Description of the anatomy 137
3.4.6.- Discussion 139
3.4.6.1.- Evaluation of molecular markers used in lymaneids 139
3.4.6.2.- Evaluation of molecular and morpho-anatomical studies in the *Galba/Fossaria* species 140
3.5.- Implications of *L. neotropica* in fascioliasis transmission in Argentina 141
3.5.1.- Geographical origin of samples studied 142
3.5.2.- Molecular procedures 143

3.5.3.- Characterisation of *L. neotropica* from Argentina 144
3.5.3.1.- Morphological and morphometric results 144
3.5.3.2.- Molecular results 147
3.5.4.- Molecular characterisation of trematode larval stages in *L. neotropica* 147
3.5.5.- Repercussions of the finding of *L. neotropica* in Argentina 149
3.5.6.- Current haplotype distribution of *L. netropica* in South America 151
3.6.- Genetic analysys of other lymnaeid species geographycally or taxonomically related 152
3.6.1.- Multigenic sequence analysys of *Lymnaea diaphana* 153
3.6.2.- Phylogenetic relationships with other *Galba/Fossaria* and other lymnaeid representing different groups of interest 162

4.- ANIMAL FASCIOLIASIS IN ANDEAN REGIONS 167
4.1.- Background 167
4.2.- Objectives 169
4.3- Liver fluke (*F. hepatica*) naturally infecting introduced European Brown hare (*Lepus europaeus*) in northern Patagonia: phenotype, prevalence and potential risk 171
4.3.1.- Samples analyzed 171
4.3.2.- Parasitological techniques 171
4.3.3- Measurement techniques and data analysis 172
4.3.4.- Coprological results 173
4.3.5.- Morphometric results 179
4.3.6.- Analysis of morphometric characteristics of *F. hepatica* in *L. europaeus* 179
4.3.7.- Importance of *L. europaeus* as *F. hepatica* reservoir 180
4.4.- Altitudinal distribution of *F. hepatica* in cattle of the province Mendoza 182
4.4.1.- Geographical origin of the samples studied 182
4.4.2.- Coprological results 183
4.4.3.- Relationships of *F. hepatica* prevalence in cattle, altitude range and transmission pattern 186
4.5.- *F. hepatica* infection and association with gastro-intestinal parasites in Creole goats from western Argentina 188
4.5.1.-Faeces collection and laboratory procedures 188

4.5.2.- Statistical analysis 188
4.5.3.- Coprological results 189
4.5.4.- Analysis of *F. hepatica* and gastrointestinal parasites prevalence in goats in western Argentina 193
4.5.4.1.- Gastrointestinal parasites 193
4.5.4.2.- Types of parasitic associations 193
4.6.- *F. hepatica* in llamas (*Lama glama*) and guanacos (*Lama guanicoe*) in the province of Mendoza 195
4.6.1.- Geographical origin of samples and coprological techniques used 195
4.6.2.- Coprological results 196
4.6.3.- Repercussions of the finding of *F. hepatica* in South American camelids 196
4.7.- *F. hepatica* in livestock associated with the presence of *L. neotropica* 198
4.7.1.- Mammal hosts sampled and study area 198
4.7.2.- Coprological results 200
4.7.3.- Analysis of prevalence of infection of *F. hepatica* 200

5.- CONCLUSIONS 205

6.- BIBLIOGRAPHY 211

7.- ABBREVIATIONS 241

8.- ANNEX: PUBLICATIONS IN CHRONOLOGICAL ORDER 244

8.1.- BARGUES (M.D.), **MERA Y SIERRA (R.)**, GOMEZ (H.G.), ARTIGAS (P.) & MAS-COMA (S.), 2006.- Ribosomal DNA ITS-1 sequencing of *Galba truncatula* (Gastropoda: Lymnaeidae) and its potential impact on fascioliasis transmission in Mendoza, Argentina. *Animal Biodiversity and Conservation*, 29 (2): 191-194.

8.2.- BARGUES (M.D.), ARTIGAS (P.), **MERA Y SIERRA (R.L.)**, POINTIER (J.P.) & MAS-COMA (S.), 2007.- Characterisation of *Lymnaea cubensis*, *L. viatrix* and *L. neotropica* n. sp., the main vectors of *Fasciola hepatica* in Latin America, by analysis of their ribosomal and mitochondrial DNA. *Annals of Tropical Medicine and Parasitology*, 101 (7): 621-641.

8.3.- BARGUES (M.D.), **MERA Y SIERRA (R.)**, GOMEZ (H.G.), ARTIGAS (P.) & MAS-COMA (S.), 2007.- Caracterización molecular de *Galba truncatula*, vector principal de la Fascioliasis, en Argentina. Implicaciones en salud pública. *Enfermedades Emergentes, Barcelona*, 9 (2): 77-82.

8.4.- **MERA Y SIERRA (R.)**, ARTIGAS (P.), CUERVO (P.), DEIS (E.), SIDOTI (L.), MAS-COMA (S.) & BARGUES (M.D.), 2009.- Fascioliasis transmission by *Lymnaea neotropica* confirmed by nuclear rDNA and mtDNA sequencing in Argentina. *Veterinary Parasitology*, 166: 73-79.

8.5.- ARTIGAS (P.), **MERA Y SIERRA (R.)**, CUERVO (P.), BARGUES (M.D.) & MAS-COMA (S.), 2009.- Scolopacidae and other aquatic migratory birds as possible dispersal agents of lymnaeid snails, in Mendoza province, Argentina. In: 6th International Symposium on Limnology and Aquatic Birds: Monitoring, Modelling and Management (Huesca, Spain, 27-30 October 2009), International Society of Limnology (SIL), Pyrenean Institute of Ecology (CSIC), Proceedings: 80-82.

8.6.- **MERA Y SIERRA (R.)**, AGRAMUNT (V.H.), CUERVO (P.) & MAS-COMA (S.), 2011.- Human fascioliasis in Argentina: retrospective overview, critical analysis and baseline for future research. *Parasites & Vectors*, 4: 104. (18 pp.).

8.7.- ARTIGAS (P.), BARGUES (M.D.), **MERA Y SIERRA (R.L.)**, AGRAMUNT (V.H.) & MAS-COMA (S.), 2011.- Characterisation of fascioliasis lymnaeid intermediate hosts from Chile by DNA sequencing, with emphasis on *Lymnaea viator* and *Galba truncatula*. *Acta Tropica*, 120 (3): 245-257.

8.8.- GAYO (V.), CUERVO (P.), ROSADILLA (D.), BIRRIEL (S.), DELL'OCA (L.), TRELLES (A.), CUORE (U.), **MERA Y SIERRA (R.)**, 2011.- Natural *Fasciola hepatica* infection in nutria (*Myocastor coypus*) in Uruguay. *Journal of Zoo and Wildlife Medicine* 42 (2): 354-356.

8.9.- BARGUES (M.D.), **MERA Y SIERRA (R.L.)**, ARTIGAS (P.) & MAS-COMA (S.), 2012.- DNA multigene sequencing of topotypic specimens of the fascioliasis vector *Lymnaea diaphana* and phylogenetic analysis of the genus *Pectinidens* (Gastropoda). *Memórias do Instituto Oswaldo Cruz*, 107 (1): 111-124.

8.10.- CUERVO (P.), SIDOTI (L.), FANTOZZI (M.C.), NEIRA (G.), GERBENO (L.), **MERA Y SIERRA (R.)**, 2013.- *Fasciola hepatica* infection and association with gastrointestinal parasites in Creole goats from western Argentina. *Revista Brasileira de Parasitologia Veterinaria*, 22 (1): 53-57.

8.11.- **MERA Y SIERRA (R.)**, CANTERO (F.). GONZALEZ (M.), 2015.- *Fasciola hepatica* en guanacos y llamas en un establecimiento de Malargue, provincia de Mendoza. In: III Congreso Panamericano de Zoonosis (La Plata, Argentina, 4-6 June 2014), published in *Revista Argentina de Zoonosis y Enfermedades Infecciosas Emergentes*, 10 (1): 46-47.

8.12.- DI CATALDO (S.D.), SOHAEFER (N.), CUERVO (P.), GONZALEZ (M.), NEIRA (G.), SIDOTI (L.), **MERA Y SIERRA (R.)**, 2015.- Factores climáticos y su relación con la presencia de *Fasciola hepatica* en caprinos de las distintas regiones productivas de la provincia de Mendoza, Argentina. In: III Congreso Panamericano de Zoonosis (La Plata, Argentina, 4-6 June 2014), published in *Revista Argentina de Zoonosis y Enfermedades Infecciosas Emergentes*, 10 (1): 45-46.

8.13.- CUERVO (P.), CATALDO (S.D.), FANTOZZI (M.C.), DEIS (E.), ISENRATH (G.D.), VIBERTI (G.), ARTIGAS (P.), PEIXOTO (R.), VALERO (M.A.), **MERA Y SIERRA (R.)**, MAS-COMA (S.), 2015.- Liver fluke (*Fasciola hepatica*) naturally infecting introduced European brown hare (*Lepus europaeus*) in northern Patagonia: phenotype, prevalence and potential risk. *Acta Parasitologica*, 60 (3): 536-543.

8.14.- **MERA Y SIERRA (R.)**, GONZALEZ (M.), NEIRA (G.), 2016.- Distribución Altitudinal de *Fasciola hepatica* en Bovinos de la Provincia de Mendoza, Argentina. Investigación, Ciencia y Universidad, 1 (1): 14-18.

8.15.- SOLANA (M.V.), **MERA Y SIERRA (R.)**, SCARCELLA (S.), NEIRA (G.), , SOLANA (H.D.), 2016.- In vivo assessment of closantel ovicidal activity in *Fasciola hepatica* eggs. *Experimental Parasitology*, 160: 49-53.

ACKNOWLEDGEMENTS

ACKNOWLEDGMENTS

First and foremost I would like to express my sincere gratitude to my directors of the Departamento de Farmacia y Tecnología Farmacéutica y Parasitología, Facultad de Farmacia, Universitat De València, Dra. MARIA DOLORES BARGUES CASTELLÓ, Dr. Dr. Honoris Causa SANTIAGO MAS-COMA and Dr. PATRICIO ARTIGAS BASCUR. Words cannot describe their knowledge, patience, guidance and motivation through all these years. They are role models and have encouraged me to accomplish this thesis, it has been a true honor not only to be directed but also advised, inspired and reassured to overcome obstacles.

My participation in international fascioliasis projects would not have been possible without the support given by Dr. Santiago Mas-Coma and his not only knowledge but also wisdom in science has been inspiring. Dra. Bargues has a contagious enthusiasm and her profound knowledge of the molecular aspects of Parasitology are responsible for great part of the results of this thesis. Dr. Artigas Bascur, with which I had the opportunity to study alongside when we were students during the Master Internacional en Enfermedades Parasitarias Tropicales, was always a friend, counselor and brilliant scientist that supported me all these years. I want to express my gratefulness to Dra. MARÍA ADELA VALERO, who contributed with her expertise and work and Dr. MESSAOUD KHOUBBANE for his collaboration and help.

I would like to thank the people at Universidad Juan Agustin Maza, RECTOR DANIEL MIRANDA, VICE RECTORA MONICA TORRECILLA, DECANO GUILLERMO GIAMBIASTIANI, DIRECTORA LIZZET VIEJLING and MARIANA GONZALEZ. They were always there to lend a helping hand and support. I want to express my gratefulness to Dr. PABLO CUERVO, even though he is not currently at the Universidad Juan Agustín Maza, much of the work of this thesis was done conjunctly.

I would also like to thank the staff of the Area de Parasitología of the Facultad de Ciencias Médicas, Universidad Nacional de Cuyo: Dra. CRISTINA SALOMON, Dra. LILIANA CARRIZO, Dr. DIEGO CARGNELUTTI, Dra. ROSA TONELLI, Dra. BEATRIZ PIETRASANTA, Dr. DANIEL BERTELLO and Dra. ADELA SARCILLO for their support and friendship.

I want to remember and thank Dr. GERRIT VILJOEN of FAO/IAEA for his support.

I am grateful to Ms.Sc. VALERIA GAYO of the Departamento de Parasitología, División de Laboratorios Veterinarios (DILAVE), "Miguel C. Rubino",Ministerio de

Ganadería, Agricultura y Pesca (MGAP), Montevideo, Uruguay, for having received and trained me during my stay in Uruguay.

I would like to thank my family, my brothers Jorge and Alberto.

My daughters, Victoria and Martina, who patiently waited for their father during his endless hours at the lab and the computer.

Even though they are not here physically, I would like to remember and thank my father and mother whom always guided, supported and loved me like only a father and mother can.

Without the help, love and support of Veterinaria GISELA NEIRA, I would never have accomplished this thesis. Not only did she help me in scientific and academic aspects, but supported me through turbulent times.

And last but not least, I would like to thank all the people that helped me in my field work, be it to allow me, many times a total stranger, access their land and livestock to do the sampling.

To be able to fulfill this thesis I had the supports of grants and help, both financial and technical, without which it would have been impossible to accomplish this work. In chronological order they are the following:

1. Project 882/07-R “Caracterización epidemiológica de *Galba truncatula*, vector de la fascioliasis de reciente hallazgo en la República Argentina”, financed by Secretaría de Ciencia y Técnica, Universidad Nacional de Cuyo (2007-2009).

2. Project RLA5049 "Fascioliasis in Humans and Animals in Latin America (financed by Regional Projects, Department of Technical Cooperation (TC), International Atomic Energy Agency (IAEA), IAEA Headquarters Vienna, Austria (2006-2010)

3. Project (resolución rectoral 630/08) “Epidemiología de la fascioliasis en equinos, con *Galba truncatula* como hospedador intermediario, en zonas de endemia de la provincia de Mendoza” financed by Área de Ciencia y Técnica de la Universidad Juan Agustín Maza, (2008-2010).

4. Project (resolución rectoral 426/10) “Descripción morfológica y molecular de huevos de *Fasciola hepática*: Su utilidad en el diagnóstico clínico y estudios epidemiológicos” financed by Área de Ciencia y Técnica de la Universidad Juan Agustín Maza, (2010-2012).

5. Project “Distribución y capacidad de transmisión de lymnaeidos vectores de *Fasciola hepatica* en zonas Andinas de la región de Cuyo” financed by Programa de Becas de Investigación “Carrillo-Oñativia: Comisión Nacional Salud Investiga, Ministerio de Salud, Presidencia de la Nación, (2012-2013).

6. Project (resolución rectoral 965/12) “Vectores implicados en la transmisión de trematodiasis zoonóticas en la provincia de Mendoza” financed by Área de Ciencia y Técnica de la Universidad Juan Agustín Maza, (2012-2014).

7. Project (resolución rectoral 965/12) “Distribución de parásitos gastrointestinales de caprinos en la provincia de Mendoza en las distintas regiones productivas” financed by Área de Ciencia y Técnica de la Universidad Juan Agustín Maza, (2012-2014).

8. Project (resolución rectoral 508/12) “Análisis espacial de la fascioliasis animal en Mendoza- sistemas de información geográfica y sensores remotos” financed by Área de Ciencia y Técnica de la Universidad Juan Agustín Maza, (2012-2014).

9. Project (resolución rectoral 766/14) “Cambios hematológicos y bioquímicos en bovinos naturalmente infectados con *Fasciola hepática* y su relación con la carga parasitaria” financed by Área de Ciencia y Técnica de la Universidad Juan Agustín Maza, (2014-2016).

10. Project (resolución rectoral 766/14) “Impacto clínico de parasitosis digestivas prevalentes en equinos de zonas andinas de la provincia de Mendoza” financed by Área de Ciencia y Técnica de la Universidad Juan Agustín Maza, (2014-2016).

11. Project (resolución rectoral 568/17) “Vigilancia de zoonosis parasitarias emergentes; Impacto clínico y productivo de parasitosis regionales emergentes” financed by Área de Ciencia y Técnica de la Universidad Juan Agustín Maza, (2017-2018).

12. Project (resolución rectoral 568/17) “Evaluación de sensibilidad antihelmíntica en ganado naturalmente infectado por *Fasciola hepatica* de la provincia de Mendoza” financed by Área de Ciencia y Técnica de la Universidad Juan Agustín Maza, (2017-2018).

CHAPTER I

FASCIOLIASIS BACKGROUND

1.- FASCIOLIASIS BACKGROUND

1.1.-FASCIOLIASIS: CURRENT SITUATION

Fascioliasis is a parasitic disease caused by trematodes of the *Fasciola* genus that affects humans and numerous species of domestic and wild animals and to complete its cycle needs small aquatic snails of the Lymnaeidae family. Even though it's a disease that has been known for centuries, it is currently expanding and poses a serious threat to livestock production and human and animal health worldwide (TORGERSON & CLAXTON, 1999; BARGUES *et al.,* 2001; KAPLAN, 2001; BARGUES & MAS-COMA, 2005; MAS-COMA *et al.,* 2009a; FAIRWEATHER *et al.,* 2011a). Nowadays it is the vector transmitted disease with the greatest latitudinal, longitudinal and altitudinal distribution (MAS-COMA, 2004). This implies that it is present in totally different scenarios with diverse climatic, ecologic, productive and even social situations. It can affect impoverished populations in Andean regions in Bolivia and residents of industrialized countries such as France, the tropical rice fields of Vietnam and in the frigid altitudes of the Patagonian Andes, goats in subsistence economies and high yield dairy cattle in the Netherlands. Being a two host cycle as it is, one must resort to different factors to explain these adaptations, related to the parasite, to its definite hosts and to its intermediate hosts, the aquatic lymnaeid snails. And it is precisely in these small aquatic freshwater snails that lie the keys to understand great part of the current epidemiological scenarios and situations (MAS-COMA *et al.,* 2009b).

Historically it has been considered a disease mainly of veterinary importance due to its effects on livestock and a cause of important economic losses to the animal industry worldwide (RANGUEL RUIZ & MARTINEZ DURAND, 1994; TORGERSON & CLAXTON, 1999; KITHUKA *et al.,* 2002; SCHWEIZER *et al.,* 2005; SARIOZKAN & YALCIN, 2011; FETENE & MEKONNEN, 2014). Yet, in recent decades it has shown beyond doubt to be an important disease in humans as well (MAS-COMA *et al.,* 1999a,b, 2005, 2009a). A literature review of human fascioliasis between 1970 and 1990, detected only 2,000 cases worldwide (CHEN & MOTT, 1990). The passing years brought a drastical change, the amount of infected persons could surpass17 million and the exposed population 91 million (MAS-COMA *et al.,* 2005; KEISER & UTZINGER, 2005).

The most important endemic regions of human fascioliasis in the world are in South America, mainly in Andean regions of Bolivia and Peru (ESTEBAN *et al.,* 1997a,b,

1999, 2002; MARCOS & TERASHIMA, 2007; GONZALEZ *et al.* ,2011) but endemic regions are also found in Chile (APT *et al*., 1992, 1993; ARTIGAS *et al.,* 2011), Ecuador and Venezuela (BARGUES *et al.,* 2011a,b).

The global and climate change taking place in recent years have contributed to the expansion of the disease. Man-made alterations of the environment, such as irrigation systems (ESTEBAN *et al.,* 2002; AFSHAN *et al*, 2014), and construction of dams (MORIENA *et al.,* 2008) can favour the colonization of the intermediate hosts. But in many regions of the world, climate change and global warming is an impotent factor behind the emergence of fascioliasis (MAS-COMA *et al* 2008, 2009a,b, 2010, 2014; AFSHAN *et al.,* 2014; QURESHI *et al*., 2016) influencing the epidemiology of fascioliasis and favouring the transmission. Also , the introduction of lymnaeid vectors can dramatically increase the transmission of the disease, such as was the case with *Galba truncatula* in the Bolivian Altiplano or *Pseudosuccinea columella* in Venezuela (BARGUES *et al.,* 2011b) and the Mesopotamia region of Argentina (MORIENA *et al.,* 2008).

In Argentina fascioliasis had been reported in cattle over 140 years ago (SAVOYAT, 1867). The disease is present in the most varied climatic, geographic, ecological situations. It is well known in rural regions as an important disease in livestock, local farmers even have autochthonous names to describe it. In the Midwestern Andean regions it is known as “Corrocho”, in Northeastern wetland it is known as “Saguaypé”, in the Andean regions of the North as “Unca” in the central regions it is known as “Choncaco” and in the province of Buenos Aires it is known as “Palomilla del hígado” (MERA Y SIERRA, 2012). This reflects not only the cultural diversity but also the profound perception local inhabitants have of the disease in livestock. Yet in humans it has always been ignored and considered a sporadic, rare disease. Even in the endemic regions of Bolivia and Peru it was considered as such previous to the studies that were started two decades ago (ESTEBAN *et al.,* 1997a,b,c, 2002; MAS-COMA *et al*, 1999a,b,c; MARCOS & TERASHIMA, 2007; GONZALEZ *et al.,* 2011).

Fascioliasis is distributed throughout great part of the vast territory in Argentina, and to date, the only region were the cycle has not been established is in extreme southern Patagonia, even though it is currently expanding in that region (AGUILAR & OLAECHEA, 2014) the presence of the lymnaeid snails has been reported up to Tierra del Fuego. Congruently to what happens at a global scale, lymnaeid snails have colonised and fascioliasis is present in totally different ecosystems in Argentina. The Andes have already been demonstrated to be suitable to establish fascioliasis endemic

regions (APT *et al.,* 1993; MAS-COMA *et al.,* 1999a,b; ESTEBAN *et al.,* 2002). Yet, the situation in Argentina has only recently starting to be addressed.

Even though there is a long history of official records o fascioliasis in cattle and sheep particularly due to veterinary inspection at official slaughterhouses, the same is not true for human fascioliasis were it is not a disease that has to be officially reported (MERA Y SIERRA, 2012). With respect to the lymnaeid snails, there is a great confusion due to the taxonomy with different species according to different authors and their distribution in Andean regions is restricted to locality reports, many over a century old and without any further confirmation or revisions.

Most texts and publications usually refer as *Lymnaea viator* as almost the exclusive vector of fascioliasis in most of the territory of Argentina without a clear methodology, almost usually only morphological, if present at all, to back up the identification of the species of snail involved in the transmission (OLAECHEA, 1994). This reflects the great difficulties that are encountered in classifying snails of the *Galba*/*Fossaria* group. It is of great importance to know what is the species of snail involved, the epidemiological heterogeneities in the diverse endemic areas are in great part determined by the lymnaeid species involved (MAS-COMA, 2005; MAS-COMA *et al.,* 2009a). The identification of the species of snail involved in the transmission is fundamental to determine the risk zones and to develop control measures since each species has different ecological characteristics. The great difficulty encountered when identifying the lymnaeid species is that there is great intraespecific variability in shell characteristics and great anatomic homogeneity in the different species with respect to their anatomy; thus the problems to identify the species of such controversial groups as *Galba/Fossaria*, *Radix* and stagnicolines (BARGUES *et al.,* 2001, 2003, 2006a). The limitations that morphological studies have to identify species make it necessary the use of molecular markers for the correct identification of which nuclear ribosomal DNA and the mitochondrial DNA appear to be the most useful tools for specimen classification purposes (BARGUES & MAS-COMA, 2005).

Great changes have occurred, usually for the worse, in the epidemiology and impact of infectious diseases at a global level. Such is the denomination of neglected tropical diseases, which compromises 20 diseases as defined by the World Health Organization in which fascioliasis is included (WHO 2010, 2013, 2015). Argentina is not the exception in this global scenario.

The overall aim of the present research is to provide knowledge on the epidemiology of fascioliasis in Andean regions of Argentina in relation to its lymnaeid vectors.

The specific objectives are to: a) Describe the distribution and characteristics of human fascioliasis and relate it to the distribution of vectors; b) Determine the species of lymnaeids present in Andean regions of Argentina; c) The molecular and phenotypic characterisation of the lymnaeids involved in the transmission of fascioliasis, d) Describe animal fascioliasis in Andean regions, determine the role as reservoirs of livestock and wildlife and their relation with the lymnaeid vectors.

1.2.-THE CAUSAL AGENT: SYSTEMATICS, PHENOTYPE, AND GENOTYPE

Liver flukes belonging to the genus *Fasciola* are amongst the causes of food-borne diseases of parasitic etiology. There are currently four species described: *Fasciola hepatica*, *Fasciola gigantica*, *Fasciola nyanzae* and *Fasciola jacksoni* (MAS-COMA *et al.,* 2009). *F. jacksoni* infects exclusively Asiatic elephants, *Elephas indicus* and *Elephas maximus* (BHALERAO, 1933; YAMAGUTI, 1971) and *F. nyanzae* infects the hippopotamus, *Hippopotamus amphibious*, (DINNIK & DINNIK, 1961). *F. hepatica* and *F. gigantica* are the species with impact in human and animal health; *F. hepatica* is of cosmopolitan distribution, found in Europe, Africa, Asia and America*, F. gigantica* is distributed exclusively in Asia, Africa and Europe (MAS-COMA, 2005; MAS-COMA *et al.,* 2009a).These flukes need aquatic snails of the Lymnaeidae family as intermediate host to complete their cycle (BARGUES *et al.,* 2001).

F. hepatica is the only liver fluke in the American continent. It has a leaf shaped body, dorsoventrally flattened and a tegument covered by small spines. Its length varies between 20-30 mm and the width between 6-13 mm. Its anterior end has a conical structure followed by two shoulders. It has two suckers; the anterior or oral sucker is located at the tip of the cephalic cone and the ventral sucker is at the limit between the cephalic cone and the body. The anterior sucker is very muscular and is also the entrance to the digestive tube. These suckers are vital for the attachment and movement of the fluke. The oral sucker is continued by a muscular pharynx and a short esophagus that divides in to branches of the caecum which has many ramifications and is directed towards the posterior part of the fluke (BEAVER, 1986).

Between the two suckers is the genital pore, the opening to both the male and female reproductive system due to the fact that liver flukes are hermaphrodites. The gonads are much ramified; there are two testicles in the mid portion and one ovary anterior to the testicles. Each testicle has a vas deferens which unites to form the

seminal vesicle which is inside the cirrus sack. The seminal vesicle ends in the ejaculatory duct (surrounded by the prostate gland), and then a protractible cirrus which can reach the exterior via the genital pore. There is a short oviduct and two vitelline glands that converge to form the vitelline reservoir. The vitelline glands lie along the lateral margins of the body. A short oviduct leads into the midline, close to where the ducts from the right and left vitelline glands converge to form the vitelline reservoir. The vitelline glands are composed of numerous follicles and are extensive, lying along the lateral margins of the body. The vitelline reservoir joins the oviduct to form the common ovovitelline duct, which, in turn, leads to the ootype. The ootype leads into the convoluted uterus which ends in the common genital pore were the eggs are released (FAIRWEATHER, 1999).

F. gigantica is larger than *F. hepatica*; 24-76 mm long and 5-13 mm. wide. The length/width ratio also differs. In *F. hepatica* it is 1.88-2.32 whereas in *F. gigantica* it is 4.39-5.20. *F. gigantica* has less pronounced marked shoulders, shorter cephalic cone, ovary with longer ramifications and a more ramified caecum (MAS-COMA & BARGUES, 1997). There are areas, particularly in Asia and Africa, were both *F. hepatica* and *F. gigantica* are present and there, morphologically, intermediate forms can be found. (MAS-COMA *et al.,* 2009a).

The eggs of *F. hepatica* and *F. gigantica* are oval in shape, with a yellowish brown colour, have an indistinct operculum and are not embryonated when passed in the faeces. Embryonation occurs outside the host. It is a large egg and its dimensions have been used to differentiate between *F. gigantica* and *F. hepatica*. In the first studies, only animals were considered and the size was for *F. hepatica* was 130-148/60-90 µm, and for *F. gigantica*150-196/90-100 µm (BORAY, 1982; DUWELL, 1982; ABROUS *et al.,* 1998). Yet, when human samples were also taken into account the size ranges differed, 100.6-162.2/65.9-104.6 µm for *F. hepatica* and 150.9-182.2/85.1-106.2 µm for *F. gigantica*. Where both species, coexist, intermediate forms can be found (VALERO *et al.,* 2009a).

1.3.- PARASITES' LIFE CYCLE AND DISEASE TRANSMISSION

The life cycle of the liver flukes is heteroxenous, thus, to be completed it needs a definite host, mainly herbivorous mammals, and an intermediate host, fresh water snails

of the Lymnaeidae family (BARGUES *et al.,* 2001).The cycle can be divided in the following five phases (ANDREWS *et al* 1999):

a) passage of the eggs from the definite host to the external environment and posterior development;
b) hatching of miracidia and subsequent search and penetration of the intermediate host (aquatic gastropod snails of the Lymnaeidae family);
c) development and reproduction inside the snail;
d) emergence of the cercariae from the snail to the water and attachment and encystment;
e) ingestion of the infective metacercariae by the definite host, migration to the liver and development to the adult worm in the bile ducts.

The adult flukes are in the bile ducts and the gall bladder, from where, the eggs travel with the bile towards the intestine and are deposited with faeces. In this moment the eggs have no embryo and it will develop exclusively if the egg is covered by water. Part of the development can occur in the faeces if they are wet but the development will not proceed inside the faeces since these have an inhibitory effect.

Embryonation occurs in 9-21 days if adequate conditions of temperature, pH, and oxygen tension are present (ANDREWS *et al.,* 1999). Development is faster at a temperature range between 23-26ºC and stops below 10ºC and above 36ºC (SALIH *et al*, 1981). Regarding pH, it has to be in the 4-9 range but the optimum is 7. Regarding oxygen tension, embryonation is faster in aerobic conditions, up to 5 times faster compared to eggs in water with lower oxygen tension (ROWCLIFFE & OLLERENSHAW, 1960). When development terminates, hatching occurs, light is the main stimulus. (ROWAN, 1956). The miracidium which is approximately 130 µm long and 28 µm wide, starts swimming by means of its cilia and actively searches for its intermediate host, the lymnaeid snail. It swims toward the shallow parts due to its positive phototropism. It has to find a suitable snail in approximately 8 hours, if it doesn't dies 24 hours post hatching.

F. hepatica and *F. gigantica* have different snail specificities. *F. hepatica* is mainly transmitted by species of small size belonging to the so called *Galba/Fossaria* group, (BARGUES & MAS-COMA, 2005, BARGUES *et al*., 2007a,b, 2011a,b,c) amongst which *G. truncatula* is the main transmitter and the only one in Europe but is also present in Africa, Asia, and South America; *Lymnaea humilis*, *Lymnaea bulimoides*, and *Lymnaea cubensis* in North America; *L. cubensis* in the Caribbean; *Lymnaea neotropica*, *Lymnaea cousini,* and *L. viator* in South America; and *Lymnaea tomentosa*

in Australia. *F. gigantica* is transmitted by species of the genus *Radix*, mainly *Radix natalensis* In Africa and varieties of *R. auricularia* and *R. viridis* in Asia (BARGUES *et al*., 2001). *Pseudosuccinea* is a monospecific genus including the species *P. columella*, which has colonised all continents and has been noted to be able to transmit both *Fasciola* species (BARGUES *et al*., 2011b). Finally, a few species have proven their *F. hepatica* transmission capacity under exceptional local natural conditions in a few areas. This is the case of amongst the lymnaeid group of the stagnicolines, of *Lymnaea (Stagnicola) palustris* and *Lymnaea (Stagnicola) fuscus* and closely related species, *Omphiscola glabra,* and amongst the *Radix* group, of *Radix balthica* (BARGUES *et al*., 2003, NOVOBILSKÝ *et al*., 2013; RONDELAUD *et al*., 2014, 2015; VIGNOLES *et al*., 2016).

The miracidium penetrates the snail, and once inside, there is a metamorphosis towards asexual reproduction stages. It loses its cilia and becomes the sporocyst in the digestive gland or hepato-pancreas of the snail. Inside the sporocyst there are bundles of germinal cells that give origin to the next stage, the redia. Eventually, due to the growth of the rediae, the sporocyst ruptures and liberates them. The redia develops a mouth and a pharynx which leads to an intestine. Inside the redia there are germinal cells that develop into the final stage, the cercaria, yet there may be a new generation of rediae before the cercariae develops (ANDREWS, 1999).

Around 4 to 7 weeks after the miracidium penetrated, the cercariae start to abandon the snail. They measure approximately 300 µm; have two suckers that correspond to those of the adult fluke and a long tail twice the length of the body. They swim during an hour with frequent direction changes but with a tendency to the surface of the water until they encyst, preferably on the leaves of aquatic plants. Some cercariae encyst in the surface of the water, forming the floating cercariae (ESCLAIRE *et al.,* 1989). The cysts are initially white, and darken as it hardens. It is infective soon afterwards and can remain so, according to adequate environmental conditions, during many months. They remain infective during longer periods if it is cold and wet, they are susceptible to high temperatures and desiccation (TORGERSON & CLAXTON, 1999).

The definite host is infected when it consumes the metacercariae, either when it eats aquatic vegetation (most frequently), or when it drinks water with floating metacercariae. Excystation occurs in the small intestine previous activation in the stomach or rumen (SUKHDEO & METTRICK, 1986).Then larvae penetrate the intestine and through the peritoneum reach the liver, which can take 4 to 6 days. Once in the liver, they migrate through the parenchyma eating mainly hepatic cells and blood to a lesser extent. This

migration takes 5-6 weeks and which causes hemorrhage and fibrosis. Then the flukes reach the bile ducts and become adults, feeding on blood and hepatic cells. Eggs are found in the faeces two to four months after infections, depending on the species; 8-15 weeks in sheep and cattle and 3-4 months in humans (MAS-COMA *et al.,* 1999b).

1.4.- THE ANIMAL RESERVOIRS

Many species of mammals, including humans, and some species of birds are the definite hosts of *F. hepatica*. The main animal reservoirs of *F. hepatica* are ruminants, sheep goats and cattle. Yet, in specific scenarios, other species can act as important reservoirs and even be the source of infection for humans, as it has been described with donkeys and swine in Bolivia (MAS-COMA *et al.,* 1997) or the case of the black rat (*Rattus rattus*), which in the isle of Corsica proved to be the main reservoir for the infection in humans (MAS-COMA *et al.,* 1988; VALERO *et al*, 2002). Fascioliasis has also been described in other domestic animals such as buffaloes, horses, camels llamas, alpacas and rabbits (SOULSBY, 1987, MAS-COMA *et al.,* 1999b).

Wild animals can also be reservoirs of *F. hepatica* and their epidemiological role is being evaluated as well as the risk the disease poses to wild populations as a potential conservation threat (CUERVO *et al.,* 2008). *F. hepatica* has been described in many wild species, particularly wild ruminants but also rodents, lagomorphs and camelids amongst others. Cervids are particularly affected and has been described in diverse species such as the European elk (*Alces alces*), Red deer (*Cervus elaphus*), White tailed deer (*Odocoileus virginianus)*, and Fallow deer (*Dama dama*) (FOREYT & TODD, 1977; MCKENNA, 1997; SHIMALOV & SHIMALOV, 2003). It also affects many rodents, it has been described in South America in capybara (*Hidrochaeris hidrochaeris*) (SANTAREM *et al.,* 2002), coypus (*Myocastor coypus*) (EL-KHOUBA *et al.,* 2009) and wild Guinea pig (*Cavia aperea*) (DITTMAR, 2002).

Amongst the rodents, particularly the coypus (*Myocastor coypus*) is worrisome since, being native of South America; it has expanded worldwide due to escapes from fur farms and effectively colonised many regions of the world. Currently it is a reservoir of fascioliasis in Corsica and the Loire-Atlantique, France (MENARD, 2001).

In Uruguay, *F. hepatica* was reported infecting coypus in a farm with an important fascioliasis problem in cattle and sheep. When parasitic burdens and total egg production, was compared, the total load of *F. hepatica* eggs in *M. coypus* (1.4 million

eggs) was surprisingly higher than that reported in naturally infected sheep, even though there were less adult liver flukes. Thus, *M. coypus* would have the capacity to disperse *F. hepatica* eggs, and due to the fact that they defecate in the water, it would be an important source of contamination thus increasing the exposure of lymnaeid snails to the miracidium of *F. hepatica* (GAYO *et al.,* 2011).

In Holland, hares have been found with triclabendazol resistant *F. hepatica* haplotypes, the same found in cattle, which would add complexity to the control of the disease since these wild populations would escape most fascioliasis control measures (WALKER *et al.,* 2011).

Birds have also been found to be definite hosts of *F. hepatica*; it has been reported in ratites. It has been reported in greater rheas (*Rhea Americana*) in Brazil (SOARES *et al.,* 2007) in farmed emus (*Dromaius novaehollandiae*) in Australia (VAUGHAN *et al.,* 1997) and both in greater rhea (*Rhea americana*) and lesser rhea (*Rhea pennata*) in Argentina (MARTINEZ-DIAZ *et al.,* 2013).

1.5.- THE SNAIL VECTORS

It is accepted that the origin of *F. hepatica* is Eurasia linked to, *G. truncatula*, its favoured vector (MAS-COMA *et al.,* 2009a). As mentioned, it needs temperate to cold climates to complete its life cycle (MAS-COMA & BARGUES, 1997). But it is not the sole potential vector and over 20 species of snails of the *Galba/Fossaria* group have been described as intermediate hosts of *F. hepatica* (TORGERSON &CLAXTON, 1999; BARGUES *et al.,* 2001). In situations such as the absence of the main vectors, members of other lymnaeid groups such as stagnicolines (BARGUES *et al.,* 2003) and *P. columella* (BARGUES *et al.,* 2001) can suitable transmit the disease.

1.5.1.- CLASSIFICATION OF LYMNAEID SNAILS

The correct classification of the lymnaeid snail species acting as vector is therefore of great importance, in order to both determine risk zones and to achieve the appropriate control measures to reduce transmission, according to the different ecological characteristics of each lymnaeid species. It is necessary to know what is/are the species involved to correctly evaluate the epidemiological situation, and thus, to be able

to develop control strategies due to the fact that different species have different capacities as vectors of *F. hepatica* due mainly to the magnitude of cercarial production. There is a relationship between a given epidemiological scenario, transmission pattern and the species of snail involved (MAS COMA *et al.,* 2005, 2009a).

Unfortunately, the correct identification of the snail species involved in the transmission of fascioliasis is not an easy task. There is great confusion regarding the taxonomy of these snails (BARGUES *et al.,* 1997). Lymnaeids are freshwater snails which show marked intraspecific variability in their shell characteristics and a surprising uniformity at the level of their anatomy. They have a remarkable morphological plasticity of the group with great intraespecific variation, particularly shell morphology. The same species can show very different shell morph types depending on the particular environmental conditions and different species can show similar shell morphology. Thus, very few species can be correctly identified solely on its phenotypic characteristics (SAMADI *et al.,* 2000). In Latin America, the only species involved in fascioliasis transmission that can be accurately identified by shell morphology and anatomy is *P. columella* (PARAENSE, 1982a; MALEK, 1985).

These features explain the great problems to distinguish between species within given controversial groups such as *Galba/Fossaria*, *Radix* and stagnicolines (BARGUES *et al.,* 2001, 2003, 2006a, 2007a,b, 2011a,b,c). Even expert malacologists have great difficulties in identifying species in these groups. In 1951 HUBENDINCK described 1,143 different species of lymnaeid snails, many of which were already considered synonyms. The taxonomical situation of the *Galba/Fossaria* group is very disconcerting and controversial (BARGUES *et al.,* 2001).

1.5.2.- MOLECULAR CHARACTERISATION

It is imperative to know what species of lymnaeid snails are present in a given site or region to be able to understand the transmission patterns and epidemiological situation. Sequences of molecular markers amongst the nuclear ribosomal DNA and the mitochondrial DNA appear to be the most useful tools for specimen classification purposes (BARGUES & MAS-COMA, 2005). For classification at a supra generic or generic level, 18S rRNA gene is useful, but it is too conserved to be used for classification at a species level. For this, the internal transcribed spacers of the rDNA, mainly ITS-2 and secondarily ITS-1, have proved most effective for lymnaeid species

and subspecies classification (BARGUES & MAS-COMA, 2005). Mitochondrial DNA can be used, but with caution in its interpretation (BARGUES *et al.,* 2011c). It is of little value for the analysis at a supra specific level or between distant species. It is useful to: a) distinguish between very closely related species of the same genus; b) to study intra specific variation and to differentiate populations; c) analyse genetic exchange between different populations of the same species; d) genetic characterisation of laboratory strains; e) perform studies of the spread of populations of a species and the genetic exchange between populations (MAS COMA *et al.,* 2009a; BARGUES, 2010).

1.5.2.1.- DNA NUCLEAR RIBOSOMAL MARKERS

Eukaryotic nuclear ribosomal DNA is constituted by a tandem repeat of a unit segment called the operon localized in the nuclear chromosomes. Each repetition is composed of the genes that code for the ribosomal RNA (18S, 5.8 S, and 28S) plus three types of spacer, the internal transcribed spacers (ITS1 and ITS2), the external transcribed spacers that flank the genes (ETS) and the non transcribed spacers (NTS) that separate one repetition from the following. The genes of ITS and the ETS are transcribed without interruptions by the rRNA polymerase I. Although their function is not clear, it is believed that they have an important role in the maturation of the ribosomal RNA (MUSTERS *et al.,* 1990; VAN DER SANDE *et al.,* 1992). Each gene of the ribosomal DNA has a different substitution rate. In general ribosomal genes have a very low variability, are highly conserved and are therefore useful to establish ancient phylogenetic relationships. ITS evolve much faster so are useful to estimate more recent evolutionary relationships. Yet, the usefulness of each marker at a determined taxonomic level varies between the different organisms (BARGUES & MAS-COMA, 1997; BARGUES *et al.,* 2000; MAS-COMA & BARGUES, 2009). Also, ribosomal DNA is transmitted by mendeleyan heredity, evolves much slower and has a concerted evolution. This implies that with time ribosomal DNA of a determined population with time homogenizes all of its copies and eliminates all of its difference as much in homologous chromosomes as in non homologous chromosomes within the genome. This results in uniformity between all the individuals of a population.

Internal spacers have the presence of micro and mini satellites. Both are repetitive sequences of whose repetitive unity has a variable number of nucleotides, up to five in the case of the microsatellites and up to one hundred in the mini satellites. Yet their

presence add a great variability to lengthen too much the sequences which may lead to erroneous interpretations of the results obtained by methods based on the length of the sequences such as the raps. (Random Amplification of Polymorphic DNA) and PCR-RFLP (Polymerase Chain - Reaction Restriction Fragment Length Polymorphism) (MAS-COMA & BARGUES, 2009).

Gene 18S of the nuclear ribosomal DNA is considered a useful marker for phylogenetic studies at the level of the greater taxons. It is the slower to evolve of all the types of rDNA and its conserved regions allow for the development of primers for its amplification by PCR. It allows for the identification of organisms that have diverged over more than 100 million years ago. It has been used for the earliest ramifications of life; and is useful for phylogenetic relations occurred during the Precambrian but is of little use for organism that have evolved since the Cretacic period.

In lymnaeid snails it has a length of approximately 1,800-2,000 nucleotides and has a balanced nucleotide composition of AT/GC of around 50% and is considered an excellent marker for the systematic classification of species. Yet, it cannot distinguish between very closely related species such as *L. viator* and *L. neotropica*. Thus, in lymnaeids this gene has to be used at a supraespecific level as is the case with other organisms (BARGUES & MAS-COMA, 1997; BARGUES *et al.,* 1997, 2002, 2006b, 2011a,b,c, 2012; MANGOLD *et al.,* 1998; STOTHARD *et al.,* 2000; ARTIGAS *et al.,* 2011).

The first internal transcribed spacer is located between18S and the 5.8S rRNA genes. Both ITS-1 and ITS-2 are the most variable of the operons of the nuclear ribosomal DNA (rDNA). They both can be easily amplified by PCR, primers can be designed from the conserved gene sequences that flank them. They are molecular markers useful to determine relationships between taxa that have diverged more recently, in the past 50 million years. Its nucleotide composition is slanted towards GC content (54-57%) The phylogeny of European stagnicolines has been determined using these genes and their complete sequence has been determined for American and European lymnaeids (BARGUES *et al.,* 2011a,b,c).

ITS-2 is shorter than ITS-1 yet its length varies greatly due to the presence or not of microsatellites and its nucleotide composition has slightly greater content of GC (56-58%). It is very useful to differentiate between problematic taxa such as cryptic species and particularly useful for inferior taxa such as at the species and subspecies level. It has been the most widely used marker for lymnaeids in different parts of the world. The complete sequence of ITS-2, used conjunctly with ITS-1, they have shown to be of

great merit for systematic and taxonomic studies of lymnaeids snails at the specific and supra-specific level (REMIGIO & BLAIR, 1997a; MAS-COMA *et al.,* 2001; BARGUES *et al.,* 2001, 2003, 2006a, 2007a,b, 2011a,b,c, 2012a,b, 2016, 2017; BARGUES & MAS-COMA, 2005; ASHRAFI *et al.,* 2007; JOUET *et al.,* 2008; PUSLEDNICK *et al.,* 2009; ARTIGAS *et al.,*2011).

The microsatellites of ITS-1 and ITS-2 furnish valuable information for the differentiation between populations, subspecies and species (BARGUES *et al.,* 2011a,b,c, 2012).

1.5.2.2.- MITOCHONDRIAL DNA MARKERS

Mitochondrial DNA is a circular molecule consisting of 36-37 genes involved in mitochondrial translation apparatus, electron transport, and oxidative phosphorylation (BALLARD & RAND, 2005). Mitochondrial DNA evolves much faster than nuclear DNA and most of the changes that occur are single nucleotide substitutions. Thus they are excellent candidates to establish phylogenetic relationships at lower taxonomic levels, such as genus, species and populations.

Mitochondrial genes have great variability, which makes it very difficult to design primers for their amplification. Only a few have been used successfully *in* phylogenetic studies. The most conserved are rDNA 12S, rDNA 16S are the most conserved. Amongst the genes that code for cytochrome oxydase, *cox*1is the most conserved, followed by *Cyt*b and then *ND*1. The choice of which to use depends on the organism under study. Their high level of saturation suggests that they should not be used to infer relationship between distant species (MORGAN & BLAIR 1998; MAS-COMA & BARGUES, 2009).

Mitochondrial DNA is maternally inherited, so the mutations that occur in one individual does not undergo recombination during sexual reproduction and will not be inherited by the offspring. In lymnaeids mtDNA have multiple substitutions in the same site, no uniformity in the nucleotide composition of its genes or certain substitutions may be favoured. Thus, this makes mtDNA in lymnaeids a molecular marker with very restricted applications when comparisons want to be made at a species or even superior level (BARGUES *et al.,* 2011c). There may be a profound heterogeneity in substitution rates indifferent regions of the mtDNA and can influence phylogenetic

hypothesis (BALLARD, 2000a,b) thus the phylogenetic and population inferences should be independently assessed (DEAN & BALLARD, 2004).

In lymnaeid snails, the mitochondrial *cox*1 gene most commonly used has a longitude of 672 bp, and its nucleotide composition is AT biased (69.2-69.8%). This marker is very useful in lymnaeids for the analysis at a sub specific or population level if the nucleotide differences in the analysed fragments are numerous (BARGUES *et al.,* 2011a). Sequences have been determined in various species of lymnaeids and has been of use for the description of haplotypes (BARGUES *et al.,* 2007a; 2011a,b,c, 2012; ARTIGAS *et al*., 2011) and also for various species of stagnicolines and radicines (REMIGIO & HERBERT, 2003; WETHINGTON & LYDEARD, 2007).

Mitochondrial DNA of animals has only 2 ribosomal genes: small unit or 12S and large unit or 16S, they don't have internal spacers and there is but one copy in each circular molecule. They have some very conserved regions intercalated with more variable regions; the first half is usually more varied than the second half (DE RIJK *et al.,* 1992; GUTELL *et al.,* 1992). In lymnaeids, the fragment of the gene 16S that is used has a length of 421-426 pb and its nucleotide composition is AT biased (69.5-70.7% biased). The sequence of this fragment was obtained in diverse species of lymnaeids and haplotypes have been described of *G. truncatula*, *L. cubensis, L. humilis, Lymnaea diaphana, L. schirazensis, P. columella* and *L. neotropica* (BARGUES *et al.,* 2011a,c, 2012, 2016). Yet, care must be taken when using this marker when studying species of lymnaeids from different genera and including distant species of the same genera due to the aforementioned effect of saturation of nucleotide positions and the limited information available when using partial sequences of both the *cox*1 mtDNA and the 16S gene (BARGUES *et al.,* 2011a). The use of mitochondrial markers should be restricted to:

i) Phylogenetic analysis of only very closely related species within the same genus.
ii) Intraespecific variability studies by comparing different individuals and populations.
iii) Genetic characterisation of laboratory strains.
iv) Dispersion studies of populations of a determined species.
v) Gene flow studies between neighbouring populations (MAS-COMA *et al.,* 2009a).

1.5.3.- LYMNAEID SNAILS DESCRIBED IN SOUTH AMERICA

There is a profuse history of description of lymnaeid species for South America, yet, three have been the malacologists that have done the most exhaustive and extensive work; B. Hubendick, L. Paraense and E. Malek.

According to HUBENDICK (1951), there are 7 valid species for South America. *Lymnaea columella* Say, 1817; *Lymnaea viator* D'Orbigny, 1835; *Lymnaea cousini* Jousseaume, 1887; *L. cubensis* Pfeiffer, 1839; *Lymnaea diaphana* King, 1830; *Lymnaea peculiaris* Hubendick, 1951; *Lymnaea pictonica* Rochebrune et Mabile, 1888.
According to PARAENSE (1976, 1982a,b, 1983, 1984) there are 6 valid species for South America: *Lymnaea viatrix*, *L. columella*, *L. diaphana*, *L. cousini*, *Lymnaea rupestris* Paraense, 1982 and *L. plicata* Scott, 1953.

According to MALEK (1985), there are 7 valid species*: L. columella*, *L. viator*, *L. cousini*, *L. cubensis*, *L. diaphana*, *Lymnaea patagonica* and *Lymnaea pictonica.*

In Argentina, 12 different lymnaeid species have been described in most of its vast territory, with rich species diversity mainly in the colder southern and mountainous regions (Table 1). These species are: *L. viator* D'Orbigny; 1835; *L. diaphana* King, 1830; *Lymnaea inelegans* Pilsbry, 1911; *Lymnaea andeana* Pilsbry, 1911; *L. patagonica* Strebel, 1907; *Lymnaea riochicoensis* Pilsbry, 1911; *L. columella* Say, 1817; *Lymnaea peculiaris* Hubendick, 1951; *Lymnaea pictonica* Rochebrune et Mabile, 1885; *L. plicata* Scott, 1954; *P. columella* Baker, 1908*; Lymnaea palustris* Müller*,* 1774*.*

They can be found in sites with very extreme weather conditions, such as the Beagle Channel, and Andean regions of Patagonia (PILSBRY, 1911; HUBENDICK, 1951; CASTELLANOS & LANDONI, 1981). Coincidentally with what occurs worldwide, there is much confusion regarding the taxonomy of the lymnaeid snails of Argentina, and different authors have take into account different morpho-anatomical parameters to classify the lymnaeids. Thus, different authors consider different valid species (Table 1).

The French naturalist Alcide D'Orbigny was the first to describe *L. viator* in Patagonia, what is now the province of Rio Negro (D'ORBIGNY, 1835).

During the Princeton University 1896-1899 expeditions to Patagonia, the malacologist Henry Pilsbry from the USA described the following species *L. viator*, *L. diaphana*, *Lymnaea diaphana inelegans*, *L. andeana*, *L. patagonica* and *Lymnaea patagonica riochicoensis* (PILSBRY, 1911).

The Swedish malacologist Bengt Hubendick considers there are 5 species in Argentina: *L. columella*, *L. viator*; *L. diaphana*, *Lymnaea peculiaris* and *Lymnaea*

pictonica. This author had profound differences with Pilsbry: *L. andeana* probably is *L. columella*; *L. diaphana inelegans* he considers a synonym of *L.*; and the following species are considered synonyms of *Lymnaea pictonica*: *L. patagonica*; *Lymnaea patagonica riochicoensis*; and *Lymnaea brunneoflavida* (Hubendick, 1951). Hubendick is the sole author to describe *Lymnaea peculiaris.*

The renowned Brazilian malacologist Wladimir Lobato Paraense considered valid for Argentina: *L. viator*, *L. columella*, *L. diaphana.* He considered *L. cubensis* a synonym of *L. viator* and *Lymnaea peregrina* a synonym of *L. columella* (PARAENSE, 1982a,b, 1984).

According to the malacologists from Argentina Castellanos & Landoni, the species valid for Argentina are *L. plicata*, *L. diaphana*, *L. viator*, *Lymnaea pictonica* and *P. columella* (CASTELLANOS & LANDONI, 1981).

According to MALEK (1985) the following species are valid for Argentina: *L. columella*; *L. viator*; *L. diaphana*, *Lymnaea pictonica* and *L. patagonica.*

L. plicata, has only been reported in Chubut province (SCOTT, 1953).

There is one sole report of the European stagnicoline, *Lymnaea palustris* in Corrientes province (LOMBARDERO *et al.,* 1979b). This is the only report for a stagnicoline in Argentina.

F. hepatica has specificity to its snail vector, thus, the species of lymnaeids present in a region determines the distribution of animal and human fascioliasis (BARGUES *et al.,* 2001). Due to the ecological characteristics that define the presence of a determined lymnaeid species, different species are associated with different transmission patterns (MAS-COMA *et al.,* 2009a). Considering the lymnaeid fauna present in Argentina, it is important to determine their roles as vectors of *F. hepatica*. If we take into account the taxonomical confusion that still prevails in many of the species, care must be taken when determining a species as vector. The exception is *P. columella* which can be differentiated on shell morphology alone (PARAENSE, 1982a).

L. viator (many authors name it *L. viatrix*) is the most frequently cited lymnaeid as vector of *F. hepatica* in Argentina (BENGOLEA *et al*., 1927; BACIGALUPO *et al.,* 1930*,* BACIGALUPO*,* 1932*;* VENTURINI, 1978; LOMBARDERO*,* 1979a; ROSSANIGO *et al.,* 1983; KLEIMAN *et al.,* 2004; RUBEL *et al.,* 2005; CUCHER *et al.,* 2006). Yet, we must bear in mind that the majority of the studies are many decades old, prior to the development of molecular techniques, proven indispensable for the identification of most of lymnaeid snails of the *Galba/Fossaria* group (BARGUES *et al.,* 2001, 2003, 2006a).

The first report describing a lymnaeid as a vector of *F. hepatica* was the pioneer work of BACIGALUPO *et al*. (1930), who experimentally infected *L. viator* collected from the site, were human cases had been reported in the province of San Luis (BENGOLEA *et al.,* 1927). The snails were infected with miracidia obtained from eggs of sheep and human origin. Later, *L. viator* from the province of Buenos Aires, naturally infected with *F. hepatica,* was reported (BACIGALUPO, 1932).There are reports of snails identified as *L. viator* naturally infected with *F. hepatica;* in the province of Corrientes (LOMBARDERO *et al*, 1979a), province of San Luis (CUCHER *et al.,* 2006) and a report from a Patagonian Andean region (RUBEL *et al.,* 2005). In this report, lymnaeid snails identified as *L. viator* are found naturally infected in the same site were a human case of fascioliasis was diagnosed and a very high prevalence in livestock the proportion of lymnaeids with *F. hepatica* was 2%.

P. columella is the next most cited vector of *F. hepatica* in Argentina. All the reports are from the province of Corrientes, a flat and wet region with a subtropical to mild climate. In the first report of *P. columella* naturally infected with *F. hepatica*, a high proportion (8.8%) of infected snails was found (PREPELITCHI *et al.,* 2003). In subsequent reports, very high proportion of infected snails was found: 51.3% (CUCHER *et al.,* 2006) and 7.4% (MORIENA *et al.,* 2008).

L. diaphana has been reported as a vector of *F. hepatica* in Perú (CORDOVA *et al.,* 1961; TANTALEAN *et al.,* 1974; LARREA *et al.,* 1994), yet there are no reports in Argentina as a vector of *F. hepatica*, even though there are many reports of the presence of this lymnaeid, in Argentina (PILSBRY, 1911; HUBENDICK, 1951; CASTELLANOS & LANDONI, 1981; PARAENSE, 1984).

Lymnaea palustris has been reported as a vector of *F. hepatica* in France (DREYFUSS *et al.,* 1994) and in the United States (WILSON & SAMSON, 1971). There is only one report indicating the presence of this lymnaeid in Argentina (LOMBARDERO *et al.,* 1979b) and it is not described as infected by *F. hepatica.*

Table 1.- Lymnaeid species recognized for Argentina according to different malacologists.

LYMNAEID SPECIES	MALACOLOGISTS & REFERENCE
Lymnaea viator D'Orbigny, 1835	Pilsbry (1911), Hubendick (1951), Paraense (1982a), Castellanos & Landoni (1981), Malek (1985)
Lymnaea diaphana King, 1830	Pilsbry, (1911), Hubendick (1951), Castellanos & Landoni (1981), Malek (1985)
Lymnaea inelegans Pilsbry, 1911	Pilsbry (1911)
Lymnaea andeana Pilsbry, 1911	Pilsbry (1911)
Lymnaea patagonica Strebel, 1907	Pilsbry (1911), Malek (1985)
Lymnaea riochicoensis Pilsbry, 1911	Pilsbry (1911)
Lymnaea columella Say, 1817	Hubendick (1951), Paraense (1982a), Malek (1985)
Lymnaea peculiaris Hubendick, 1951	Hubendick (1951)
Lymnaea pictonica Rochebrune et Mabile, 1885	Hubendick (1951), Castellanos & Landoni (1981), Malek (1985)
Lymnaea plicata Scott, 1954	Scott (1954), Castellanos & Landoni (1981)
Pseudosuccinea columella Baker, 1908	Scott (1954), Castellanos & Landoni (1981)
Lymnaea palustris Muller, 1974	Lombardero (1979b)

1.6.- EPIDEMIOLOGY

1.6.1.- WORLDWIDE GEOGRAPHICAL DISTRIBUTION

Whereas *F. hepatica* is cosmopolitan and found in all continents except Antarctica, *F. gigantica* is restricted to Africa and Asia. Due to its ample distribution, *F. hepatica* is considered more relevant than *F. gigantica*, yet, both are currently rapidly expanding. Thus far, *F. hepatica* has been considered more pathogenic than *F. gigantica*, but present-day studies in animal models and clinical observations are indicating that *F. gigantica* may have a higher pathogenic potential than previously supposed (VALERO *et al.,* 2016).

Both *Fasciola* species share many of its main definite hosts, mainly herbivorous (particularly ruminants) and omnivorous mammals, including humans, thus the distribution of the disease, is dictated by the availability of its snail intermediate hosts. These can also be defined as vectors (in a *sensu lato* conception), although not arthropods as defined in a strict sense of the concept, but in fact share most of the characteristics that define a vector. For *F. hepatica* the vectors are snails of the *Galba/Fossaria* group; *F. gigantica* demands snails of the *Radix* genus to complete its cycle (BARGUES & MAS-COMA, 2005; MAS-COMA *et al.,* 2009a). Both *F. gigantica* and the *Radix* genus requisite a warmer temperature range than *F. hepatica* for its development, thus explaining the absence of *F. gigantica* in colder regions (MALONE *et al*., 1998; YILMA & MALONE, 1998; BARGUES *et al.,* 2001).

At present, the World Health Organization includes fascioliasis amongst the important diseases requiring global efforts for its control and is in the roadmap for the control of neglected tropical diseases (WHO 2010, 2013, 2015). Human reports are increasing in number and so are the description of human endemic areas, comprising hypo to hyper endemic situations in many countries of Latin America, Africa, Europe and Asia (MAS-COMA *et al.,* 1999a,b, 2005, 2009a). The reasons for this emergence are varied, but climate change and global change surely can be accounted for (MAS-COMA *et al.,* 2008; 2009b, 2010). Climate change has recently been demonstrated to be associated to human fascioliasis in Pakistan (AFSHAN *et al.,* 2014; QURESHI *et al*., 2016). Increasingly important are the anthropogenic modifications of the environment and the boost of short and long distance travel and import/export facilities available nowadays, thus currently it should be also considered a disease of importance in Travel Medicine (ASHRAFI *et al.,* 2014). Due to the aquatic and amphibious nature of the

intermediate hosts of fascioliasis, modifications on the distribution of surface water, such as manmade irrigation channels, have shown to increase the risk of transmission of fascioliasis in such diverse scenarios as Bolivia or Pakistan (ESTEBAN *et al.,* 2002; AFSHAN *et al.,* 2014).

In the American continent, fascioliasis is only caused by *F. hepatica,* at present there are no reports of ongoing transmission of *F. gigantica* due to the absence of its potential vectors, snails of the *Radix* group (MAS-COMA *et al.,* 2009a). This disease affects livestock throughout all countries of South America, causing great economic losses in the animal husbandry industry (BUNDY *et al.,* 1984; PIZZI *et al.,* 1984; QUIJADA *et al.,* 2005; GONZALEZ *et al.,* 2007; ESPINOZA *et al.,* 2010; DAS CHAGAS *et al.,* 2011; CEDEÑO *et al*., 2012). Moreover, human infection has been described in most of the South American countries (MAS-COMA *et al.,* 1999a, 2005), with important human endemic areas in Andean areas of countries such as Chile, Bolivia and Perú (APT *et al.,* 1993; MAS-COMA *et al.,* 1999b,c; ESTEBAN *et al.,* 2002; ARTIGAS *et al.,* 2011; BARGUES *et al*., 2012). Human fascioliasis has also been reported in all the other Andean countries: Venezuela, Colombia, Ecuador (BARGUES *et al.,* 2011a,b) and Argentina (CHEN & MOTT, 1990; ESTEBAN *et al.,* 1998), albeit with much less cases than in the aforementioned countries. Nevertheless the doubt arises as to whether there may be a possibility of under diagnosis. Non Andean countries have few human cases even though the prevalence in cattle can be extremely high, such the case of Uruguay (BARGUES *et al*., 2017), that has a reported prevalence in cattle of over 57%, yet historically there are less than 100 human cases (LOPEZ LEMES *et al.,* 1996).

As in all other vector-borne diseases, in fascioliasis the snails of the Lymnaeidae family are crucial with regards to the epidemiological situations and transmission patterns of the disease. Epidemiological and transmission heterogeneities in the different endemic areas are related to the lymnaeid vector species involved (MAS-COMA, 2005; MAS-COMA *et al.,* 2009a).

In Argentina, production of mostly cattle, but also sheep and goats, is a main national economic activity, with wide areas mainly or totally dedicated to livestock management. Therefore, animal fascioliasis has always been a target of major concern in this country, due to its high pathogenicity, losses it causes, and economic efforts which shall be made for its control (LOMBARDERO *et al.,* 1979a; PIZZI *et al.,* 1982; OLAECHEA, 1994). In Andean regions, important prevalence in livestock has been described. In goats, a prevalence of 33% was described in the mountainous regions of the provinces of Mendoza, San Juan and La Rioja (CUERVO *et al*., 2013). In the province of

Mendoza, the Andean Valley region of Tupungato a prevalence of 67.5% was found in the slaughterhouse in cattle (MERA Y SIERRA *et al.,* 2005a), and 32.7% prevalence in goats of the same region by coprological exams (DI CATALDO *et al*., 2015). Camelids, both domestic and wild, such as llamas *(Lama glama*) and guanacos (*Lama guanicoe*), have also been described in the Andean valleys of Mendoza (MERA Y SIERRA *et al*., 2015).

The situation of human fascioliasis has never been the subject of an adequate analysis. Only short reports within large worldwide reviews may be specifically acknowledged (CHEN & MOTT, 1990; ESTEBAN *et al.,* 1998). This is surprising when taking into account that: (i) neighboring countries such as Bolivia and Chile reported hyper endemic areas of human fascioliasis long ago (APT *et al.,* 1992, 1993; ESTEBAN *et al.,* 1997a,b, 1999, 2002); (ii) the country presents a very widely distributed veterinary problem of fascioliasis in livestock; (iii) it includes Andean environmental characteristics appropriate for fascioliasis transmission to humans (MAS-COMA *et al.,* 2001).

Argentina is a country of high livestock production, where sheep and cattle but also equines constitute important economic sources. All these different domestic species are important reservoirs of fascioliasis and represent similar sources of infection for humans, given the results obtained in experimental studies which have demonstrated that snail-borne infective metacercarial stages originating from different animal species do not significantly differ in their infection capacity (VALERO & MAS-COMA, 2000; VALERO *et al.,* 2001a).

1.6.2.- TRANSMISSION PATTERNS OF FASCIOLIASIS

Regarding human fascioliasis, different transmission patterns can be clearly identified (MAS-COMA, 2005):

a) A very-high-altitude pattern in South America exclusively in Andean countries with transmission of *F. hepatica* by imported *G. truncatula*. Two sub-patterns can be recognized.

i) The altiplanic pattern in which transmission occurs continuously year round, such as in the Northern Bolivian and Peruvian Altiplano.

ii) The valley pattern in which transmission has a seasonal variation such as in the Peruvian valley of Cajamarca.

b) The Caribbean insular pattern which occurs in permanent human hypo-endemic regions with epidemic episodes which affect not too many people (a few hundred at the most), as happens in Pinar del Rio, Cuba. The lymnaeid snail involved is *L. cubensis*.
c) Afro-Mediterranean pattern in which both fasciolid species*, F. hepatica* and *F. gigantica*, are involved. The lymnaeid snails responsible for the transmission are mainly *G. truncatula* and *Radix natalensis*, secondarily *P. columella.* There is seasonality such as in the Nile delta of Egypt.
d) Pattern surrounding the Caspian Sea, such as in the Gilan province of Iran, which is a hypo-endemic region but large epidemics occur, affecting up to 100,000 persons. Both fasciolid species are involved and the vectors are several *Galba/Fossaria* and *Radix auricularia* and other stagnicoline lymnaeids.
e) South East Asia pattern, in Vietnam, in lowland areas but may originate large epidemics. Even though both fasciolid species may co-exist, it is related mostly to *F. gigantica* and *Radix* snails (DE *et al.,* 2006; LE *et al.,* 2008; NGUYEN *et al.,* 2011).
f) A new pattern has very recently described in Andean regions of the province of Catamarca, Argentina, were isolated foci were found in desert arid and semi arid conditions were there is a concentration of transmission factors and the seasonal transmission depends on the conjunction of appropriate temperature and river water availability (BARGUES *et al*., 2016).

The variety of the aforementioned transmission patterns clearly demonstrates the great adaptability of *F. hepatica* and *F. gigantica*. The enormous expansion they have experienced from its Eurasian and African origins, for *F. hepatica* and *F. gigantica,* respectively, would not have been possible if it were not also for the colonization capacity of the lymnaeid vectors that adapt both to the tropics of Cuba or Vietnam thrive even in such extreme situations as the ones experience in the Andean altitudes.

1.6.3.- EPIDEMIOLOGICAL SITUATIONS OF HUMAN FASCIOLIASIS

Human fascioliasis can be characterized according to different epidemiological situations. The following classification proposed by MAS-COMA (1999a) to the WHO is still currently valid:
a) Imported cases: Human cases that are diagnosed in a region where fascioliasis is absent in human and animals, yet the person has a history of travel to an area were fascioliasis transmission in known to occur.

b) Autochthonous, isolated, non-constant cases: cases that occur in an area were animal fascioliasis is present yet human cases are very sporadic.

c) Endemic: there are three different distinct situations according to the prevalence in the population as diagnosed by coprological exams:

Hypo-endemic: the prevalence is less than 1%., the intensity, measured in mean eggs per gram (epg) shed is less than 50.There are usually acceptable sanitation procedures with adequate elimination of faeces by latrines or sewage. Humans would not participate in the transmission of the disease.

Meso-endemic: Prevalence ranges from 1% to 10%, although they may be higher in children 5-15 years of age. The mean intensity is 50-300 epg, although there may be individual cases shedding more. The hygienic sanitary situation is not optimal and there may be outdoor defecation.

Hyper-endemic: Prevalence exceeds 10% and higher values may be found in children 5-15 years of age (holo-endemic). The mean intensity in human communities exceeds 300 epg and there may be individuals shedding very large amounts, up to more than 1,000 epg. There are deficient sanitary conditions with absence of waste and sewage disposal and latrines, outdoor defecation is currently practiced.

Epidemic: There are different situations according to the endemic or non endemic status of the region.

Epidemics in animal but not human endemic areas, in regions that human cases are very sporadic, the epidemic occurs as family or small group outbreaks that have ingested the same contamination source.

Epidemic in human endemic areas: the outbreaks concern a greater amount of subjects, usually associated with a climatic condition that favours the fluke and the snail lifecycles such as an increase in rains.

1.6.4.- HUMAN INFECTION SOURCES

The infective form of *F. hepatica* and *F. gigantica* is the metacercaria, the result of the cercaria which is shed by the lymnaeid snail and swims briefly before contacting a solid support, mainly leaves of plants, just above or below the waterline, where they will lose their tails, and encyst. Some cercariae encyst at the waterline and become floating metacercariae (VAREILLE-MOREL *et al*., 1993). After 24-72 hours they become infective. Hence, fascioliasis is a disease that can be transmitted to humans both by the

ingestion of plants and of water. Additionally, even though it is not the usual infection source, humans can also become infected by the ingestion of fresh raw liver which may have early migrating flukes which pursue their migration and restart intraorganic migration (MAS-COMA *et al*., 2018).

Humans can become infected by a vast array of sources that reflects the diversity of the potentially affected populations; geographic, ethnic, religious, social and cultural factors determine the possible infections sources. Traditionally, the ingestion of watercress was considered as almost the sole source of infection (MAS-COMA, 2005) yet, the reality is far more complex and reflects the profound epidemiological heterogeneity of human fascioliasis (MAS-COMA *et al*., 2018). Infection can occur due to the ingestion of freshwater wild plants, freshwater cultivated plants, terrestrial plants, (both wild and cultivated), beverages and juices made from local plants due to the metacercaria attached to them and by drinking water with floating metacercariae. Many of these gastronomic activities have cultural and even religious roots, which means they are deeply incorporated behaviors. Floating metacercariae con infect humans not only drinking the water that contains them but also but by washing of vegetables, fruits, tubercles and kitchen utensils with contaminated water.

Plants that are not used for feeding can also be a source of infection, such is the case of khat, a plant native to the horn of Africa and the Arabian Peninsula that is chewed and used as a stimulant. The infectivity of the metacercariae is prolonged due to the fact that it is usually transported in damp conditions. Cases have been reported not only in the African countries where it is produced and consumed, but also in European countries due to the uncontrolled importation of khat to be used by immigrants (CATS *et al*., 2000; DE BREE *et al*., 2013).

The great diversity of potential infection sources for humans implies that generalizations cannot and should not be made and the situation has to be assessed in each site. Not doing so can result in simplifications of sometimes very complex situations winch need control strategies to be tailored to each particular situation (MAS-COMA *et al*., 2009a).

1.7.- DIAGNOSIS

Fascioliasis can be suspected based on epidemiological and clinical information, but since there are no pathognomonic symptoms the confirmation has to be done by

diverse laboratory exams. The epidemiological background that suggest the possibility of fascioliasis is if the patient lives in or even if it has travelled to an endemic region (ASHRAFI *et al.,* 2014), the consumption of wild or commercial watercress (*Nasturtium officinalis*) (MAILLES *et al.,* 2006) or other aquatic plants (MAS-COMA *et al.,* 1995, 2018).

Fascioliasis can be diagnosed either by observing the characteristics egg in faeces or visualization of mature or immature fluke in the liver or other organs (in erratic presentations).

Coprological exams have the advantage of being very low cost and do not require sophisticated equipment. The disadvantage is that it will only detect fascioliasis if eggs are being eliminated, so it is of no use in the acute phase since eggs are eliminated after a prepatent period of 2 to four months, depending on the species.

Direct examination of faeces has very low sensitivity. The WHO recommends the Kato-Katz technique, that, even though it has low sensitivity, it can be increased repeating the technique, but it allows for a quantification of the eggs per gram.

Different concentration techniques are used, mainly sedimentation since *F. hepatica* and *F. gigantica* are heavy eggs. A technique widely used in South America is Lumbreras rapid sedimentation that is on the sedimentation the sample in water during four minutes which allows for the rapid sedimentation of the *F. hepatica* eggs and not the rest (LUMBRERAS *et al.,* 1962.). Originally designed for use in humans but it is currently also used for diagnosis of fascioliasis in ruminants (CUERVO *et al.,* 2013). In cattle other sedimentation techniques are used, such as the Dennis, Stone, and Swanson technique (DENNIS *et al.,* 1954), and some, to increase sensitivity, implement the use of fine (170 µ) sieves (SUAREZ *et al.,* 1997).

In many regions of the world, there is a tradition of consuming raw liver, thus exists the possibility of consuming *Fasciola* spp. eggs that will be visualized in the coprological tests and give rise to the equivocal diagnosis of fascioliasis (MAS-COMA *et al*., 2018). To differentiate between the eggs in transit that are somewhat degenerated from the normal eggs, certain expertise is needed. To avoid this possible misdiagnosis, the patient must be placed on a liver free diet and perform repeated follow ups to verify if the eggs persist in the faeces (MAS-COMA *et al*., 2014).

Necropsy has the greatest sensitivity, but obviously it is used in animals mainly at the slaughterhouse. The liver has to be inspected, observing hemorrhage or fibrosis that may indicate the presence of the flukes. The biliary ducts and the gall bladder (except in the horse due to its inexistence) are opened and inspected in search of the flukes. The

liver parenchyma has to be inspected to identify juvenile forms (MERA Y SIERRA, 2012).

Inmunodiagnosis has the great advantage of detecting fascioliasis when eggs not eliminated in faeces, particularly during the acute face of the disease during to the prepatent period (which can be from two to four months depending on the species) or of the sometimes irregular elimination of eggs. It is also very useful for surveys due to the greater ease of collecting blood samples rather than stools. Yet the disadvantages are a notorious lack of consensus as to what are the reference tests, uniformity between tests and scarcity of commercial kits available for routine diagnosis. Also, compared to coprological techniques, better equipped laboratories are necessary, many times not available in field situations.

Assays have been developed both for the detection of *F. hepatica* antigens or antibodies, the latter are the most widely used. High sensitivity and specificity have been achieved using as antigenexcretion/secretion (ES) products of live adult flukes incubated in culture media. Cysteine proteases secreted by juvenile and adult worms have shown great antigenicity in animals and humans and provide markers for the diagnosis of great sensitivity and specificity (MAS-COMA *et al.,* 2007). Tests using cysteine proteases have been developed and successfully used for diagnosis, including mass surveys in Perú (O´NEIL *et al.,* 1999; ESPINOZA *et al*., 2007). The test FASCIDIG® uses ES antigens and detects coproantigen in faeces and is also used to evaluate success in treatment (ESPINO & FINLAY 1994). Another test that detects coproantigen is MM3 COPRO ELISA, (MEZO *et al.,* 2004) which has recently been enhanced and has greater sensitivity with the capacity to detect even if there is only one fluke in the cow´s liver (MEZO *et al.,* 2016). This test has its serological counterpart, MM3 SERO ELISA (VALERO *et al.,* 2009b).

1.8.- TREATMENT

1.8.1.- ANTIPARASITIC DRUGS AVAILABLE

Up to now all trials to develop an effective vaccine have failed so chemotherapy is the main tool for treatment and prevention of fascioliasis. Even though there are multiple drugs that are used or have been used for the treatment of human and animal fascioliasis, the only single drug that is effective for early immature, immature and adult

flukes is triclabendazol. Other drugs, such as closantel, nitroxinil, clorsulon and albendazol are effective, with minor differences, to late immature or adult flukes (KELLEY *et al.,* 2016).

In humans, triclabendazol (Egaten ®) is currently the only first line drug available and its efficacy has been demonstrated (MAS-COMA *et al.,* 2001).The dosage is 10mg/kg administered orally (with food for a better absorption) two times separated by 12-24 hours (total dose 20mg/kg). Some patients may require a third dose. There are no collateral effects of significance and it should not be used in pregnant women. In patients shedding more than 400 eggs per gram, a high fluke burden is suspected and they should not be administered the total dose of 20 mg/kg to prevent colic and possible biliary obstruction due to the dead flukes. Hospitalization, particularly in children, is recommended 7 days post-administration (MAS-COMA *et al.,* 2007).

In the different animal species, triclabendazol is also the drug of choice, particularly for the treatment of acute fascioliasis due to the fact that it is the only drug that kills the immature flukes. The dosage is 12 mg/kg in cattle and equids, 10 mg/kg in sheep and goats orally. If chronic fascioliasis present and there are mainly adult flukes in the liver, other drugs can and should be used to deter the appearance of resistance. The most frequently used are closantel (cattle 3mg/kg, sheep 5mg/kg, goats 12 mg/kg), nitroxinil (cattle, sheep and goats 10 mg/kg), clorsulon (cattle and goats 7mg/kg), and albendazol (cattle 10mg/kg, sheep 7.5 mg/kg, goats 20 mg/kg) (MERA Y SIERRA, 2012).

Yet, due to the widespread and more often than not abusive use mainly in sheep and cattle, has lead to the appearance of resistance. Since its first report in sheep in Australia (OVEREND & BOWEN 1995), there have been multiple reports in South America, Europe, Australia, New Zealand and Turkey (KELLEY *et al.,* 2016). In Argentina, resistance to triclabendazol has been reported in cattle in Andean regions of the province of Neuquén, in Patagonia (OLAECHEA *et al.,* 2011).

With the threat of resistance in mind, other drugs are being evaluated for use in humans as alternative treatments. One of them is nitazoxanide which has already been used for the treatment of chronic fascioliasis, with varied but promising results. In Mexico an efficacy rate of 94% after one dose and 100% after two doses was reported (ZUMAQUERO *et al*., 2013); in Egypt a 97% efficacy was reported in children (KABIL *et al*., 2000). The first report of human fascioliasis in Nepal was successfully treated with nitazoxanide (SAH *et al*., 2017). Yet, in Cuba, no response to nitazoxanide treatment was found (DEL RISCO *et al*. 2005). There could exist regional differences due to geographical strains (ZUMAQUERO *et al*., 2013).

1.8.2.- RESISTANCE OF *FASCIOLA HEPATICA* TO ANTIPARASITIC DRUGS

Resistance to triclabendazole has also been reported in humans in The Netherlands (WINKELHAGEN *et al.,* 2012), Chile (GIL *et al.,* 2014), Turkey (GÜLHAN *et al.,* 2015) and recently Perú (CABADA *et al.,* 2016). The resistance found in Peru is particularly worrisome due to the hyper-endemicity of the area. If resistance to triclabendazol extends, it would be a very serious problem due to the lack of effective alternatives.

It should also be considered that there may be an overestimation of the resistance since there is a lack of consensus as to what method is used to determine resistance. Many instances of resistance are actually treatment failures due to other causes such as incorrect dosing, product failure, reduced metabolism of the drugs due to liver damage, etc. (FAIRWEATHER, 2011b).

One of the tests most widely used is the fecal egg count reduction test (FECRT) in which it is considered successful if there is a 95% reduction in fluke egg counts. Yet, results can be misleading, since *F. hepatica* eggs can be stored in the gall bladder for several weeks (CHOWANIEC & DARSKI, 1970), so the coprological exam can result positive even though there are no more flukes in the liver.

Another test that can be used when evaluating resistance to triclabendazol is the coproantigen reduction test (CRT) (GORDON *et al.,* 2012). The problem with both FECRT and the CRT is when flukicides that affect only adults are evaluated, is that at the time of retesting (21 days post treatment), the younger flukes not targeted by the drug will have matured and release eggs or coproantigen and thus give false positive results even in susceptible populations. Another test that is being developed to evaluate resistance of *F. hepatica* to flukicides is the egg hatch assay (EHA). The EHA has been adapted to be used in *F. hepatica*, since flukicides can affect not only egg production and formation but also egg development and hatching (viability). Testing closantel, significative differences were found in egg morphometry, embryonation and hatching 36 hours post treatment compared to untreated sheep. Even though closantel treated animals may eliminate eggs the first days after treatment, these are not viable, and thus, from an epidemiological point of view the treatment will have been effective even if eggs are being shed (SOLANAS *et al.,* 2016).

1.9.- PREVENTION

To be able to control fascioliasis it is of utmost importance to know the epidemiology of each particular region. This is especially important in Argentina due to the great ecological and climatic diversity of the country. Too many control programs are based solely in the administration of triclabendazol on a routine basis, regardless of the characteristics that determine de dynamics of the disease. A protocol for administration to the drug in livestock that is in a subtropical region as can be in Northeast Argentina cannot be the same as the one used in southern Patagonia. The moment of infection depends on the emission of cercariae by the snails and these depend on the climate. The protocols will vary in the different regions and also in the same region depending of the climatic characteristics in any given period. Properly used, antiparasitic drugs can be centrally important in control and prevention of fascioliasis by preventing the infection of the snails and consequently the contamination of plants and water.

Other control methods are destined to prevent the ingestion of the metacercariae. In humans these are orientated towards the control of the consumption of aquatic plants that can be contaminated. Also, in many places the contamination is not by eating the plants with the metacercariae but by drinking water with the floating metacercariae. This can only be prevented by a correct treatment of the water to make it potable by filtering techniques (MAS-COMA, 2004). In the case of animals, were feasible, access should be limited to the sites were the snail populations and thus the metacercariae are present. For example, by fencing off springs were the snail populations thrive or the implementation of artificial water systems (KNUBBEN-SCHWEIZER & TORGERSON, 2015).

With respect to the control of the snail populations, many systems and methods have been used with variable success. Incrementing the drainage of pastures will reduce the sites were the vectors can proliferate but this is usually a costly operation beyond the scope of most production systems (WILSON *et al.,* 1982). Molluscicides have also been used, but due to the great reproduction capacity of lymnaeids the surviving snails very rapidly re colonise the site. Since the molluscicides, such as copper sulfate, are not selective and will affect much of the aquatic fauna of a given site including potential predators of the snails, so its use is being discontinued (MERA Y SIERRA, 2012).

CHAPTER II

HUMAN FASCIOLIASIS IN ARGENTINA

2.- HUMAN FASCIOLIASIS IN ARGENTINA

2.1.- RETROSPECTIVE OVERVIEW,CRITICAL ANALYSIS AND BASELINE FOR FUTURE RESEARCH

In Argentina, human fascioliasis has never been adequately analysed, although having a physiography, climate, animal prevalence and lymnaeids similar to those of countries where the disease is endemic such as Bolivia, Perú and Chile (APT *et al.,* 1993; ESTEBAN *et al.,* 1998; MAS-COMA *et al.,* 1999b).The purpose of the present ten-year research work is to provide an in-depth analysis of the results obtained in a thorough bibliographical search of human fascioliasis cases in Argentina. In that country, even though there are national data on animal fascioliasis, where slaughterhouse reports have been submitted to the authorities for practically a hundred years, there are, however, no official reports on human fascioliasis, because human infection by the liver fluke is not of obligatory declaration. Thus, published and unpublished written reports are the only source of information, whether they may be articles in scientific journals, books, university theses, communications at scientific meetings, or internal reports of agencies, ministries, hospitals, and health centres.

2.2.- MATERIALS AND METHODS

2.2.1.- INFORMATION SOURCES FOR CASE REPORTS

The literature review has been made from sources including databases, national and multinational web-entries and free collections, multititle packages or web platforms, libraries, and through personal e-mail requests when appropriate.

Human fascioliasis case reports were obtained from the following sources: a) local medical and veterinary articles published in Argentina: 38 references; b) local medical and biological publications from Uruguay: 2 references (BACIGALUPO *et al.,* 1930; BACIGALUPO, 1942); c) a publication in a medical journal from Spain: 1 reference (CARENA *et al.,* 1972); d) scientific communications in medical and veterinary congresses and meetings (abstract books): 7 references; e) a parasitology book published in Argentina: 1 reference (NIÑO, 1965); and f) doctoral theses made in universities from Argentina: 2 references (BOTO, 1939; SICILIANO, 1982).

Permission to use and refer to the contents of the unpublished papers such as doctorate theses and certain meeting abstract books was previously obtained in all cases. In the very few cases in which permission was denied for different reasons, neither is the content reviewed nor is the paper included in the references.

Review methods: Different key words were used when searching in digital sources. Special efforts were made to obtain old references published in local journals or very secondary, non-digitalized journals, unpublished reports, abstracts of meetings, symposia, congresses or similar (usually produced by simple photocopying and in very reduced number of copies) and Master's and PhD Theses. The scope on human fascioliasis was in need to be widened to non-medical journals, because this disease in humans was so neglected in the past that getting acceptance for a paper dealing with human reports in a medical journal was sometimes difficult time ago.

Due to the fact that most of the references are from local publications, the majority of them were not to be found in electronic databases. As an illustrative example, only two local articles (CARENA *et al.,* 1972; RUBEL *et al.,* 2005) could be retrieved from MEDLINE.

Therefore, manual searches had to be done in libraries in many instances. More than half of the references were more than 40 years old, giving rise to a long and difficult process to obtain them, since most are not kept at hand and thorough searches had to be performed. The difficulties encountered doing the bibliographical survey were numerous, principally due to the fact that most of the older material is outdated and the databases are still manual and in many instances incomplete. This is the main reason for the ten-year duration this overview and analysis have taken.

Quality assessment: In front of a bibliography of such characteristics, a great care had to be taken not to repeat the same case, since in many instances, duplications could be ascertained and the same patient accounted for in successive publications. Additionally, most of the old papers were published in local, non-peer-reviewed journals and several were made by non-specialists in fascioliasis. Consequently, data of such papers were originally not appropriately reviewed by referees and shall therefore be considered in many cases only at informative or suggestive level. This does not mean, however, that data contained in such literature may furnish more or less valuable information in one or another aspect.

Because of the peculiar nature of several of the papers analysed, some references of this article will be hardly available for the reader. Nevertheless, we consider that justice must be done to everybody who in one way or another contributed to the knowledge of

human fascioliasis in Argentina. Some references could never be found, such as for example the doctoral thesis that Andrada presented in 1970, and which could not be found even in the library of the university where it was made, presented and supposed to have been archived. Similarly, the abstract of the meeting communication by URRUTIA & FERRARIS (1962) could be obtained nowhere.

Owing to the impossibility to even know about the existence of internal reports in both private and official institutions, it cannot be assured that this review covers all studies carried out on human fascioliasis in Argentina. Anyway, the thorough search performed and the exhaustive information compiled is sufficient as to correctly estimate both history and present situation of knowledge on human fascioliasis in the country.

2.2.2.- INFORMATION SOURCES FOR CLIMATIC DATA

For the analysis of the seasonal and yearly distributions of human cases with regard to the climatic characteristics, only the province of Córdoba was selected due to the relatively high number of human reports from that province. No other province presented a sufficient number of cases as to allow a significant comparative analysis. Apart from sporadic reports, specific information of the date the patients got infected, started with symptoms and were diagnosed that allows for the correlation with the climatic variables is available in a doctoral theses (SICILIANOS, 1982) that describes 101 human cases in the province of Córdoba during the decades of 1960's and 1970's.

Mean monthly data of maximum and minimum temperatures, precipitation and humidity were obtained from the Servicio Nacional de Meteorología, Buenos Aires. Data analysed only concerned the decades of 1961-1970 and 1971-1980, during which the patients were infected as explained previously. The aforementioned climatic variables were furnished by ten different meteorological stations throughout the province of Córdoba, strategically selected according to the completeness of the data per station and the appropriate coverage of the geographical distribution of human cases. Geographical coordinates of the ten stations are as follows: Córdoba Aero (31º 19' S, 64º 13' W); Córdoba Observatorio (31º 24' S, 64º 11' W); Dique Cruz del Eje (30º 45' S, 64º 45' W); Dique La Viña (31º 53' S, 65º 02' W); Dique Pisco Huasi (30º 20' S, 64º 00' W); Embalse (32º 11' S, 64º 23' W); Huerta Grande (31º 05' S, 64º 29' W); Rio Cuarto Aero (33º 05' S, 64º 16' W); Rio Tercero (32º 10' S, 64º 08' W); and Villa Dolores Aero (31º 57' S, 65º 08 W).

To investigate the correlation between the moment of diagnosis of the human cases in Córdoba province between the years 1961 and 1981, and different climatic parameters (precipitation, relative humidity, maximum temperature, and minimum temperature), Pearson's correlation coefficient (*r*) was used by means of Infostat 2008 software. The human cases where correlated with the climatic parameters on a month per month basis and considering time lags of 1, 2, 3, 4, and 5 months considering the incubation period of fascioliasis which can be of up to two to four months. A correlation analysis was also applied to assess the potential relationship between annual precipitation and the number of cases per year in the aforementioned years.

2.3.- RESULTS

2.3.1.- FIRST HUMAN CASES IN ARGENTINA

The first case published in Argentina concerned an Arab immigrant who had just arrived in Argentina (ROFFO, 1913). The onset of symptoms was upon arrival, indicating that the disease was most probably acquired in his homeland. The patient died and the diagnosis was made during autopsy when numerous flukes were found in the liver. Since this case was not autochthonous, it is not considered in this review.

This overview begins from the first autochthonous case in 1924 diagnosed by coprology (GREENWAY, 1924). More cases followed soon, such as a coprological diagnosed patient from San Luis province (BENGOLEA *et al.,* 1927), another diagnosed during surgery (DEL VALLE & DONOVAN, 1928) and yet another one from San Luis province (BACIGALUPO *et al.,* 1930). Fifty three cases had already been published prior to 1960.

The first WHO review (CHEN & MOTT, 1990) includes only 13 human reported cases in Argentina for the 1970-1990 period, namely only those reported by CARENA *et al*. (1972). This number of human cases was increased to 85 in the following extensive WHO initiative (MAS COMA *et al.,* 1999a). The present review offers a completely new picture of human fascioliasis in Argentina, including a total of 619 autochthonous cases in 58 reports of different kinds analysed up to 2010 (Table 2) which means that the number of human cases published is more than seven times greater than previously noted. Such a pronounced difference seems to be due to the great amount of overlooked local publications (and also communications at scientific

meetings with abstract books of restricted dissemination). When considering that human fascioliasis infection is in Argentina of non-obligatory declaration, similarly to the rest of the world, one may conclude that the number of infected patients should be even greater than that. Interestingly, the need for Argentinean health authorities to warn about this disease was already noted long ago when fascioliasis was cited in animals in the country for the first time (SAVOYAT, 1867).

Both report and case numbers follow a parallel evolution with quite important fluctuations (Figure 1). This result is most likely to be linked to particular circumstances encouraging physicians to publish their diagnosed cases rather than a real reflection of the annual evolution of the epidemiological situation. For instance, only two authors, C. Rodriguez and C. Siciliano (RODRIGUEZ, 1952, 1954, 1961; SICILIANO, 1982; SICILIANO *et al.,* 1989) are responsible for 51% of the cases reported in Argentina, the decades when they were active publishing appearing as those decades with the greatest amount of cases (1960's and 1980's).

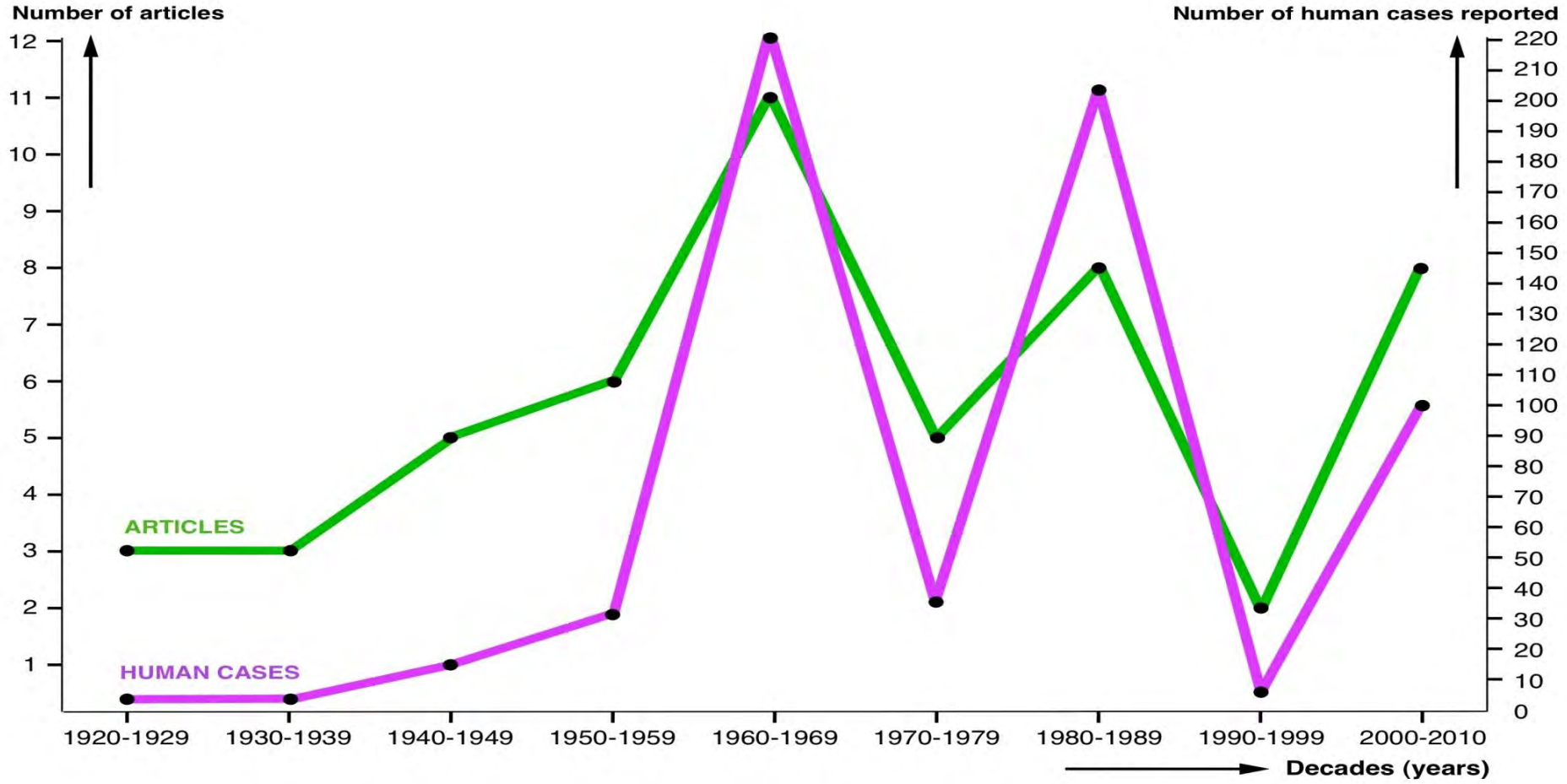


Fig. 1.- Reports on human fascioliasis in Argentina. Evolution of the number of reports on human fascioliasis and number of human infection cases in Argentina according to decades (MERA Y SIERRA *et al*., 2011).

2.3.2.- CHARACTERISTICS OF THE INFECTED POPULATION

In only 267 cases did the authors specify the gender of the patients: 120 (44.94%) were male and 147 (55.06%) were female. A somewhat higher preference for females (68.52% out of 54 subjects) was also found in the serological study performed with ELISA by MALANDRINI *et al.* (2009a) in the locality of Taton, Tinogasta, Catamarca, the only randomized survey carried out in Argentina so far. Unfortunately, no studies on eggs per gram of faeces (epg) have been performed in Argentinean patients, so that a gender relationship with intensity could not be assessed. Although that apparent preference for females is not statistically significant in Argentina, this trend is consistent with what has been described in human endemic areas: significantly higher intensities in females in Bolivia and Perú (ESTEBAN *et al.,* 1997a, 1997b, 1999, 2002), and significantly higher prevalence in Chile (APT *et al.,* 1993) and Egypt (ESTEBAN *et al.,* 2003). However, such a female preference rule does not always appear to be significant at prevalence level (ESTEBAN *et al*., 1997a,b, 1999, 2002; MOGHADDAM *et al.,* 2004; ESPINOZA *et al.,* 2007).

Age in years was specified in 219 (35.38%) patients and noted for guidance only (e.g., child, adult) in another 12. The range was from 3 (SICILIANO, 1982) to 95 years of age (MALANDRINI *et al.,* 2009a) (average 37.09 ± 17.07 years) (Figure 2). In Argentina, the only random survey detected positivity from small children to old individuals, without age preference (MALANDRINI *et al.,* 2009a). This study was performed in an Andean locality in the department of Tinogasta, province of Catamarca. This absence of age correlation contrasts with other countries, where prevalence and intensities peak in the 9-11 age group, although adults and old age groups may also show high infection rates, as is the case of Bolivia (ESTEBAN *et al.,* 1997a,b, 1999) Perú (ESTEBAN *et al.,* 2002) and Egypt (ESTEBAN *et al.,* 2003). However, no differences between age groups were found in Chile (APT *et al.,* 1993) or Iran (MOGHADDAM *et al.,* 2004).

In Argentina, several outbreaks presenting typical food borne characteristics appear related to the most common risk factor: ingestion of watercress naturally growing along the river- and stream-beds picked during recreational, weekend or vacation activities. Many of these field excursions are undertaken by a family or as a group activity. This explains why family outbreaks have been noted to be common, whereas isolated cases seem to be rare in the country (NIETO SOSA *et al.,* 2008). Outbreaks described in eleven families involving a total of 63 people (SICILIANO *et al.,* 1989; NIETO SOSA *et*

al., 2008; SALOMON *et al*. 2006; OSSOLA *et al.,* 1972; PEIRETTI & MORALES, 1973; MINOPRIO *et al.,* 1995; BACIGALUPO, 1942), including a maximum of up to 15 family members affected at once (NIETO SOSA *et al.,* 2008) are good examples. However, results obtained in a recent serological survey of a local resident population in Catamarca (MALANDRINI *et al.,* 2009a) illustrate that not all situations including several infected subjects are in fact family outbreaks.

2.3.3.- BIOGEOGRAPHICAL ASPECTS

2.3.3.1.- GEOGRAPHICAL DISTRIBUTION ACCORDING TO PROVINCES

The geographical origin of the patients was specified in 587 patients (94.83%), whereas in 32 cases (5.17%) not even the province of origin was noted. Sometimes only the province where the infection occurred was mentioned, in other instances the specific locality was also added.

Human cases have been found in 13 out of the total of 23 provinces plus Buenos Aires capital, covering more than half (51.48%) of the total surface of continental Argentina. Córdoba, Catamarca, San Luis and Mendoza include the largest number of patients detected, the remaining provinces being only rarely affected (Table 3, Figure 3). Human infection has most probably been completely overlooked in La Rioja and San Juan provinces, with no case reported despite being surrounded by the aforementioned provinces. Adequate studies are needed to assess whether human infection occurs in La Rioja and San Juan, given their physiographic and climatic characteristics, which suggest infection risks similar to those in the aforementioned neighbouring ones.

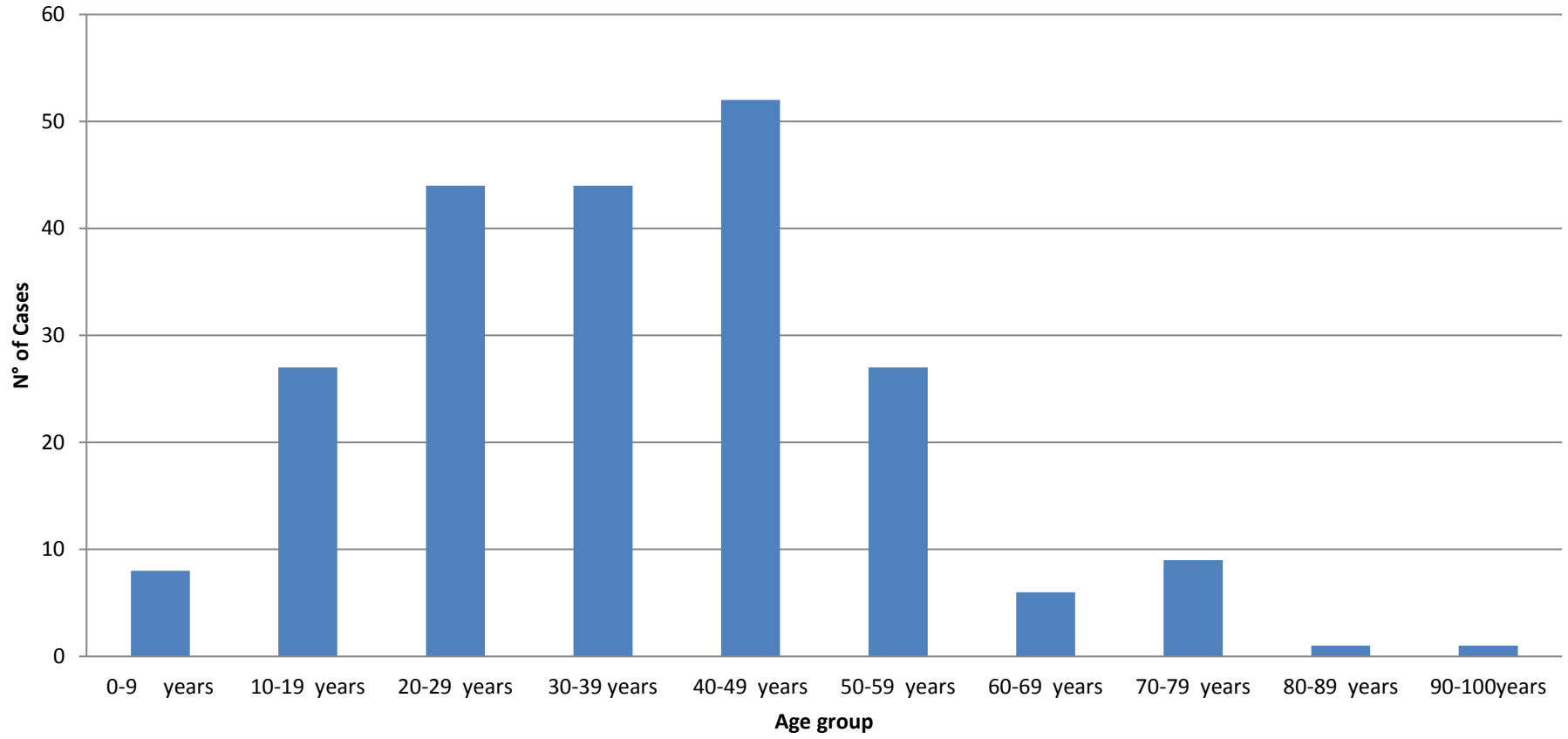


Fig. 2.- Age of fascioliasis patients. Age distribution in 219 patients in whom fascioliasis was diagnosed (MERA Y SIERRA *et al*., 2011).

The pronounced concentration of cases in the Córdoba and Andean areas is worth mentioning, although not in absolute numbers, at least with regard to the proportion of human case distribution. Unfortunately, human community surveys (active detection) have not been undertaken. All reports concern symptomatic subjects who voluntarily seek medical assistance (passive detection), except the only survey performed (MALANDRINI *et al*., 2009a) whose results increased the human case number for Catamarca province from 9 to 73.

The very high case number in Córdoba results from patients diagnosed by different physicians throughout many decades (see Table 3), mainly at the end of the 50s and beginning of the 60s, as noted in the compilation, made by one active Cordobese author, of the many patients diagnosed by himself plus the 150 patients diagnosed by other Cordobese colleagues, and which he presented at the Primer Congreso Médico Sanitario de la Provincia de Córdoba, held in La Falda on August 1957 (RODRIGUEZ, 1961). Although of course such a pronounced case number difference when comparing Córdoba with other provinces may in part be the consequence of a bias due to the absence of similarly actively publishing authors in the other provinces, the very high case number in Córdoba merits an analysis. The literature review suggests an explanation related to the very large number of villages and towns playing an important role in recreational, weekend or holiday activities. These recreational areas attended by thousands of tourists, campers or weekend visitors overlap with areas in which lymnaeids and animal fascioliasis are present (Figure 4) (NIETO SOSA *et al.,* 2008). In such places, the infection risk for a large amount of people is greater than in scarcely inhabited Andean areas. Moreover, the social, cultural and economic level of people spending holidays in such areas is high and the likelihood for them to seek appropriate medical assistance and obtain a correct diagnosis is greater than for people living in rural areas.

This highlights that people may become infected in a place different, sometimes even far or very far away, from the place where they live. Several family outbreaks described in Córdoba province support this assumption: the first such outbreak in La Calera (BACIGALUPO, 1942), another family infected during a picnic, another during a few days camping, another family buying uninspected watercress sold by a street vendor (OSSOLA *et al.,* 1972), and finally an eight-member family in La Punilla (NIETO SOSA *et al.,* 2008). In San Luis province, an outbreak involved two families that camped together in El Volcán (PEIRETTI & MORALES, 1973), and another affected 15 family members in Merlo (NIETO SOSA *et al.,* 2008). Similar family outbreaks occurred in

Mendoza province, one involving five members (MINOPRIO *et al*., 1995) and another with five members infected during a trip to the Andean region of San Carlos (SALOMON *et al*., 2006; LLORET *et al*., 2005).

2.3.3.2.- DISTRIBUTION ACCORDING TO ALTITUDE

Even though human infection risk is present in many geographical regions of the country, data indicate higher probabilities in given high altitude areas. Indeed, the great majority of cases including information on infection place (574: 97.79%) are from hilly or mountainous areas. Human reports appear concentrated in: (i) the central mountainous areas of Córdoba and San Luis, and (ii) the Andes mountains, mainly in Andean valleys.

Córdoba, the province with the greatest amount of cases, is a good example, with practically all human cases coming from its western mountainous areas (Sierras de Córdoba), despite higher precipitation rates and livestock abundance in its eastern lowland plains. Nevertheless, a possible sample bias cannot be ruled out in this case concentration due to the extensive patient record publication by two local authors (RODRIGUEZ, 1952, 1954, 1961; SICILIANO, 1982; SICILIANO *et al.,* 1989). Similar situations are found in San Luis and Mendoza provinces, both with all human cases from their western mountainous areas, instead from their eastern plains.

Only 13 cases (2.21%) originated from areas near sea level and flat terrain, namely from Buenos Aires province and city, Santa Fe, Chaco and Formosa. These few reports were all related to important rivers. One interesting case was a patient from Buenos Aires city who declared not having left the city in the previous 17 years (CACERES, 1955).

Such a case concentration in mountainous areas is not unlike Bolivia, Perú and Chile, where human endemic areas are linked to altitude areas, as a consequence of both (i) geographical distribution of the main lymnaeid vectors involved in transmission to humans restricted to or preferring such altitude areas (MAS-COMA *et al.,* 1999c), and (ii) the greater liver fluke transmission capacity in high altitude areas (MAS-COMA *et al.,* 2001). This suggests the appropriateness of verifying whether human fascioliasis endemic situations may also exist in high altitude areas of Argentina.

2.3.3.3.- LINKS TO LIVESTOCK INFECTION

The distribution of human infection does not appear to fit the one of animal fascioliasis, which covers the whole country according to official slaughterhouse records (Figure 3). In this respect certain areas are worthy of note, such as Corrientes province with absolutely no human case reported in the literature but high prevalence in livestock (MORIENA *et al.,* 2001, 2002; LOMBARDERO *et al.,* 1979a,b), and Neuquén province with only two human cases reported (RUBEL *et al.,* 2005; RIOS *et al.,* 2008) despite a very high prevalence in cattle (KACZORKIEWICZ, 1983).

A similar lack of geographical fit between human and animal fascioliasis has already been seen in other countries (MAS-COMA *et al.,* 2005, 2009a; BARGUES *et al*., 2017). Unfortunately, in Argentina it becomes impossible to ascertain whether this is the real situation or only a distorted picture due to incomplete data. Lack of appropriate human community surveys in areas with high prevalences in animals, absence of human-case reporting due to non-obligatory declaration, and overlooked human infection related to misdiagnosis or to inhabitants of rural areas not attending health centres for diagnosis may explain such situations.

However, the lack of geographical fit in question may also be due to an altitude factor, in its turn related to both (i) altitudinal selection of lymnaeid vector species with ecological characteristics adequate for transmission to humans and (ii) altitudinal climatic factors enhancing *F. hepatica* life cycle development, as already demonstrated in other Andean countries (MAS-COMA *et al.,* 2001; ESPINOZA *et al*., 2007). Concentration of human cases in hilly or mountainous areas supports this altitude explanation.

2.3.3.4.- SEASONALITY

Both fasciolid life cycle and lymnaeid population dynamics are markedly dependent on climate, mainly temperature and rainfall (FUENTES *et al.,* 1999; MAS COMA *et al*., 2008, 2009b; OLLERENSHAW, 1971). This climatic influence is evidenced by three different transmission patterns which define human and animal infection characteristics

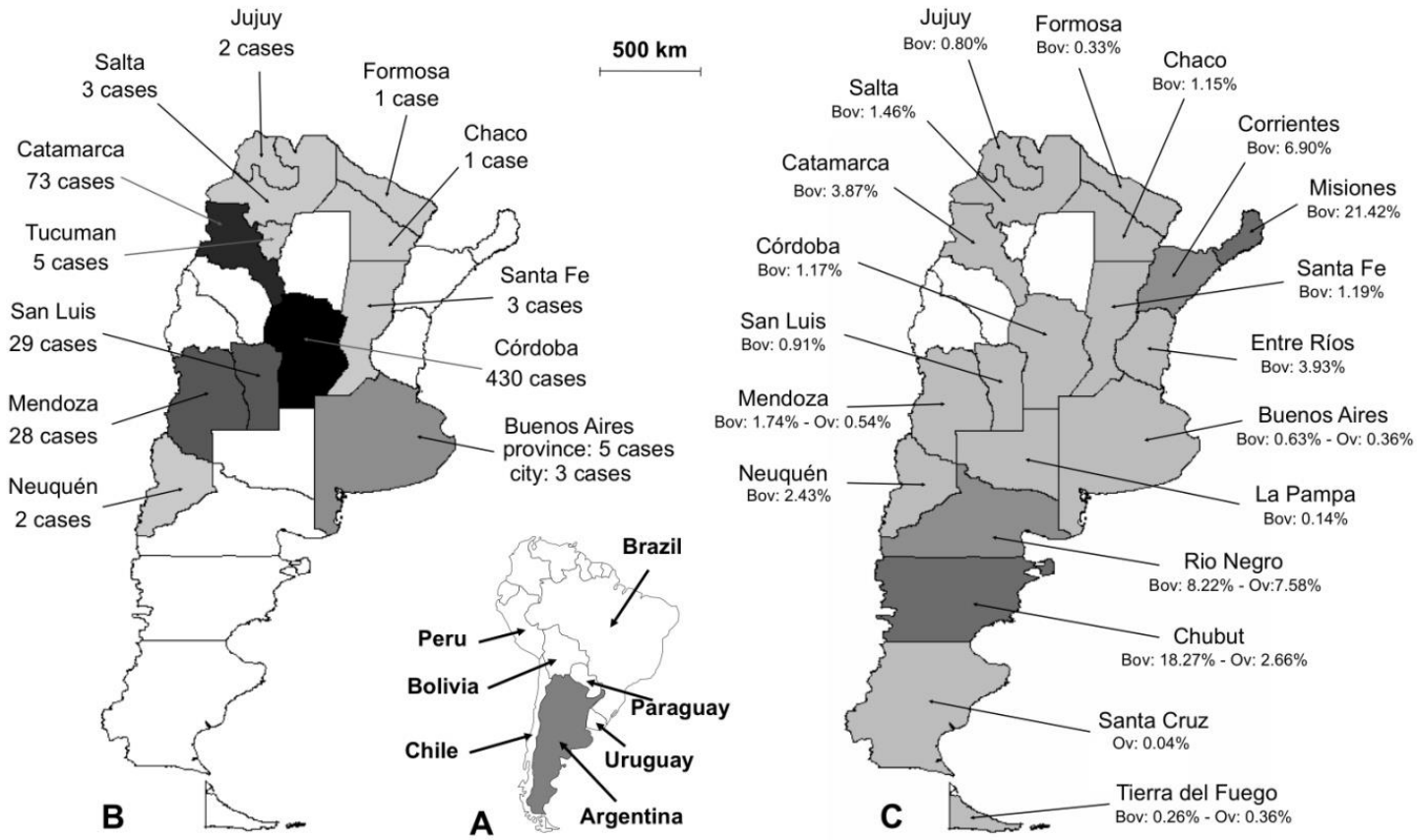


Fig. 3.- Geographical distribution of fascioliasis in Argentina. A) country location in the southern cone of South America; B) distribution of human fascioliasis infection reports (number of cases), according to provinces where infection was presumed to have occurred; C) distribution of fascioliasis in livestock including province prevalences according to slaughterhouse condemnation data for the 2006-2009 period provided by the Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA) (bov = bovines; ov = ovines) (MERA Y SIERRA *et al*., 2011).

(MAS-COMA, 2004; MAS-COMA *et al.,* 2005) monoseasonal, biseasonal and annual, permanent depending on the existence of one rainfall concentration period per year, two of them, or appropriate water body availability throughout the year (FUENTES *et al.,* 1999; MAS-COMA *et al.,* 1999c). Sometimes seasonality is related to the ingestion of contaminated plants, with most human cases occurring during the watercress season (MAS-COMA *et al.,* 1999b, 2018).

To estimate the moment when metacercariae were ingested, a prepatent period of 2-4 months should be considered. This overlaps with the acute phase, egg appearance in faeces marking the beginning of the chronic phase (MAS-COMA, 2004). For instance, in Europe, human infection takes place in summer and autumn and symptoms appear in winter, and a prolonged and wet summer has often been followed by an outbreak (MAS-COMA *et al.,* 1999b, 2018).

In Argentina, a total of 110 case reports were found in which the month of the first appearance of symptoms was noted. Most of them (97) corresponded to reports from Córdoba province, of which 93 had already been analysed from that point of view (SICILIANO, 1982).

A first pronounced January-April peak appears in the monthly distribution of these 110 cases. Interesting results arise when comparing case distribution with the annual distributions of mean monthly of precipitation (m.m.p.) and humidity (m.m.h.) and with mean monthly data of maximum (m.m.max.temp.) and minimum temperatures (m.m.min.temp.) for Córdoba province. There is a significant correlation when a 2 month time lag is applied between the presentation of the disease with m.m.p. (p=0.013) (Figure 4), m.m.max.temp. (p=0.008), and m.m.min.temp. (p=0.0069). These are even more significant when a 3 month time lag is applied (p values of 0.003, 0.0049 and 0.0035, respectively).

.

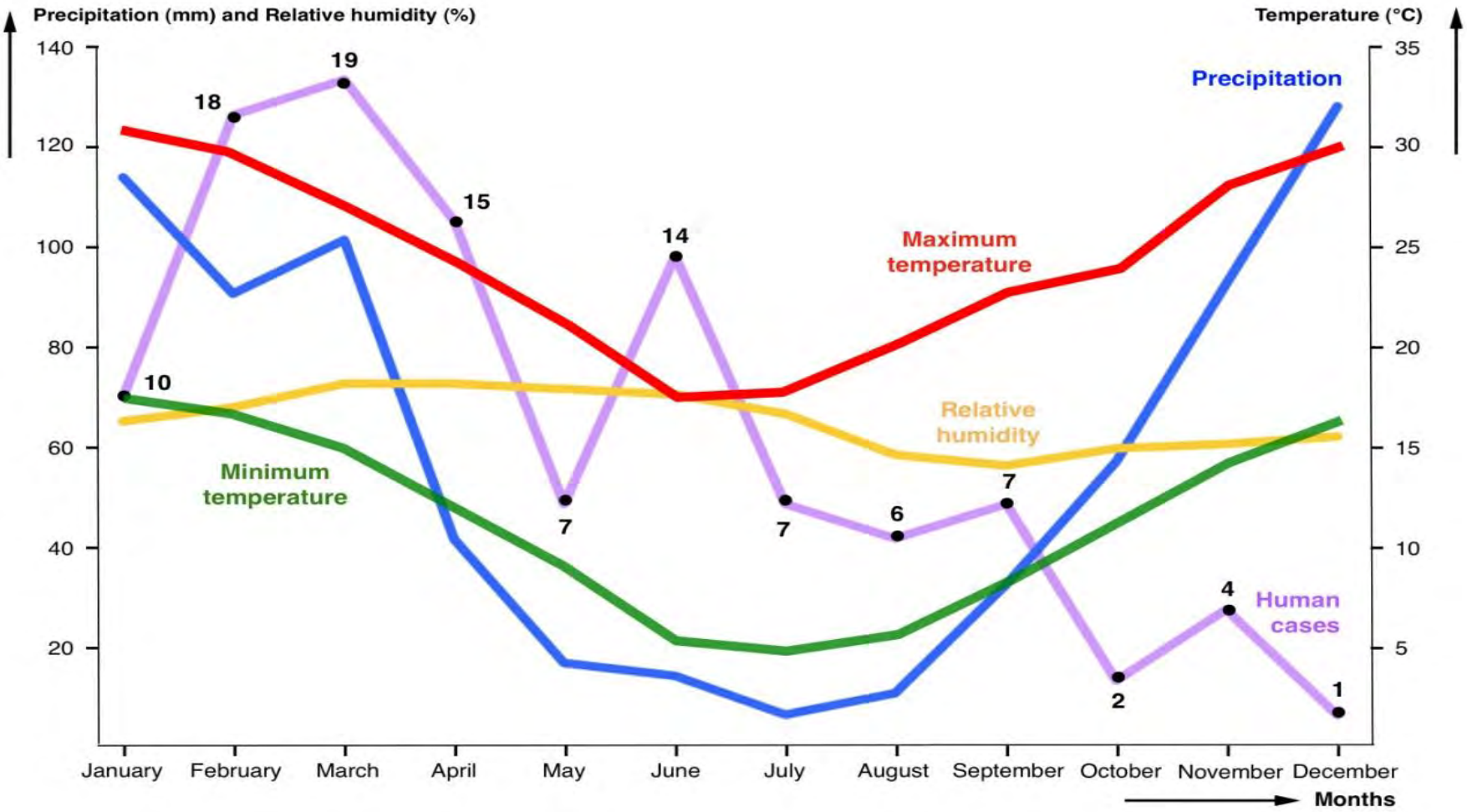


Fig. 4.- Human fascioliasis monthly incidence. Distribution of human fascioliasis incidence according to the month, in which symptoms appear, compared with the annual distributions of mean monthly data of precipitation, humidity, and maximum and minimum temperatures (MERA Y SIERRA *et al*., 2011).

Data concerns the province of Córdoba in the 1960's and 1970's when the patients were infected and 4 months (*p* values of 0.0389, 0.0293 and 0.0358, respectively) were considered. This fits with the logical delay between infection moment and symptom appearance and diagnosis. However, this largest peak of January-April may not only be due to higher precipitation and temperature data. Many people enjoy summer holidays in January and February in Córdoba province, so that the increase of recreational field activities may also interact with rainfall and temperature increases in inducing this monthly January-April incidence peak.

There is a second peak in June which poses a question mark, as no such climatic correlation appears. Other factors may be involved, such as the second yearly increase of recreational field activities during the Easter holidays. Metacercariae are known to keep their infectivity for months (even up to two years) under adequate environmental conditions (VALERO & MAS-COMA, 2000) so that remaining metacercariae, namely those already available but not having been ingested from January, may be the cause of that delayed incidence peak.

Human infection, mainly during the first months of the year, is also suggested in another 15 case reports from Córdoba, in which the first month with symptoms was unfortunately not specified (therefore not included in the aforementioned statistical analysis) but the season of autumn (i.e., 21 March to 21 June) was noted (SICILIANO *et al.,* 1989).

However, in Argentina other transmission patterns and human infection risk seasonality's/periodicities may not be ruled out according to the large physiographical heterogeneity of the different endemic areas of such a vast country.

2.3.3.5.- RELATIONSHIPS WITH ANNUAL CLIMATE CHANGES

The climatic dependence of fascioliasis is also known to modify the inter annual distribution of human case detection, with increases in years with heavy rainfall (MAS-COMA *et al.,* 1999b; MAS-COMA, 2004; AFSHAN *et al*., 2014).

When analyzing the evolution of the human case number in the whole country according to decades, one peak appears in the 1960s and another one in the 1980s (Figure 1). The annual human case number for a more detailed study was, however, only sufficient when restricted to Córdoba province during the 1960's and 1970's. The comparative analysis with annual precipitation (Figure 5) shows that the 1972 outbreak

may have been caused by the sudden increase of rainfall in that year in Córdoba province, as already highlighted a long time ago (SICILIANO, 1982). However, we could not find any significant correlation.

The most pronounced outbreak in 1977 does not appear to have any apparent climatic causal origin, although it was argued that this year was the one with the largest amount of precipitation during the 1973-1981 period (SICILIANO, 1982). We have confirmed that the rainiest year in the Córdoba area in question was 1978 and not 1977. Thus, this second human report increase may have been linked to a passing fad or temporary professional trend for reporting.

2.3.4.- SOURCES OF HUMAN INFECTION

Humans become infected when ingesting the metacercariae that are attached to edible and chewable plants or the floating metacercaria, either directly from drinking water or when using contaminated water to wash food or kitchen utensils (MAS-COMA *et al*., 2018). The ingestion of freshwater plants carrying infective metacercariae, amongst which preferentially watercress (*Nasturtium officinalis*), is known to constitute one of the most frequent fascioliasis infection source in humans (MAS-COMA, 2005; ASHRAFI *et al.,* 2006). The consumption of aquatic plants other than watercress, as described in the hyperendemic area of Bolivia (MAS-COMA *et al.,* 1995) and other endemic regions of the world, such as the Guilan province in Iran or south-eastern Asian countries is not a common practice in Argentina (MAS-COMA *et al*., 2018). Watercress is almost the only aquatic wild plant regularly consumed. In this country, with the highest beef consumption in the world, it is traditional to accompany barbecue meat (“asado”) with salad. When camping or doing outdoor activities, it is very common to go to a stream and collect watercress to cover for the absence of salads. Physicians should ask if the person has visited recreational waters in endemic areas, since it is frequent that the patients come in contact with the infection sources during vacation or recreational activities.

Previous watercress ingestion has been described in 214 patients. A relationship between watercress and fascioliasis was described already long ago (BENGOLEA *et al.,* 1927; BACIGALUPO *et al.,* 1930) and has recently been highlighted again in an editorial article (BARCAT, 2005). Amongst a total of 101 cases reported from Córdoba, 94 (93.07%) had a history of watercress consumption (SICILIANO, 1982).

Besides watercress, there are a very few reports which relate fascioliasis to other plants. In two cases from Córdoba province, ingestion of dandelion (*Taraxacum officinale*) was noted as a possible infection source (SICILIANO, 1982). Two other patients aren't mentioned to have chewed on grass that grew on a riverbank (BOTO, 1939).

Water consumption has also been involved as a human infection source (MAS-COMA, 2004). In Argentina, natural water was already presumed to be an infection source for livestock as early as two centuries ago (SAVOYAT, 1867). With regard to humans, contaminated water as a possible infection source was noted in only two patients who mentioned having drunk natural water from mountain streams (MINOPRIO *et al.,* 1995; BOTO, 1939).

2.3.5.- DIAGNOSTIC ASPECTS

2.3.5.1.- METHODOLOGICAL AND TECHNICAL PROBLEMS

The diagnosis of human fascioliasis poses well-known methodological and technical problems (ESTEBAN *et al*., 1998; HILLYER, 1999; MAS-COMA *et al*., 2005). The different epidemiological and clinical situations imply that the diagnostic methods have to be decided upon the particular circumstances. Since the incubation phase (few days to 2-3 months) is inferior to the prepatent period (three to four months) there will be many weeks in which the patient may show clinical signs yet the coprological results will be negative. In such cases, the serological tests become essential, yet, the usual situation is that these tests are not available in the endemic areas and have to be done in reference centers that are usually very far away with all the complications that this implies. There are even regions of the world that apparently common procedures such as obtaining blood or faecal samples is sometimes not viable. In Aymara communities in Bolivia there were serious problems to obtain blood samples and in regions of Argentina, such as Catamarca, the same is true for faecal samples (MAS-COMA *et al*., 2014).

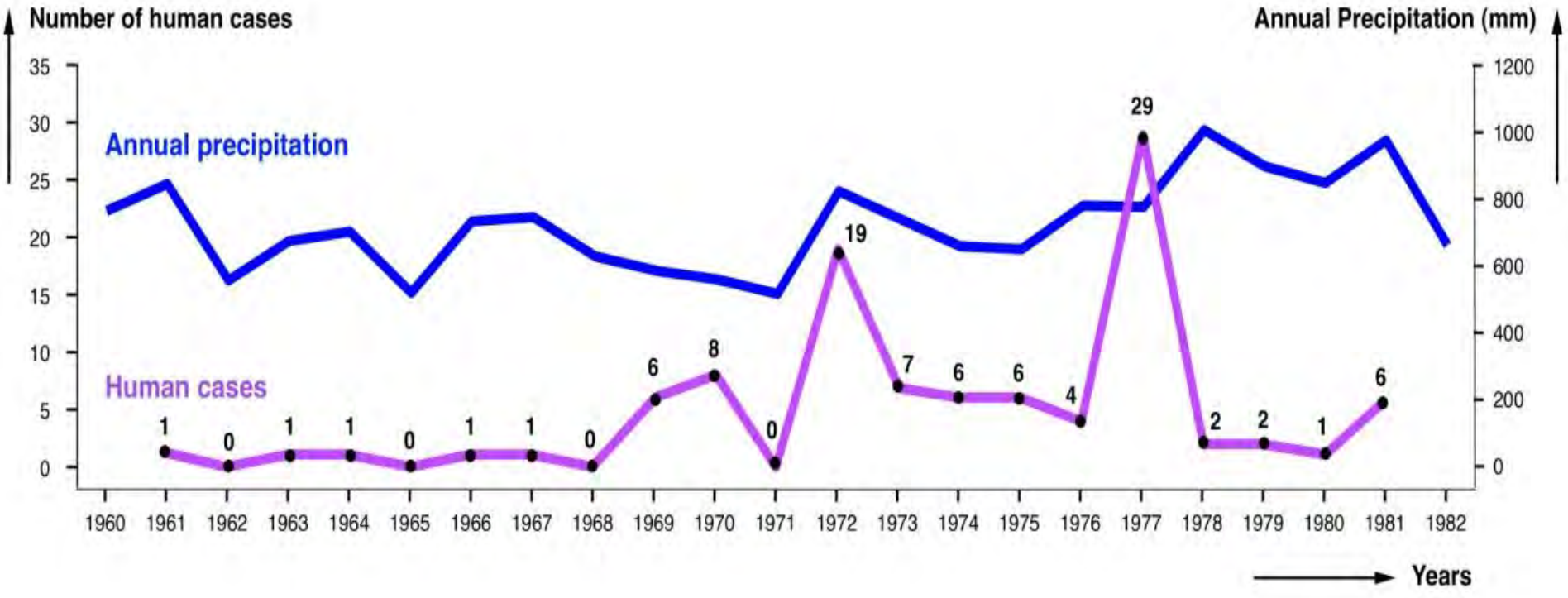


Fig. 5.- Human fascioliasis annual incidence. Distribution of human fascioliasis incidence according to the year, in which symptoms appear, compared with annual precipitation data. Data concerns the province of Córdoba in the 1960-1982 period during which the patients were infected (MERA Y SIERRA *et al*., 2011).

In Argentina, the diagnostic method used was specified in the majority of the human patient reports analysed (454 cases: 73.34%) (Table 2). In several cases, even more than one method/technique was used. The diagnostic method was not specified when referring to 150 unpublished cases detected by other colleagues (RODRIGUEZ, 1961) and it was insufficiently clarified in 15 other cases mentioned in an endnote as “patients having been diagnosed while in press” (STRADA, 1961).

Fascioliasis diagnosis in Argentina has usually relied on traditional techniques, mainly egg detection (288 positive patients), followed by common serological techniques (82), intradermal reaction (63), fluke finding during surgery (45), and erratic fluke observation (6). Sensitivity and specificity of these techniques are far from the standards in more modern ones (O’NEILL *et al.,* 1998; ESPINOZA *et al.,* 2007; UBEIRA *et al.,* 2009). Additionally, the absence of the use of quantitative methods, such as the standardized Kato-Katz technique to assess both pathogenicity and intensity for adequate treatment dose selection (WHO, 2007), is worth mentioning.

An historical account (see Table 2) shows that diagnosis by egg identification was the method used from the very beginning and, although still used in the last decade, surgical findings were common during the 1969-1989 period and serology from 2005 onwards. The intradermal test appears to have been used only during the 60’s and at the beginning of the 70’s and abandoned after 1982. Other methods based on clinical/epidemiological observations, non-invasive techniques, and ectopic presentations have been only sporadically applied.

The long delay with which many patients were diagnosed should be emphasized. In given reports on 26 patients, the time elapsed between the appearance of symptoms and confirmation of infection by appropriate diagnosis is noted in days or sometimes years. Calculated delay average is very high, of 1,262 days, nearly three and a half years, and there are references about patients having suffered from symptoms for ten or more years without diagnosis (BENGOLEA *et al.,* 1927; DEL VALLE & DONOVAN, 1928; CAMES, 1940, 1947; CID, 1947; SOLARI & CANEPA, 1943; GIFFONIELLO *et al.,* 1983;). This suggests either infected subjects not looking for professional diagnosis due to mild symptoms of low fluke burdens and/or misdiagnosis of patients due to the non-pathognomonic clinical picture, easily confused with other diseases when the patient attends a health centre not used to dealing with fascioliasis (LA POOK *et al.,* 2000).

2.3.5.2.- COPROLOGICAL ANALYSES

Coprological analyses were performed in 278 patients, including positive egg finding in 221 (79.50%). Details about the coprological technique used were very rarely described. Where noted, the Charles Barthelemy sedimentation technique, M.I.F., flotation, Baerman and multiple concentrations were mentioned, although with-out giving further specifications (RODRIGUEZ, 1961). Other techniques, such as the veterinary Lumbreras rapid sedimentation technique, (SICILIANO *et al.,* 1989) and those of Faust and Sheather (SALOMON *et al.,* 2006), have been also used in Argentina.

Duodenal sondage was performed in 90 patients, yielding egg detection in 67 (74.44%). Interestingly, nine cases appeared negative in a coprological examination, while positive in duodenal sondage.

Despite egg finding still being the gold standard today, problematic situations giving rise to overlooking an infection may be taken into account, such as (i) absence of egg shedding during the acute phase, (ii) no egg production by given fluke strains when in humans, and (iii) lack of sufficient sensitivity in light infections. These are common in human sporadic infections in animal endemic areas, as appears to be the case in many reports from Argentina, e.g. in travelers, weekend family outings, and tourists. Thus, the widespread use of an egg finding technique as the single method for patient diagnosis may have been the reason for overlooking human infection in Argentina in the past.

Unfortunately, no study on egg size variability in human stools has been performed in the country. Consequently, given the absence of *F. gigantica*, the recently corrected egg size ranges to be henceforth used in Argentina for *F. hepatica* are 100.6-162.2/65.9-104.6 µm in humans and 73.8-156.8/58.1-98.1 µm in animals (VALERO *et al*., 2009a) which are pronouncedly different from the range of 130-150/63-90 µm previously used both for humans and animals worldwide.

2.3.5.3.- SEROLOGY

Its use in Argentina has been reported only recently, despite serological techniques having been available for this disease for a long time (HILLYER, 1999). However, an ELISA developed locally by means of recombinant procathepsin L cystein proteinase was successfully assayed in test serum samples from 16 coprological positive patients

(CARNEVALE *et al.,* 2001a), and a Micro-ELISA method showed a sensitivity of 100% and a specificity of 97% when applied to 22 test serum samples from patients with fascioliasis infection previously verified by coprology, surgical observation or retrograde cholangiopancreatography, thus proving to be highly useful, mainly for the previous screening of a large amount of samples (CARNEVALE *et al.,* 2001b).The technique specified was such an ELISA in 69 cases (RUBEL *et al.,* 2005; SALOMON *et al.,* 2006; RIOS *et al.,* 2008; MALANDRINI *et al.,* 2009a,b). In another case, an ELISA without further details specified was employed (CORTI *et al.,* 2006).

Table 2.- Human fascioliasis reports in Argentina, arranged chronologically, including details on infection according to number of cases, gender, province, diagnostic method, treatment, and clinical data (MERA Y SIERRA *et al*., 2011).

Year	Author	N° of cases	Gender	Province	Diagnostic Method	Treatment	Clinical Data
1924	Greenway	1	$NS_{(1)}$	$NS_{(1)}$	$Ec_{(1)}$	$NS_{(1)}$	$NS_{(1)}$
1927	Bengolea *et al*.	1	$F_{(1)}$	$SL_{(1)}$	$Ec_{(1)}$ $Es_{(1)}$	$NS_{(1)}$	$AP_{(1)}$
1928	Del Valle & Donovan	1	$F_{(1)}$	$NS_{(1)}$	$Surg_{(1)}$	$NS_{(1)}$	$AP_{(1)}$ $Nau_{(1)}Ic_{(1)}$ $HA_{(1)}$
1930	Bacigalupo *et al*.	1	$F_{(1)}$	$SL_{(1)}$	$Ec_{(1)}Es_{(1)}$	$NS_{(1)}$	$NS_{(1)}$
1933	Mascheroni	1	$F_{(1)}$	$BA_{(1)}$	$Ec_{(1)}$ $Es_{(1)}$	$NS_{(1)}$	$NS_{(1)}$
1933	Scrimaglio (In Bacigalupo *et al*. 1943)	1	$M_{(1)}$	$SFe_{(1)}$	$Ec_{(1)}$ $Es_{(1)}$	$NS_{(1)}$	$NS_{(1)}$
1937	Castex & Greenway	1	$M_{(1)}$	$Cba_{(1)}$	$Ec_{(1)}$ $Ect_{(1)}$	$NS_{(1)}$	$AP_{(1)}$ $Lks_{(1)}$
1939	Boto	1	$F_{(1)}$	$Tuc_{(1)}$	$Es_{(1)}$	$Em_{(1)}$	$Eo_{(1)}AP_{(1)}Fev_{(1)}Lks_{(1)}Urt_{(1)}$
1940	Cames	2	$M_{(2)}$	$Cba_{(1)}$ $SFe_{(1)}$	$Es_{(1)}$ $Surg_{(1)}$	$Em_{(2)}$ $MFE_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$ $Fev_{(2)}$ $WL_{(1)}$ $Ic_{(1)}$
1942	Bacigalupo	$5_{(fo1)}$	$F_{(2)}$ $M_{(3)}$	$Cba_{(4)}$ $BA_{(1)}$	$Ec_{(2)}$ $Es_{(1)}$ $CE_{(2)}$	$Em_{(4)}$ $NS_{(1)}$	$Eo_{(4)}$ $AP_{(4)}$ $Fev_{(3)}$ $WL_{(1)}$
1943	Solari & Canepa	1	$F_{(1)}$	$Cba_{(1)}$	$Es_{(1)}$	$Em_{(1)}$	$AP_{(1)}$
1943	Bacigalupo *et al*.	1	$M_{(1)}$	$Cba_{(1)}$	$Ect_{(1)}$	$Em_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$
1944	Cuenya	3	$F_{(1)}$ $M_{(2)}$	$Tuc_{(1)}$ $Ju_{(1)}$ $Sal_{(1)}$	$Ec_{(3)}$ $Es_{(1)}$	$NS_{(3)}$	$AP_{(1)}$
1952	Rodríguez	16	$F_{(3)}$ $M_{(1)}$ $NS_{(12)}$	$Cba_{(15)}$ $Cat_{(1)}$	$Ec_{(16)}$	$NS_{(16)}$	$Eo_{(5)}$ $Ap_{(1)}$ $Lks_{(4)}$ $Ic_{(1)}$

Table 2. Continuation

Year	Author	N° of cases	Gender	Province	Diagnostic Method	Treatment	Clinical Data
1953	Longo & Daraio	1	$F_{(1)}$	$Sal_{(1)}$	$Surg_{(1)}$ $NI_{(1)}$	$Em_{(1)}$	$AP_{(1)}$ $Urt_{(1)}$ $Nau_{(1)}$ $Lith_{(1)}$ $Vo_{(1)}$
1954	Petraglia	1	$M_{(1)}$	$Cha_{(1)}$	$Ec_{(1)}$	$Em_{(1)}$	$AP_{(1)}$ $Eo_{(1)}$ $Fev_{(1)}$
1954	Rodríguez	10	$F_{(1)}$ $M_{(2)}$ $NS_{(7)}$	$Cba_{(9)}$ $Cat_{(1)}$	$Ec_{(10)}$	$NS_{(10)}$	$Eo_{(4)}$ $Lks_{(1)}$
1955	Cáceres	1	$F_{(1)}$	$BAcity_{(1)}$	$Surg_{(1)}$	$Em_{(1)}$	$AP_{(1)}$ $Fev_{(1)}$ $Ic_{(1)}$ $Lith_{(1)}$ $Vo_{(1)}$
1955	Logaldo	1	$F_{(1)}$	$Mza_{(1)}$	$Surg_{(1)}$	$Em_{(1)}$	$Ap_{(1)}$ $Nau_{(1)}$ $Lith_{(1)}$
1961	Ahualli & Arias	1	$F_{(1)}$	$Tuc_{(1)}$	$Es_{(1)}$	$Em_{(1)}$	$AP_{(1)}$ $WL_{(1)}$ $Nau_{(1)}$ $Vo_{(1)}$ $HA_{(1)}$ $Dia_{(1)}$
1961	Rodriguez	23	$NS_{(23)}$	$Cba_{(22)}$ $Cat_{(1)}$	$Ec_{(23)}$	$NS_{(23)}$	NS
1961	“Other authors” cited in Rodriguez 1961	150	$NS_{(150)}$	$Cba_{(150)}$	$NS_{(150)}$	$NS_{(150)}$	NS
1961	Strada	19	$F_{(1)}$ $M_{(1)}$ $NS_{(17)}$	$Cba_{(19)}$	$Ec_{(1)}$ $Es_{(2)}$ $Surg_{(1)}$ $NS_{(15)}$	$Em_{(2)}$ $NS_{(17)}$	$Eo_{(2)}$ $AP_{(3)}$ $Fev_{(3)}$ $Lks_{(1)}$ $WL_{(1)}$ $Vo_{(1)}$ $HA_{(1)}$
1962	Urrutia & Ferraris (in Correa *et al* 1969	1	$NS_{(1)}$	$NS_{(1)}$	$Surg_{(1)}$	$NS_{(1)}$	NS
1964	Cornejo & Castillo	1	$M_{(1)}$	$Sal_{(1)}$	$Ec_{(1)}$ $Es_{(1)}$	$Em_{(1)}$	$Eo_{(1)}$ $Lks_{(1)}$
1964	Simon *et al.*	1	$M_{(1)}$	$Mza_{(1)}$	$Es_{(1)}$ $ID_{(1)}$	$Em_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$ $Fev_{(1)}$ $Lks_{(1)}$ $WL_{(1)}$ $Vo_{(1)}$
1964	Cañas *et al.*	1	$M_{(1)}$	$Ju_{(1)}$	$Es_{(1)}$	$Em_{(1)}$	$AP_{(1)}$ $Asth_{(1)}$ $Nau_{(1)}$ $Dia_{(1)}$ $Cst_{(1)}$

Table 2. Continuation

Year	Author	N° of cases	Gender	Province	Diagnostic M*ethod*	Treatment	Clinical Data
1965	Niño	4	$NS_{(4)}$	$BAcity_{(2)}$ $NS_{(2)}$	$Ec_{(2)}$ $Es_{(2)}$	$NS_{(4)}$	NS
1967	Sosa & Romero	2	$F_{(1)}$ $M_{(1)}$	$Cba_{(2)}$	$Es_{(2)}$	$Em_{(2)}$	$Eo_{(2)}$ $AP_{(1)}$ $Fev_{(2)}$ $WL_{(1)}$ $Nau_{(1)}$ $Vo_{(1)}$ $Dia_{(1)}$
1969	Peiretti *et al*.	17	$NS_{(17)}$	$Mza_{(15)}$ $SL_{(2)}$	$Es_{(5)}$ $ID_{(17)}$	$NS_{(17)}$	NS
1969	Trossero & Nocetti	1	$F_{(1)}$	$SFe_{(1)}$	$Surg_{(1)}$	$Em_{(1)}$	$AP_{(1)}$ $Fev_{(1)}$ $Ic_{(1)}$ $Vo_{(1)}$
1969	Correa *et al*.	1	$F_{(1)}$	$Cba_{(1)}$	$Surg_{(1)}$ $Ect_{(1)}$	$NS_{(1)}$	$AP_{(1)}$ $Fev_{(1)}$ $Urt_{(1)}$ $Nau_{(1)}$ $Ic_{(1)}$ $Vo_{(1)}$ $HA_{(1)}$
1970	Padilla Antoni *et al*.	1	$NS_{(1)}$	$Tuc_{(1)}$	$Surg_{(1)}$	$NS_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$ $Vo_{(1)}$ $HA_{(1)}$
1972	Carena *et al*.	13	$F_{(4)}M_{(9)}$	$Cba_{(11)}$ $Cat_{(1)}$ $Tuc_{(1)}$	$Es_{(13)}$	$Em_{(13)}$	$Eo_{(13)}$ $AP_{(8)}$ $Fev_{(1)}$ $Asth_{(3)}$ $Urt_{(2)}$ $HA_{(2)}$ $Cst_{(1)}$ $Dia_{(1)}$
1972	Ossola *et al*.	$12_{(fo3)}$	$F_{(7)}$ $M_{(5)}$	$Cba_{(12)}$	$Ec_{(2)}$ $Es_{(2)}$ $ID_{(12)}$	$NS_{(12)}$	$Eo_{(11)}$ $AP_{(12)}$ $Fev_{(9)}$ $WL_{(8)}$ $Asth_{(10)}$ $Urt_{(4)}$ $Ic_{(3)}$ $Cst_{(3)}$ $Dia_{(2)}$
1973	Sonzini Astudillo *et al*.	5	$F_{(4)}$ $M_{(1)}$	$NS_{(5)}$	$Es_{(1)}$ $Surg_{(4)}$	$Em_{(1)}$ $NS_{(4)}$	$Eo_{(1)}$ $AP_{(4)}$ $Asth_{(1)}$ $Lith_{(3)}$ $Ic_{(4)}$ $HA_{(1)}$
1973	Peiretti & Morales	$4_{(fo1)}$	$F_{(1)}$ $M_{(3)}$	$SL_{(4)}$	$ID_{(4)}$	$Em_{(4)}$	$Eo_{(4)}$ $AP_{(4)}$ $Fev_{(2)}$ $Lks_{(2)}$ $WL_{(1)}Ic_{(1)}$ $Cst_{(1)}$ $Vo_{(1)}$
1981	Majul *et al*.	6	$NS_{(6)}$	$NS_{(6)}$	$Es_{(2)}$ $Surg_{(6)}$	$Em_{(6)}$	$Eo_{(2)}$ $AP_{(6)}$ $Lith_{(6)}$
1981	Alaggia (in Andrada *et al.,* 1983)	16	$NS_{(16)}$	$NS_{(16)}$	$Surg_{(16)}$	$NS_{(16)}$	SD
1982	Pizzi *et al*.	54	$NS_{(54)}$	$Cba_{(54)}$	$Ec_{(54)}$	$NS_{(54)}$	$NS_{(54)}$

Table 2. Continuation

Year	Author	N° of cases	Gender	Province	Diagnostic M*ethod*	Treatment	Clinical Data
1982	Siciliano	101	$F_{(50)}$ $M_{(51)}$	$Cba_{(101)}$	$Ec_{(61)}$ $Es_{(16)}$ $ID_{(29)}$	$Em_{(97)}$ $Clq_{(4)}$	$Eo_{(87)}$ $AP_{(78)}$ $Fev_{(58)}$ $Lks_{(69)}$ $WL_{(56)}$ $Anrx_{(53)}$ $Asth_{(67)}$ $Urt_{(37)}$ $Nau_{(31)}$
1983	Andrada *et al*.	5	$F_{(5)}$	$Cat_{(5)}$	$Surg_{(4)}$ $NI_{(5)}$	$Em_{(5)}$	$Eo_{(3)}$ $AP_{(5)}$ $Urt_{(1)}$ $Ic_{(2)}$ $Lith_{(2)}$
1983	Giffoniello *et al*.	2	$F_{(1)}$ $M_{(1)}$	$Cba_{(2)}$	$Es_{(1)}$ $Surg_{(1)}$ $NI_{(1)}$	$Em_{(2)}$	$Eo_{(2)}$ $AP_{(2)}$ $Fev_{(2)}$ $WL_{(1)}$ $Asth_{(1)}$ $Lith_{(1)}$ $Vo_{(1)}$ $HA_{(1)}$
1985	Miguel *et al*.	5	$NS_{(5)}$	$BA_{(3)}$ $Mza_{(1)}$ $For_{(1)}$	$Es_{(5)}$	$Em_{(5)}$	$Eo_{(1)}$ $AP_{(5)}$ $Lks_{(4)}$ $Ic_{(2)}$
1989	Siciliano *et al.*	$15_{(fo2)}$	$F_{(9)}$ $M_{(5)}$ $NS_{(1)}$	$Cba_{(15)}$	$Ec_{(12)}$ $Es_{(1)}$ $Surg_{(2)}$	$Em_{(15)}$	$Eo_{(14)}$ $AP_{(15)}$ $Fev_{(15)}$ $Lks_{(10)}$ $Urt_{(8)}$ $Ic_{(2)}$
1991	Melero *et al*.	1	$M_{(1)}$	$SL_{(1)}$	$Es_{(1)}$ $NI_{(1)}$	$Tcl_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$ $Fev_{(1)}$ $Lks_{(1)}$ $WL_{(1)}$
1995	Minoprio *et al*.	$5_{(fo1)}$	$F_{(3)}$ $M_{(2)}$	$Mza_{(5)}$	$Ect_{(1)}$ $CE_{(4)}$ $NI_{(3)}$	$Tcl_{(5)}$ $Pzq_{(1)}$	$Eo_{(3)}$ $AP_{(2)}$ $Fev_{(2)}$ $Lks_{(1)}$ $Asth_{(2)}$ $Urt_{(3)}$ $Ic_{(1)}$
2000	Ale *et al*.	1	$F_{(1)}$	$SL_{(1)}$	$Ec_{(1)}$	$NS_{(1)}$	$Eo_{(1)}$ $Lks_{(1)}$
2005	Carnevale (in Rubel *et al*.	4	$NS_{(4)}$	$SL_{(4)}$	$Ser_{(4)}$	$NS_{(4)}$	NS
2005	Rubel *et al*.	1	$F_{(1)}$	$Neu_{(1)}$	$Ser_{(1)}$	$Tcl_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$ $Fev_{(1)}$ $Lks_{(1)}$
2006	Salomon *et al*.	$5_{(fo1)}$	$NS_{(5)}$	$Mza_{(5)}$	$Ec_{(2)}$ $Es_{(1)}$ $Ser_{(2)}$ $CE_{(2)}$	$NS_{(5)}$	$Eo_{(5)}$ $Lks_{(5)}$
2006	Corti *et al*.	1	$F_{(1)}$	$Cba_{(1)}$	$Ser_{(1)}$ $NI_{(1)}$	$Tcl_{(1)}$	$Eo_{(1)}$ $Fev_{(1)}$

Table 2. Continuation

Year	Author	N° of cases	Gender	Province	Diagnostic Method	Treatment	Clinical Data
2008	Rios *et al*.	1	$M_{(1)}$	$Neu_{(1)}$	$Ec_{(1)}$ $Ser_{(1)}$	$Alb_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$ $Fev_{(1)}$ $Lks_{(1)}$ $Asth_{(1)}$ $Urt_{(1)}$
2008	Nieto Sosa *et al*.	$23_{(fo2)}$	$NS_{(23)}$	$Cba_{(8)}$ $SL_{(15)}$	$Ec_{(23)}$ $Ser_{(9)}$	$Em_{(15)}$ $Tcl_{(8)}$	$Eo_{(23)}$ $AP_{(23)}$ $Fev_{(23)}$ $Lks_{(23)}$
2009	Malandrini *et al*.	54	$F_{(37)}$ $M_{(17)}$	$Cat_{(54)}$	$Ser_{(54)}$	$NS_{(54)}$	NS
2009	Malandrini *et al*.	10	$NS_{(10)}$	$Cat_{(10)}$	$Ser_{(10)}$	$NS_{(10)}$	NS

Abbreviations: F: female; M: male; NS: not specified; Fo: family/group outbreaks.
Provinces: BA: Buenos Aires; BAcity: Buenos Aires city; Cat: Catamarca; Cha: Chaco; Cba: Córdoba; For: Formosa; Ju: Jujuy; Mza: Mendoza; Neu: Neuquén; Sal: Salta; SL: San Luís; SFe: Santa Fé; Tuc: Tucumán.
Treatment: Em: emetine; Tcl: triclabendazole; Alb: albendazole; MFE: male fern extract; Pzq: praziquantel; Clq: Cloroquine.
Diagnostic Method: Ec: egg observation in coprological sample; Es: egg observation in sample obtained by sondage; ID: intradermal test; Surg: surgical; CE: clinical/epidemiological; Ect: ectopic presentation; NI: non invasive image-based diagnosis; Ser: serology.
Clinical Data: AP: abdominal pain; Anrx: anorexia; Asth: asthenia; Cst: Constipation; Dia: diarrhea; Eo: eosinophilia; Fev: fever; HA: headache; Ic: ictericia; Lks: leucocitosis; Lith: lithiasis; Nau: nausea; Urt: urticaria; Vo: vomiting; WL: weight loss.
Note: number of cases noted in parenthesis.

In 13 patients the serological technique applied was not mentioned (RIOS *et al.,* 2008; NIETO-SOSA *et al.,* 2008). In three patients diagnosed by coprology or duodenal sondage, serology appeared positive, being negative in two other patients in whom eggs were not found (SALOMON *et al.,* 2006). Surprisingly, when applying serology to 11 patients shedding eggs in their stools, only nine of them gave a positive serological result (NIETO SOSA *et al.,* 2008). The two coprological positive although serologically negative patients suggest either spurious cases (fluke eggs in transit after infected livestock liver ingestion) or lack of sufficient sensitivity of the serological test applied. The opposite, that is negative coprology and positive serology, was found in another case (CORTI *et al.,* 2006). In many human case reports, serology was the sole diagnostic method used (RUBEL *et al.,* 2005; MALANDRINI *et al.,* 2009a,b).

Serological diagnosis was mentioned to have been used in the only article dealing with a random human survey performed in Argentina up to the present. In 148 randomly selected subjects from Tinogasta, province of Catamarca, ELISA was positive in 54 of whom (36.48%), as well as in ten amongst other 14 patients from the same locality (MALANDRINI *et al*., 2009a,b).

2.3.5.4.- INTRADERMAL TESTS

This diagnostic method, is today known to be insufficiently specific (ESTEBAN *et al.,* 1998) and considered obsolete for the diagnosis of individual patients, although still potentially useful as a quick indicator within broad field screening. It seems to have been quite commonly used in Argentina in the sixties, seventies and eighties (in chronological order): 1 case (SIMON *et al.,* 1964), 17 cases (PEIRETTI, 1969), 12 cases (OSSOLA *et al.,* 1972), 4 cases (PEIRETTI & MORALES, 1973), and 29 cases (SICILIANO, 1982). Since there has never been a commercial or standardized test, details on antigen and correlation with other diagnostic tests noted in the aforementioned articles need to be taken into account.

2.3.5.5.- DIAGNOSIS BY NON-INVASIVE TECHNIQUES

Many image-based diagnostic techniques are useful for fascioliasis (CHEN & MOTT, 1990; ESTEBAN *et al.,* 1998; MAS-COMA *et al.,* 1999b; MAS-COMA *et al*., 2014) and

have also been applied in Argentina since the eighties, although mainly for initial detection followed by confirmation by another more specific diagnostic technique (LONGO & DARAIO, 1953; ANDRADA *et al.,* 1983; GIFFONIELLO *et al.,* 1983; MELERO *et al.,* 1991; MINOPRIO *et al.,* 1995; CORTI *et al.,* 2006).

2.3.5.6.- ECTOPIC CASES

In a 25 year old male patient, a fluke was eliminated through the urethra (CASTEX & GREENWAY, 1937). In a female patient undergoing surgery for appendicitis, a fluke was removed from the appendix (PALADINO & GALARCE, 1039). A *F. hepatica* specimen was found when surgically opening a tumor at the level of the last rib in a 32 year old man who lived in the endemic zones of Córdoba, San Luis and Mendoza (BACIGALUPO *et al.,* 1943). In a 35 year old woman from Tucumán, *F. hepatica* eggs were found in peritoneal granulomas adhered to the gall bladder and near to the transverse colon (CAMES *et al*., 1947; CID, 1947). Another case of intracranial fascioliasis was reported in a 44 year old female from Córdoba (RUGGIERI *et al*., 1967; CORREA *et al*., 1969). More recently, a case of cutaneous fascioliasis was described in a 26 year old male from Mendoza (MINOPRIO *et al.,* 1995).

2.3.5.7.- CLINICAL/EPIDEMIOLOGICAL DIAGNOSIS

In some cases, patients were diagnosed based on clinical and epidemiological characteristics compatible with fascioliasis even though coprological or serological analyses yielded negative results.

In an outbreak, four family members living in the same dwelling had shared meals including watercress and presented compatible symptoms, although eggs were only found in two of them (BACIGALUPO, 1942; BACIGALUPO *et al.,* 1943). One of the coprological negative patients had eosinophilia, fever and generalized pain. Symptoms disappeared after emetine treatment and in two months the patient had gained 7 kg weight. The other negative patient also showed eosinophilia (52%) and suffered generalized pain but no temperature. After treatment, pain disappeared and eosinophilia diminished to 14%.

Table 3.- Evolution of human fascioliasis infection reports in Argentina, according to provinces where infection was presumed to have occurred, number of cases and respective articles furnishing the information (MERA Y SIERRA *et al*., 2011).

Province (nº of cases)	**Number of cases per individual references (in parenthesis nº of cases)**
Córdoba (430)	Castex & Greenway, 1937(1); Cames, 1940 (1); Bacigalupo, 1942 (4); Bacigalupo *et al*., 1943 (1); Solari & Canepa, 1943 (1); Cuenya, 1944 (1); Rodriguez, 1952 (5); Rodríguez, 1954 (9); Rodríguez, 1961 (22); Rodriguez, 1961 (150); Strada, 1961 (19); Sosa & Romero, 1967 (2); Correa *et al*., 1967 (1); Ossola *et al*., 1972 (11); Carena *et al*., 1972 (12); Pizzi *et al*., 1982 (54); Siciliano, 1982 (101); Giffoniello *et al*., 1983 (2); Siciliano, 1989 (15); Corti *et al*., 2006 (1); Nieto Sosa *et al*., 2008 (8)
Catamarca (73)	Rodríguez, 1952 (1); Rodríguez, 1954 (1); Rodríguez, 1961 (1); Carena *et al*., 1972 (1); Andrada *et al*., 1983 (5); Malandrini *et al*., 2009a (54); Malandrini *et al*., 2009b (10)
San Luis (29)	Bengolea *et al*., 1927 (1); Bacigalupo *et al*., 1930 (1); Peiretti, 1969 (2); Pieretti & Morales, 1973 (4); Melero *et al*., 1991 (1); Ale *et al*., 2000 (1); Rubel *et al*., 2005 (4); Nieto Sosa *et al*., 2008 (15)
Mendoza (28)	Logaldo, 1955 (1); Simon *et al*., 1964 (1); Peiretti, 1960 (15); Miguel *et al*., 1985 (1); Minoprio *et al*., 1995 (5); Lloret *et al*., 2005, Salomon *et al*., 2006 (5)
Tucumán (6)	Boto, 1939 (1); Cuenya, 1944 (1); Cid, 1947 (1); Cames *et al*.,1961 (1); Ahualli & Arias, 1970 (1); Padilla Antoni *et al*., 1972 (1)
Buenos Aires (5)	Mascheroni, 1933 (1); Bacigalupo, 1942 (1); Miguel *et al*., 1985 (3)
City of Buenos Aires (4)	Paladino & Galarce, 1939 (1); Caceres, 1955 (1); Niño, 1965 (2)
Salta (3)	Cuenya, 1944 (1); Longo & Daralo, 1953 (1); Cornejo & Castillo, 1964 (1)
Santa Fe (3)	Cames, 1940 (1); Bacigalupo *et al*., (1943); Trossero & Nocetti, 1969 (1)
Neuquen (2)	Rubel *et al*., 2005 (1); Rios *et al*., (2008)
Jujuy (2)	Cuenya, 1944 (1); Cañas *et al*., (1964)
Chaco (1)	Petraglia, 1954 (1)
Formosa (1)	Miguel *et al*., 1985 (1)
Not specified (32)	Greenway, 1924 (1); Del Valle & Donovan, 1928 (1); Urrutia & Ferraris, 1962 (1); Niño, 1965, (2); Sonzini Astudillo *et al*., 1973 (5); Allagia, 1981 (6); Majul *et al*., 1981 (6)
Total: 13 provinces	58 reports

In another family outbreak (MINOPRIO *et al.,* 1995), the wife of a male patient showing a cutaneous fluke, had pruritic skin lesions, eosinophilia and compatible images in the gall bladder during ecography later confirmed by CATScan; her symptoms disappeared after triclabendazol treatment. The mother had asthenia, urticaria, and multiple gall-stones that did not allow for the visualization of parasites through ecography; her symptoms also subsided after treatment. The brother-in-law had eosinophilia and fever, and became asymptomatic after triclabendazol self treatment. A fifth patient, a 14-year-old girl who participated in the same family outing, had hepatomegalia and urticaria, compatible images upon ecography, and also recovered after triclabendazol medication.

After consuming wild watercress during an outing, five persons showed compatible symptoms, including eosinophilia and leucocytosis. In three of them diagnosis was confirmed by coprology and/or serology. In the remaining two patients, analyses yielded negative results but they were considered as having been infected when recovering after fascioliasis treatment (SALOMON *et al.,* 2006).

2.3.6.- MEDICAL ASPECTS

2.3.6.1.- CLINICAL FINDINGS

Symptoms, laboratory results and their frequency do not appear to differ from those described elsewhere. Articles reviewed are very diverse in nature. Some include detailed clinical descriptions, but many are merely an enumeration of human cases with very vague or sometimes even no accompanying clinical data at all. Thus, information has to be treated with caution. Establishing prevalence of symptoms in the total population of patients becomes impossible, and assumptions may only be obtained from the few articles in which the symptomatology was sufficiently described (Table 2).

Of 225 patients in whom eosinophil counts where performed, 198 (88.00%) had eosinophilia. In 143 patients, authors did not note the eosinophil level. Amongst those in whom it was quantified, the mean was 28.00% (± 21.33%). Very high counts were found in some patients, with a maximum of 84% (BOTO, 1939).

Leucocytosis was found in 128 of 167 patients (76.65%) in whom leukocyte counts were analysed, with a mean of 1,2478.72 (± 7,917.89), the lowest value of 4,400, and

the maximum of 52,600 found in the afore-mentioned patient in whom eosinophilia of 84% was also detected (BOTO, 1939).

Fever was described in 138 amongst 153 patients (90.20%) in whom temperature was analysed. In the great majority it was just referred to as simply presenting fever. In the only 10 patients in whom the exact temperature was described, the mean was 39 °C (± 0.62 °C). In the article in which a higher number (69) of patients was recorded in whom temperature analysis was performed, 58 (84.06%) had fever (SICILIANO, 1982).

Abdominal pain, noted in 200 patients, appears to be the most frequent finding. Unfortunately, it appears not always adequately described. Thus, in most instances only terms as “diffuse”, “right hypocondrial pain”, “epigastrial”, or similar are mentioned.

Weight loss due to the disease was found in 76 of 91 patients (83.52%) in whom a weight control follow-up was made. In the majority of cases only presence or absence of weight loss is stated. It was quantified in only 8 patients, with a range from 4 to 21 kg lost (mean 13.00 ± 5.48 kg). In two articles, the weight loss was classified according to its degree (OSSOLA *et al.,* 1972; SICILIANO, 1982) in 20 patients it was slight, in 18 moderate, and in 29 intense or very intense.

Anorexia was described in 53 out of 74 patients (71.6%) in whom this symptom was evaluated in a study (SICILIANO, 1982). In the literature analysed, anorexia appeared described only in one other patient (BOTO, 1939). Asthenia was described in only 86 patients in Argentina, whereas weakness is commonly associated with mild to moderate anemia during the acute phase elsewhere (MAS-COMA *et al.,* 1999b).

Urticaria was only found in 62 Argentinean patients, although this symptom is considered a distinctive feature in the early stage of fluke invasion (MAS-COMA *et al.,* 1999b). Other less diagnosed symptoms in the country include nauseas in 38 patients, jaundice 23, lithiasis 17, vomiting 11, headache 9, diarrhea 8, and constipation in 6.

2.3.6.2.- CO-INFECTION WITH OTHER PARASITES

Co-infections of fascioliasis with other protozoan and helminth species have recently proved to be the usual rule in human fascioliasis endemic areas (MAS-COMA *et al.,* 2005; ESTEBAN *et al*. 1997a,b, 1999, 2002, 2003; ZUMAQUERO-RIOS *et al.*, 2013). Clinical synergistic associations of fascioliasis with other pathogens are believed to be at the base of high morbidity and mortality rates in children (MAS-COMA, 2004),

immunological responses being markedly suppressed and concomitant infection being exacerbated following liver fluke infection (BRADY *et al.,* 1999; GIRONES *et al.,* 2007).

In Argentina, unfortunately patient analyses do not appear to have particularly focused on co-infections. The most frequently reported parasite co-infecting with *F. hepatica* appears to be *Echinococcus granulosus*, including a total of 14 patients reported to simultaneously present both parasites. All of these cases were from a hydatidosis endemic zone in Mendoza province (PEIRETTI, 1969; MIGUEL *et al.,* 1985). This relative high number of fascioliasis-hydatidosis co-infected patients is outstanding, as such a co-infection has never been detected in human endemic areas of other countries so far.

Other parasite species found in the coprological diagnosis of fascioliasis patients were: *Entamoeba coli* (6 patients), *Giardia intestinalis* (5 patients), *Blastocystis hominis* (4 patients) and *Entamoeba histolytica* (2 patients) amongst protozoans, and *Enterobius vermicularis* (1 patient), *Strongyloides stercolaris* (1 patient) and *Hymenolepis nana* (1 patient) amongst helminths. Only two patients have been described to present more than one parasite species additional to *F. hepatica*, namely *G. intestinalis* and *E. vermicularis* in one case (PETRAGLIA, 1954), and *E. coli* and *G. intestinalis* in another patient (STRADA, 1961). In total, only 30 patients had co-infections with other parasites, of which only 16 had intestinal parasites. This low amount of co-infection reports may perhaps partly be explained by the geographical origin of the fascioliasis patients, usually dry regions where other helminth diseases are relatively difficult to find, and also by the age of the patients, since most were adult subjects in whom intestinal parasitic diseases are not so prevalent.

2.3.6.3.- TREATMENT

This was specified in 212 patients. Drugs mentioned to have been used were emetine (in 186 patients), triclabendazole (21), cloroquine (4), praziquantel (1), albendazole (1) and male fern extract (1). In two patients, more than one drug was used. A historical analysis shows that emetine was the only drug used up to the end of the 80's, except for the sporadic use of chloroquine (SICILIANO, 1982). From the beginning of the 90's, triclabendazole became the drug of choice, although praziquantel, albendazole and again emetine were applied in given cases (Table 2).

The first successful treatment report was with emetine intravenous administration in a 38 year old female patient. Fever and abdominal pain subsided shortly after treatment and coprological analyses showed the disappearance of eggs (BENGOLEA *et al.,* 1927). Emetine was also successfully used a few years later (BACIGALUPO *et al.,* 1930) and in another patient to solve the lack of effectiveness of male fern extract (CAMES, 1940). In Argentina, only a few treatment failures with emetine have been reported (RODRIGUEZ, 1954). An efficacy of 92.4% was obtained in 65 patients in whom *F. hepatica* eggs ceased to be found after treatment (RODRIGUEZ, 1954). Emetine was even recently used in a family outbreak involving 15 patients, amongst whom two presented severe hypotension. Coprological analyses became negative 60 days after a 10-day-long treatment (NIETO SOSA *et al.,* 2008).

Emetine derivatives were the classic drugs and continue to be used today, administered intramuscularly or subcutaneously, at doses of 1-10 mg/kg emetine/day for ten days (PADILLA *et al.,* 1970; CHEN & MOTT, 1990; SICILIANO *et al.,* 1989). Worldwide, dehydroemetine, at a dose of 1 mg/kg daily for 10-14 days, was considered the therapy of choice a few decades ago (CHEN & MOTT, 1990; ESTEBAN *et al.,* 1998; MAS COMA *et al.,* 1999b). However, emetine derivatives cause a variety of toxic manifestations involving the heart, liver and digestive tract. A cardiac counter indication led to the ineffective treatment with chloroquine of four patients to whom emetine could not be prescribed: all of these patients continued to shed fluke eggs after cloroquine administration (SICILIANO, 1982).

Even though there was no triclabendazole formulation for humans available in Argentina, it was, nevertheless, given to patients in its veterinary form, usually with prior consent. The first report of triclabendazole treatment concerned a dose of 10 mg/kg in a 40 year old male patient with prolonged high temperature and eosinophilia, including a repeated dose 9 weeks later. Clinical symptoms and eggs in stools disappeared thereafter (MELERO *et al*., 1991).

Another report of triclabendazole use concerned a family outbreak. First, a 26-year-old male patient with fever, eosinophilia, hepatic abscesses, and an ectopic subcutaneous fluke in the abdominal region, was treated with 900 mg/day praziquantel for three consecutive days, with initial disappearance of symptoms. After relapsing two weeks later, the patient was re-treated with 750 mg praziquantel every 8 hours for two days. As symptoms persisted, 10 mg/kg triclabendazole were applied three weeks later. Symptoms disappeared within 48 hours, and normalization of clinical and laboratory parameters was obtained. Four other patients of the same outbreak were also

successfully treated with triclabendazole (MINOPRIO *et al.,* 1995). Triclabendazole was also used at a dose of 400 mg/day for two days in a 20-year-old female (SICILIANO, 1982), well as with a single 10 mg/kg dose in a 58 year old woman (CORTI *et al.,* 2006), both with remission of symptoms. In an eight person family outbreak, six patients received triclabendazole (dosage and protocol not specified) with elimination of egg shedding. Another patient had to receive a second dose to stop egg shedding, and three triclabendazole doses were needed for the last patient (NIETO SOSA *et al.,* 2008).

Albendazole at a dose of 400 mg/12 hours was used in a 54 year old man with remission of clinical manifestations within days. This report stated that albendazole was used because it was impossible to obtain triclabendazole (RIOS *et al.,* 2008). Although Egaten® (triclabendazole, the drug of choice for human treatment at present) has been recently available from WHO (WHO 2006, 2008), it has been still never applied in Argentina. Nitazoxanide has been approved for human use in Argentina for fascioliasis, but no reports have been found in the literature.

2.3.6.4.- SURGICAL CASES

Even though the number of patients in whom surgery was involved is small (6.9%), publications dealing with surgical cases appear to be proportionally important (15; 26.7%). Surgical description was indeed the main objective of several articles. In the first surgical case reported, actually the third autochthonous case of the country (DEL VALLE & DONOVAN, 1928) the intervention was described in great detail, but no epidemiological information was provided, not even the province of origin of the patient.

In 45 cases, a surgical procedure contributed to the diagnosis when flukes were unexpectedly found upon liver exploration. The largest number of surgical cases described within one article is 16 (ALAGGIA, 1981). Unfortunately, sometimes no details were given and cases were merely referred to as surgical (STRADA, 1961).

In the majority, surgery was indicated due to abdominal pain and biliary obstruction suggestive of lithiasis. Indeed, gallstone disease has recently proved to be one of the effects of advanced chronic fascioliasis, since *F. hepatica* is able to survive up to 9-13.5 years within a human host (VALERO *et al.,* 2003; MAS-COMA *et al*., 2005, 2014). Hence, such a high lithiasis proportion suggests that long-term infected patients having been overlooked for a long time has been a relatively frequent situation in Argentina.

This agrees with the not unusual long delay in diagnosis already emphasized before, and both observations together pose a question mark about human fascioliasis detection in the country.

In most of these surgical cases with lithiasis suspicion, the patient inhabited a large city (Buenos Aires, Córdoba, Mendoza, and Tucumán) as opposed to a rural area where attending a health centre is less usual due to economic reasons or at least complicated due to the long journey that has to be made. This additionally suggests a far greater underestimation of the problem in rural areas.

The importance of intraoperative cholangiography was highlighted in cases in which, even though gallstones were removed, evidence of obstruction observed during the cholangiography led to the finding of flukes (CACERES, 1955; LONGO & DARAIO, 1953; LOGALDO, 1955). Gallstones were found and concomitant fascioliasis diagnosis made while performing choledoctomy in another six cases (MAJUL *et al.,* 1981). Two more patients with lithiasis in whom *F. hepatica* was diagnosed upon surgery were described later (SICILIANO *et al.,* 1989).

In a patient in whom a gallstone was suspected, intraoperative cholangiography showed that in fact, instead of a lithiasis problem, a *F. hepatica* specimen was involved (GIFFONIELLO *et al.,* 1983). In another patient operated due to lithiasis suspicion and to resolve a hiatus hernia, stones were neither found at cholecystectomy but flukes were after undertaking an intraoperative cholangiography that indicated estenosis altering normal bile flow to the duodenum (TROSSERO & NOCETTI, 1969). Similarly, no stones were observed but parasites found when performing choledoctomy in another patient diagnosed shortly after (PADILLA *et al.,* 1970).

Amongst four operated patients, fluke infection was detected in two only after a second surgical intervention. In the first operation, cholecistectomy was performed to remove stones but flukes were not detected since intraoperative cholangiography had not been applied. Upon re-operation, fascioliasis was diagnosed when *F. hepatica* was found in the common bile duct (SONZINI ASTUDILLO *et al.,* 1973).

The usefulness of intraoperatory cholangiography for fascioliasis diagnosis was highlighted for cases in which preoperatory diagnosis was difficult (ANDRADA *et al.,* 1983). This was concluded when operating five cases due to severe abdominal pain and lithiasis suspicion in only three of them.

In an interesting case, diagnosis was made after surgery to the brain when an expansive parasagital process was diagnosed by means of a carotid arteriogram performed in a patient with memory loss, nominal aphasia and discrete right facial paresia

(CORREA *et al.,* 1969). Two cysts containing *F. hepatica* eggs were extracted from the cortex. The patient died 24 hours after surgery. This is one of the few fatal cases known to be due to fascioliasis worldwide (MAS-COMA *et al.,* 1999b). In Argentina, the other reported fatal case was the first ever to be diagnosed in the country (ROFFO, 1913) but since it was an imported case it is not accounted for in the present study.

2.3.7.- RECENT REPORTS OF HUMAN FASCIOLIASIS IN ARGENTINA

After the precedent search was finalized, 10 new cases were published that will be dealt upon since not only new cases have been reported but a new epidemiological situation detected. The actual number of cases published currently is thus 629.

SAWICKI *et al*. (2017) reports 5 cases of fascioliasis in which the diagnosis was made by ultrasound, in two of them serologic diagnosis was also made (technique not specified). According to gender, 4 were females and one male, aged from 42 to 58 years old (mean 51 years). The province where they got infected was specified in only two cases, which was during a trip to Córdoba that they consumed watercress and still another reported its consumption. Three of them had abdominal pain, one was asymptomatic, four of them had eosinophilia, two had fever and one had jaundice due to an elevation of hepatic enzymes. Unfortunately the province or region where three of the cases they lived or could have acquired the disease is not specified.

In a mountainous region of the province of San Luis, a survey was performed in 42 persons. At the coprological studies one patient (2.38%) was positive and 5 (11.90%) were positive to serology, including the one that tested positive at the faecal exam. The serological test performed was an ELISA for the detection of anti-recombinant procathepsin L1 of *F. hepatica* (FhrproCL1) antibodies (CARNEVALE *et al*. 2001a). According to gender they were 3 females and 2 males, aged from 22 to 58 years old (mean 44 years). Four of them had the habit of consuming watercress. With respect to the clinical signs, two of them were asymptomatic and three had abdominal pain; three had diarrhea, two had vomiting, two had hepatomegalia and one had jaundice; one presented important eosinophilia (34% of eosinophils) and one had mild eosinophilia (7%), the other three patients had a normal proportion of eosinophils. The snail species involved was identified as *L. viator*, but molecular studies were not performed and identification was on morphological grounds.

In the province of Catamarca, in the sites were a very high prevalence (36.5%) was found in humans (MALANDRINI *et al*., 2009a,b) a thorough epidemiological study was performed (BARGUES *et al*., 2016). The climatic characterisation resulted that the two endemic sites were extreme desertic arid conditions and semiaridity aridity respectively. The habitats of the snails are in lateral riverside flooding and small irrigation channels. The molecular studies identified two species, *L. neotropica* and *L. viator*. Due to the extremely low precipitation, the water in these sites depends on the rainfall, snowmelt and melt off from glaciers in very distant sites and in much higher altitudes in the mountains. There has to be a concurrence of factors in time, such as temperature and water availability. This favours the concentration of snails, infected livestock and humans in the transmission sites.

2.4.- DISCUSSION

2.4.1.- LESSONS FROM THE THOROUGH LITERATURE SEARCH

This review highlights the pronounced difficulties in the literature search when dealing with a highly neglected disease in humans as it is the case of fascioliasis. Obtaining old publications, mainly in local journals or non regular publications, has proved to be especially difficult and time consuming. The information furnished by these old published reports becomes very important to assess areas where fascioliasis transmission may follow characteristics enabling human infection. In many of these potentially important endemic areas, the absence of additional human reports or the very low number of patients diagnosed may be simply due to the lack of appropriate surveys and/or sometimes due to misdiagnosis of other patients.

Before the 1990s, human fascioliasis was so neglected that human case reports were published in veterinary journals instead of in medical journals. Publications as those of PIZZI *et al*. (1982) are illustrating examples, the aforementioned one being even a reference including a high number of human reports in Argentina. The fact of articles included in publications not related to human health increases the difficulties in finding references about human reports considerably.

The problem of being a neglected, usually non fatal, clinically mild, and non reportable infection explains why human cases are simply not published or notified to national health responsible, neither reported anywhere. Liver fluke infected patients

described in university theses and afterwards never published demonstrate this problem. Even so more if we consider the difficulties sometimes posed to find copies of old theses, even in the libraries of the university and department where it was made and presented. Anyway, the literature on human fascioliasis in Argentina does not appear to be so problematic with regard to unpublished reports as it is in other countries as Bolivia (MAS-COMA *et al.,* 1995). In the latter country, the numerous non-published reports stocked in ministries, national and international agencies and NGOs, seem to be mainly due to the fact of the human endemic area being in the neighbourhood of the country capital La Paz, which is not the case in Argentina, where human cases diagnosed in Buenos Aires appear to be very few.

Worth mentioning also is the almost total uselessness of internet for the finding of most of the references about human infection by a neglected disease such as fascioliasis. This problem does not only concern old papers having never been digitalized and put available in the net, but also reports in local publications, non-regular journals, internal papers, etc., whose entity will most probably never be considered of sufficient merit as to carry out the effort to include them in internet.

2.4.2.- A CRITICAL ANALYSIS OF PAST AND PRESENT HUMAN FASCIOLIASIS KNOWLEDGE IN ARGENTINA

2.4.2.1.- COUNTRY SITUATION

This review offers a complete new picture of human fascioliasis in Argentina. General aspects of liver fluke infection in humans from this country had been only very rarely the focus of previous analyses. The number of cases in the present review increases manifold the amount accounted for in previous instances. Such a high number of overlooked cases seem to be due to the great amount of local publications (and also short communications to scientific meetings with abstract books of very restricted dissemination) where much of the case reports could be found.

When considering that human fascioliasis infection is in Argentina of non-obligatory declaration, similarly as in the rest of the world, one may easily conclude that the real number of liver-fluke-infected patients detected should most probably be pronouncedly greater than that.

Interestingly, the recent and present situation may not differ much from the one described in the old paper in which the presence of fascioliasis, found in animals, was mentioned in Argentina for the first time (SAVOYAT, 1867). This author already stated to be convinced about many human symptoms found in that period to indeed be attributable to *F. hepatica*, highlighting the need for health authorities to warn about this disease.

What is most alarming is the results of the surveys done, even though there are very few, they could be highlighting a much more serious situation. The great majority of cases published are because of persons that seek and have access to medical attention and diagnostic resources that most rural populations have great difficulty, if not impossibility, of accessing. This is particularly true in Andean regions, when many times the person has to travel up to days on horse or mule just to reach the basic medical attention. The few surveys that have been done in rural and remote regions have produced alarming results. CARNEVALE *et al*., (2013) described a prevalence of 11.9% in the province of San Luis and MALANDRINI *et al.,* (2009a) the alarming prevalence of 36.5% in the province of Catamarca. What characterizes these sites and population sampled is that they are in remote places, composed of exclusively rural inhabitants and if it were not for the active search done, quite probably these patients would have had very low probability of being diagnosed. If the same or similar situations occur all throughout Andean and mountainous regions of Argentina, the prevalence and amount of human cases could be of a great magnitude.

2.4.2.2.- GEOGRAPHICAL DISTRIBUTION OF HUMAN CASES

The geographical origin of the patients includes 13 provinces, covering more than half of the total surface of continental Argentina, with Córdoba, Catamarca, San Luis and Mendoza including the highest number of patients detected, the remaining provinces being only rarely affected. Human infection should most probably have been fully overlooked in La Rioja and San Juan provinces, with no case reported despite being surrounded by the aforementioned provinces. As already known in other countries (MAS-COMA *et al.,* 2005, 2009b), this distribution does not fit the one of animal fascioliasis, which covers whole Argentina, except southern Patagonia.

Human infection risk is in fact present in many geographical areas of this country, with higher probabilities in given altitude areas. The great majority of cases are from

hilly or mountainous areas, concentrating in central mountainous areas and Andes Mountains, mainly valleys, remembering other Andean countries in which human endemic areas are linked to altitude areas. This may be related to both (i) geographical distribution of the main lymnaeid vectors involved in transmission to humans restricted to or preferring such altitude areas, and (ii) the greater liver fluke transmission capacity in altitude areas, as already proved in the Northern Bolivian Altiplano human hyperendemic area at 3,800-4,100 m altitude (MAS-COMA *et al.,* 2001). This suggests the appropriateness to verify whether in Argentina fascioliasis situations exist similar to those known in high altitude human endemic areas of the neighboring Chile, Bolivia and Perú.

2.4.2.3.- EPIDEMIOLOGICAL CHARACTERISTICS

The higher prevalences in females than in males suggest epidemiological characteristics similar to those found in human endemic areas in other countries. Patient age ranges from 3 to 95 years old (average 37), differing from other Andean countries where children are the most affected subjects, although the lack of studies on infection intensity and of appropriate surveys in high risk rural areas of the country does not allow for an adequate evaluation.

Watercress consumption is the most frequently cited possible human infection source, other plants and water consumption having been only sporadically involved. The most common risk factor appears to be ingestion of watercress during recreational, weekend or vacation activities. Field excursions undertaken by a family or as a group activity explain the numerous family outbreaks. However, a 36% seroprevalence found in a recent random survey shows that not all situations including several infected subjects are in fact family outbreaks.

Seasonality appears related to human incidence, human infection occurring mainly during the first months of the year in which higher precipitation and temperatures interact with an increase of recreational field activities during the summer vacations in January and February, with a second lower June peak perhaps related to the Eastern vacations, at least in the province of Córdoba. However, different transmission patterns may occur and different human infection risk seasonalities/periodicities may exist according to the large physiographical heterogeneity of the country.

The number of human cases reported shows two peaks in the 1960 and 1980 decades, with the 1972 outbreak probably related to the sudden increase of rainfall in that year in Córdoba province, although other outbreaks do not show any apparent climatic causal origin.

The new transmission pattern identified in the province of Catamarca (BARGUES *et al.*, 2016) could very well be occurring in countless remote rural sites along the thousands of kilometers that compromise the Andes of Argentina. Vast extensions of land in regions with very low precipitation have the similar characteristic that the presence of water in rivers and streams depend on the melt off of snow higher in the mountains and human and domestic animal populations are centered along the scant water resources. The epidemiological scenario of fascioliasis could be of unexpected proportions.

2.4.2.4.- DIAGNOSTIC AND TREATMENT PROBLEMS DETECTED

We today know that the diagnosis of fascioliasis in humans poses many problems: (i) the clinical picture may not be pathognomonic and consequently easily confused with other diseases, mainly liver diseases and other helminth infections, when the patient attend a health centre not used to deal with fascioliasis infection (LA POOK *et al.,* 2000); (ii) in human endemic areas the clinical picture does sometimes not appear to be sufficient as for the infected subject to attend an hospital for diagnosis and/or treatment (ESTEBAN *et al*., 1997a,b, 1999, 2001, 2003); (iii) children with even heavy infections do not use to go to health centres, as seen in the neighboring countries of Chile (APT *et al.,* 1992, 1993) and Bolivia (ESTEBAN *et al.,* 1997a,b, 1999).

Additionally, the specific diagnosis of human fascioliasis poses well-known methodological and technical problems. Egg finding in coprology (gold standard) may be impossible due to the absence of eggs not only in the acute phase (fluke metacercariae still tissue migrating or only shortly in the biliary canals and thus absence of mature egg-laying fluke adults), but also in the chronic phase (as described in areas where the liver fluke strain may be not sufficiently adapted to human infection due to the very sporadic infection in humans, e.g. France) (MAS-COMA *et al.,* 1999b). Moreover, other problematic situations may not be discarded, such as the lack of sufficient sensitivity in light infections. This is common in human sporadic infections in animal

endemic areas, as it appears to be the usual case in many reports of Argentina, e.g. in travelers, weekend family movements, and tourists.

Human fascioliasis diagnosis in Argentina has in general relied on old techniques, mainly egg finding in coprology or duodenal sondage, followed by common serological techniques, intradermal reaction, surgical diagnosis, and erratic fluke observation. The efficacy of these techniques for individual patient diagnosis is far from the modern ones offering pronouncedly higher sensitivity and specificity, such as CL1 (O'NEILL *et al.,* 1998) and Fas2 (ESPINOZA *et al.,* 2007), for serological diagnosis or MM3Copro (UBEIRA *et al.,* 2009) for specific coproantigen detection in human stools, or similar techniques. And this without forgetting that a quantitative diagnosis enabling egg counting in fascioliasis, such as the standardized Kato-Katz technique (ESTEBAN *et al.,* 1997a,b, 1999, 2001, 2003) has never been applied in that country, despite the importance of parasite burden in both the pathogenicity and intensity assessment for the adequate treatment dose.

Noninvasive, image based diagnostic techniques have been rarely applied, opposite to other countries. Symptoms, laboratory results and their frequency do not appear to differ from those described elsewhere. The studies performed do not appear to have focused on co infections as needed, owing to their importance as the consequence of the immunosupression capacity of the liver fluke (GIRONES *et al.,* 2007). Anyway, the relative high number of patients diagnosed as concomitantly infected by *F. hepatica* and *Echinococcus granulosos* shall be highlighted, as such a co infection has never been detected in human endemic areas of other countries so far (ESTEBAN *et al.,* 1997a,b, 1999, 2001, 2003).

Publications on surgical cases appear to be proportionally important (26.7%), with lithiasis suspicion as the main reason to justify intervention. This suggests that long term infected patients having been overlooked during long time has been a relatively frequent situation and poses a question mark about human fascioliasis detection in the country.

The old emetine has been the drug most used in Argentina, even for patients being detected very recently. Triclabendazole, the drug of choice for human treatment at present, has been always given to patients under the veterinary form, usually with prior consent, due to unavailability of a human formulation in the country. This situation should be solved as soon as possible.

2.4.2.5.- PRESENT SITUATION AND FUTURE PERSPECTIVES

All aforementioned aspects suggest that, in Argentina, human fascioliasis may have been overlooked in the past and its real epidemiological situation might be underestimated in the present, mainly in high risk rural altitude areas. In Andean regions of Argentina, the presence of three different species of lymnaeids with vectorial capacity has been confirmed: *G. truncatula, L. neotropica* and *L. viator* (BARGUES *et al.,* 2006b, 2007, 2016; MERA Y SIERRA *et al.,* 2009). These species are linked to high prevalences and intensities of human fascioliasis in neighbouring countries such as Bolivia Perú and Chile. This diversity of species makes the current situation more alarming. The very high prevalences recently obtained in a survey in Catamarca province (MALANDRINI *et al.,* 2009a), of the level of human hyperendemic situation (MAS-COMA *et al.,* 1999a), also indicate in the same direction.

Thus, the need for appropriate epidemiological studies in the field, in selected areas where lymnaeid vector species of high liver fluke transmission are present, shall be emphasized. Health centres in these areas should be prevented about human infection probabilities, main clinical picture characteristics, adequate diagnosis techniques and the need for Egaten® (triclabendazole for human use) availability. Triclabendazole resistance recently detected in Argentinean cattle from the province of Neuquén (OLAECHEA *et al.,* 2011), where human infection has already been reported twice (RUBEL *et al.,* 2005; Rios *et al.,* 2008), and its capacity to spread to other areas of the country, pose a serious question mark for human treatment in Argentina in the future.

The results of this retrospective overview conform an extremely valuable baseline on which to design adequate multidisciplinary studies on fascioliasis in humans, animal reservoirs and lymnaeid vectors to assess up to which level and in which areas of this very large and environmentally heterogeneous country, human fascioliasis may represent a public health problem in Argentina.

CHAPTER III

MOLECULAR AND MORPHO ANATOMICAL CHARACTERISATION OF LYMNAEIDS FROM ARGENTINA

3.- MOLECULAR AND MORPHO ANATOMICAL CHARACTERISATION OF LYMNAEIDS FROM ARGENTINA

3.1.- BACKGROUND

The susceptibility of a given lymnaeid species to fasciolid infection represents a crucial factor in establishing not only the geographical distribution of the disease in both animals and humans, but also prevalences and intensities due to more or less appropriate ecological characteristics (population dynamics, anthropophilic characteristics, type of water bodies, etc.) of the different lymnaeid vector species. This is why different lymnaeid species appear linked to the different transmission patterns (MAS-COMA, 2005) and epidemiological scenarios (MAS-COMA *et al*., 1999a, 2018) of this very heterogeneous disease in humans (MAS-COMA *et al*., 2009a). The continental differences in lymnaeid faunas also explain that in the Americas fascioliasis is only caused by *F. hepatica*, due to the absence of lymnaeids of the genus *Radix* which act as vectors of *F. gigantica* (BARGUES *et al*., 2001). Similarly as in other vector-borne diseases, this relationship supports the use of lymnaeids as biomarkers of the disease at both local and large scales (BARGUES *et al*., 2011a). Distribution, both in space (latitudinal, longitudinal and altitudinal) and time (seasonal, yearly), of fascioliasis depends on the presence and population dynamics of the specific mollusc species, which in turn is linked to the presence of the appropriate water bodies and on adequate climate characteristics enabling fluke development (BARGUES *et al*., 2016, 2017).

As in all other vector-borne diseases, in fascioliasis the transmitting snails of the family Lymnaeidae are crucial with regard to the epidemiological situations and transmission patterns of the disease. Epidemiological and transmission heterogeneities in the different endemic areas are related to the lymnaeid vector species involved (MAS-COMA, 2005; MAS-COMA *et al*., 2009a). The correct classification of the lymnaeid snail species acting as vector is therefore of great importance, in order to both determines risk zones and to achieve the appropriate control measures to reduce transmission, according to the different ecological characteristics of each lymnaeid species. Unfortunately, lymnaeid species are freshwater snails which show marked intraespecific variability in their shell characteristics and a surprising uniformity at the level of their anatomy. These features explain the great problems to distinguish between species within given controversial groups such as *Galba/Fossaria*, *Radix* and stagnicolines (BARGUES *et al*., 2001, 2003, 2006a, ARTIGAS *et al*., 2009, 2011). For

such groups in which not even expert malacologists are sometimes able to correctly classify specimens, sequences of molecular markers amongst the nuclear ribosomal DNA and the mitochondrial DNA appear to be the most useful tools for specimen classification purposes (BARGUES & MAS-COMA, 2005).

In the Americas, except *P. columella*, all lymnaeid species involved in the transmission belong to the *Galba/Fossaria* group. Species differentiation within *Galba/Fossaria* is crucial given (i) their different disease transmission capacities to humans; (ii) the difficulties in species differentiation due to their pronounced morphological similarities; and (iii) the fact that there is a snail species of this group which, although usually present in high transmission areas, does not transmit (BARGUES *et al*., 2011c). The abundant species of lymnaeid snails described for Argentina were classified according to morphological features (Table 1). The incorporation of molecular studies not only has identified the existence of introduced species into Argentina, but is also profoundly revising the current taxonomy of the lymnaeid fauna of this country.

3.2.- OBJECTIVES

The present study aims to identify the lymnaeid vector species involved in human and animal fascioliasis endemic areas of Argentina by means of molecular tools (sequence and analysis of ribosomal and mitochondrial DNA molecular markers) and by their shell morphometry and genital anatomy; develop molecular tools for the future identification, to species and haplotype level of lymnaeid populations; and subject to phylogenetic analyses both, nuclear ribosomal DNA and mitochondrial DNA markers sequenced to determine valid species and to assess phylogenetic relationships by different tree reconstruction methods.

The specific aims of the present study are:

a) Describe the presence of *G. truncatula* in Argentina and molecular confirmation, by analysis of the complete sequence of the first and second internal transcribed spacer, ITS-1 and ITS-2 respectively, of the nuclear ribosomal DNA (rDNA).

b) Describe the evidence that supports that *L. viato*r var. B elongata, be reclassified as *Lymnaea neotropica* n. sp. by means of a combined genetic and phenetic analysis of specimens from "terra typica" and an exhaustive comparison with other *Galba/Fossaria* species such as *L. cubensis*, *L. viator* and *G. truncatula*, as

representative species frequently involved in fascioliasis transmission in Central and South America.

c) Describe the presence of *L. neotropica* in Argentina and its natural infection by *F. hepatica*, by means of molecular identification of both, the intramolluscan trematode larval stages found in naturally infected snails and the lymnaeid species carrying the trematode larval stages.

d) Genetic analysis of other lymnaeid speciesgeographycally or taxonomically related as the case of *L. diaphana*, by means of sequencing of combined rDNA and mtDNA markers from topotypic specimens.

e) Analysis of phylogenetic relationships of lymnaeid species from Argentina with other *Galba/Fossaria* species described in new and old world as well as with other selected lymnaeid species representing the different lymnaeid groups of interest.

3.3.- MOLECULAR CHARACTERISATION OF *GALBA TRUNCATULA*

3.3.1.- GEOGRAPHICAL ORIGIN OF THE SPECIMENS STUDIED

For the identification of *G. truncatula* in Mendoza province, lymnaeid specimens were collected in the locality of El Salto, a small town in the Department of Lujan de Cuyo in the province of Mendoza, Argentina. The snails where collected from a small irrigation channel (Figure 6) that diverted water from a mountain river, Río Blanco, of the Río Mendoza basin. The coordinates of the collection site were 32°57'19.61"S-69°17'55.08"W and at an altitude of 1,966 m.a.s.l. Upon collection the snails were fixed in 70% ethanol.

Mendoza province has the Andes Mountains as a backbone in the west, with a medium altitude of 4,500 m.a.s.l. and towards the east a transition to a plains region. The rivers have their origin at great altitudes in the Andes and they flow eastward conforming the Desaguadero River basin, which receives water from the following rivers: Mendoza, Tunuyán, Diamante and Atuel. The climate in the mountains is cold with precipitation as snow during the winter. The eastward plains have a temperate climate with scarce precipitation mainly during the summer rarely exceeding 250 mm. According to the National Census, El Salto has a population of 361 inhabitants, but this number increases manifold during weekends, holydays and vacations due to its tourist and recreational activities.

Fig.6.- Irrigation cannel (arrow) in El Salto (32°57'19.61"S 69°17'55.08"W), Lujan de Cuyo department, where the snails were found. In the background, oriental view of the Andes Mountains (Cordón del Plata).

.

3.3.2.- METHODOLOGICAL PROCEDURES

DNA extraction procedure steps were performed according to methods outlined previously (BARGUES *et al.,* 2001).Total DNA was isolated according to the phenol-chloroform extraction and ethanol precipitation method. After dissection under a microscope with sterile scissors, half of each foot was suspended in 400 µl lysis buffer [10 mM Tris-HCl (pH 8.0), 100 mM EDTA, 100 mM NaCl, and 1% sodium dodecyl sulphate] containing 500 mg proteinase K (Promega, Madison, WI)/ml, and digested for 2 h at 55ºC, shaking every 15 min.

Total extraction of DNA was done in three steps according to the phenol-chloroform method. First, 400 µl of phenol were added and centrifuged during 5 min at 1,300 rpm. Then the aqueous phase with the DNA is recovered. In the second step, 200 µl of phenol, and 200 µl chloroform: isoamyl alcohol (24:1, by vol.) is added and mixed gently. Again, it is centrifuged and the aqueous phase is recovered. In the third and final step, 400 µl of the same chloroform: isoamyl-alcohol mixture was added, mixed, centrifuged and the supernatant recovered. The final aqueous phase was precipited with 4M sodium acetate and 100% ethanol. The resultant DNA pellet was suspended in 30 µl of TE buffer (Tris-HCl 10mM, pH=7.6, EDTA 0.1 mM, pH=8,0) and conserved at -20ºC until used.

The fragments corresponding to ITS-1 and ITS-2 were amplified using primers designed to match the conserved regions of genes 5.8S and 28S (BARGUES *et al.,* 2001; MAS-COMA *et al*, 2001). PCR was done using a MiniCycler™ PT-150 (MJ Research, Watertown, MA, USA) thermal cycler using 4-6 µl of genomic DNA from each snail for each 5 µl of PCR reaction with the following program: 94°C during two minutes; 30 repetitive cycles of 30 seconds at 94°C, 30 seconds at 50°C and 30 seconds at 72° C; then 2 minutes at 72°C and a final cooling of 4°C. After, 10 µl of each PCR product were separated by electrophoresis in 1% agarose and visualized by staining with ethidium bromide. In each reaction a positive and negative control was added as well as a molecular-weight marker.

Purification was done with UltraClean (MoBio Laboratories, CA, USA). The final DNA concentration was determined by measuring absorbance at 260 and 280nm in a Spectrogenics Genesis 5 (Spectronic, NY, USA) spectrophotometer. The sequencing of the amplified regions was done using the ABI Prism™ dRhodamine Terminator in the automatic ABI Prism 377A (Perkin-Elmer) sequencer and the PCR primers were employed.

Sequences were aligned using version 1.8 of the CLUSTAL-W program (THOMPSON *et al.,* 1994). Alignments were done including the lymnaeids from Argentina and also with sequences of other known haplotypes of *G. truncatula*. The following ITS-1 and ITS-2 sequences present in GenBank were used for comparisons: *G. truncatula* haplotype A (AJ243018), from Spain, Portugal, Switzerland and Corsica, haplotype B (AJ296270), from Morocco, and haplotype C (AJ272052), from Bolivia (MAS-COMA *et al.,* 2001); *G. truncatula* Haplotype 1 (AJ296271), from Spain, Portugal and Corsica, Haplotype 2 (AJ243017), from Spain, Portugal and Switzerland, and Haplotype 3 (AJ272051), from Bolivia (BARGUES *et al.*, 2001; MAS-COMA *et al.,* 2001) (= *L. viator* sensu UENO *et al.*, (1975); = *L. cubensis* sensu UENO *et al.*, (1975); = *Lymnaea* sp.morph I y morph II sensu OVIEDO *et al.*, (1995a). Homologies were investigated using the BLAST program, via the website of the United States National Center for Biotechnology Information (www.ncbi.nlm. nih.gov/BLAST).

3.3.3.- RESULTS OBTAINED IN THE STUDY OF rDNA MARKERS

ITS-1: The ITS-1 sequences from the lymnaeid specimens analysed presented the same length of 504 base pairs (bp) and a scarcely biased GC content of 57.5%. The nucleotide sequence was in all cases the same and it is shown in Table 4. The Argentinean lymnaeids presented an ITS-1 sequence showing a 99.6% similitude with the *G. truncatula* haplotype A (HA) from Europe. The nucleotide differences detected were only two mutations: the transition G / A in position 74 and the transversion T / G in position 75. Concerning *G. truncatula* haplotype B (HB) from Morocco, the similitude was of 99.4% and a total of three mutations were detected: two transitions G / A and T / C in positions 74 and 132, respectively, and one transversion T / G in position 75 (Table 4). When comparing the ITS-1 sequence of the Mendoza lymnaeid specimens with that of *G. truncatula* haplotype C (HC) from the Northern Bolivian Altiplano, no nucleotide differences appeared (Table 5).

ITS-2: All the lymnaeid specimens analysed presented the same length of 401 base pairs (bp) and a GC content of 58.6%. The nucleotide sequence was in all cases the same and it is shown in Table 6. The comparison with the sequences available in GenBank, by means of the BLAST program, immediately showed great similitude with the sequences of *G. truncatula.* Compared to haplotype H1 of Europe there is a

Table 4.- Complete nucleotide sequence of the first internal transcribed spacer ITS-1 of the nuclear ribosomal DNA of lymnaeids from the population of El Salto, Mendoza, Argentina (BARGUES *et al*., 2006b) .

```
1   ATCATTAACG AGCAGCCAAC CGAGCGTTGA CTACTTTGTT GTCTCAGTCA GTCAGTCAGT
61  CAGGCCCCGC GGCGTGCACG CATGAAGCGC TGTCGCGGGG CTGTGTCCGC TTCGTCTTTC
121 GGGGTACCTA TTACTGTCCT CGATGCGACC CACGGTGACG GCTTAGAGCC CGTGTGCTCG
181 CCGGGTCGCG ACGGTTCAAA GAGTGGCCGG CTTGGCTCAG CTCGAGAGTC AGCCGGCGAC
241 CGCCCCGCCG TCGCAAAAAA ACAGGAGGTT AGTCCGGGGT ACCTATGCCC TGCCCGCGCT
301 CGCTCTCGCG CCGGCAAGGC GGTAGCTCCA GCTCGCTATT TGGCCGCGAG GTTCAAAGAG
361 ACGACCGTGC CTTAACTTGC TCTCTCCGTG GGCAACGGTC GCCGCCCCGG GCCTCCTAAA
421 ATTTCCTTTA ATAAAACGAA ATTATTTTTT AAAAATGTGT GTCGGCTCGA TCGTGGCACA
481 CGAAAAACAA ACAAAAGTC  TTAT
```

Table 5.- Sequence length, nucleotide contents and differences found in the comparison of the sequences of ITS-1 of the nuclear ribosomal DNA of lymnaeid populations from Argentina and known haplotypes of *G. truncatula* (BARGUES *et al*., 2006b).

.

G. truncatula haplotype	Origin	Length	%GC	Positions 74	75	132	GenBank Acc. No.
HA	Spain, Switzerland, Corsica, Portugal,	504 bp	57,5	A	G	T	AJ243018
HB	Morocco	504 bp	57,5	A	G	C	AJ296270
HC	Northern Bolivian Altiplano	504 bp	57,5	G	T	T	AJ272052
HC	El Salto, Mendoza, Argentina	504 bp	57,5	G	T	T	present work

Table .6.- Complete nucleotide sequence of the second internal transcribed spacer ITS-2 of the nuclear ribosomal DNA of lymnaeids from the population of El Salto (32°57'19.61"S 69°17'55.08"W) , Mendoza, Argentina (BARGUES *et al*., 2007b).

```
          GCTAGTCACA AAGCATTCGT GTCCTTGCAG CTCTCGCAAA AACCGAAGCC 50
          TTGCTGCGTG AGCTCTCACG CTGCTCGGCG ATGGTTGGAT ACGCCCTGGA100
          CCCTCGCGGC CAAAGCTGTC GTTGCCTGCT CGGCGGCGAC GGTGACGGTC150
          CCGTGGTCTT AAGCGCAAGC CGCGCCGTTG TCCGTTCATC TCGTAACGTC200
          TTCGACGCTG CCCTGCTCTT GGCGGCCTGT CCGTTTTCTC TACCGCCAGG250
          CAGGACCCGG CTCGCTTACT TTATTTATTA TCGTGGCGTT CTCGGGCCTG300
          CAGTCCATGG CATCGCAGCT CGTGGGTGGA GAACAAGGGG CTCTAAGACG350
        CTACGTGGTC GGCGCCCGTC GTTGAATGAA ACATTATTTG TTTCTTTTCTC 401
```

Table 7.- Sequence length, nucleotide contents and differences found in the comparison of the sequences of ITS-2 of the nuclear ribosomal DNA of lymnaeid populations from Argentina and known haplotypes of *G. truncatula* (BARGUES *et al*., 2007b) .

G. truncatula haplotype	Origin	Length	%GC	Positions 56 149	GenBank Acc. No.
H1	Spain, Portugal, Corsica	401 bp	59,1	G C	AJ296271
H2	Spain,Portugal, Switzerland	401 bp	58,8	G T	AJ243017
H3	North Bolivian Altiplano	401 bp	58,6	T T	AJ272051
H3	El Salto, Mendoza, Argentina	401 bp	58,6	T T	present work

97% similitude in the sequence, it has two mutations: a transversion of T for G in position 55 and a transition T for C in position 149. Compared with haplotype H2 of *G. truncatula* there is only one mutation: transversion of T for G in position 55 (Table 6).

When Compared the ITS-2 sequence of these lymnaeids from Argentina with the ITS-2 haplotype H3 of *G. truncatula* from the Northern Bolivian Altiplano, not one nucleotide difference was detected, thus the similitude is 100% (Table 7).

3.3.4.- IMPACT OF *GALBA TRUNCATULA* FINDING IN ARGENTINA IN THE TRANSMISSION OF FASCIOLIASIS

The analysis of sequences of ITS-1 and ITS-2 of rDNA of the lymnaeids from Mendoza has provided that these sequences were identical, both in length (504 and 401 bp, respectively) and nucleotide sequence, to the previously described for the same markers in *G. truncatula* from the Northern Bolivian Altiplano (GenBank Accession Numbers AJ272052 and AJ272051, for ITS-1 and ITS-2, respectively). The lymnaeid species detected originally in Mendoza, Argentina, in the year 2001 are, therefore, *G. truncatula* haplotype HC referring to ITS-1 and haplotype H3 referring to ITS-2 (BARGUES *et al*., 2006b, 2007a).

Lymnaeids of the *Galba/Fossaria* group have exhibit great intraespecific variability with overlapping morphology and morphometry which not only difficults but also sometimes does not permit a thorough classification of the specimens (BARGUES *et al.,* 2001). Many techniques both genetic and phenotypic have been proposed for the definitive species classification. Amongst them, the ITS-1 and ITS-2 have demonstrated to be good molecular markers; ITS-2 has demonstrated not only to be useful to differentiate between species but also between different populations by means of the SNP (single nucleotide polymorphisms) (BARGUES *et al.,* 2005).

The case of the Northern Bolivian Altiplano is an excellent example. This region was widely surveyed from a veterinary standpoint and it was evident that animal fascioliasis was an important issue and that the disease in cattle was transmitted by two American species: *L. viator* and *L. cubensis* (UENO *et al.,* 1975). However, studies performed on intraespecific variability showed that both species overlapped morphologically (OVIEDO *et al.,* 1995a). Also, both species showed an identical experimental capacity to transmit *Fasciola hepatica* in the laboratory, which is not coincident with a disease transmitted by a vector that, in the presence of more than one vector species in the same region, the

parasite is always more adapted to determined species and thus is better transmitted by one species than by others. This information raised the doubt if the classification of these species was correct. Molecular markers demonstrated that they were not two species but only one with a great intraespecific morphological variability and that this species was neither of the American species previously described (UENO *et al.,* 1975) but the introduced European species *G. truncatula.*

G. truncatula is a species of European origin that is very uniform genetically speaking. In Europe, by means of ITS-2 classification, the only two haplotypes known are H1 and H2 that differ only by 1 nucleotide mutation due to a transition in position 149; C in H1 and T in H2 (MAS-COMA *et al.,* 2001). This genetic uniformity seems to be related with the capacity for self-fertilization which bestows the ability to generate clonic or monomorphic populations (MEUNIER *et al.,* 2001). Up to now, only one other haplotype of the ITS-2 besides the two European is known: it is named H3 and was found in the Northern Bolivian Altiplano. It is characterized by a transfer in 55 (G in H1 and H2; T in H3), besides a transition in position 149 (C in H1 and T in H3) (MAS-COMA *et al.,* 2001).

If we take into account that the haplotype of *G. truncatula* described in the present work is responsible for the human endemic zone of the world with the highest prevalence, intensity and child mortality in the world, the result of the molecular confirmation of the classification of this lymnaeid snail in Mendoza, Argentina is quite meaningful. The province of Mendoza is also located in the Andean area and although the inhabited altitudes are not as high as those of hyperendemic zones in Bolivia and Perú, temperatures are similar because of the southern latitude (FUENTES *et al.,* 1999). This suggests a high disease transmission capacity in Mendoza. Besides the well known great capacity for transmission of *Fasciola hepatica* of this snail, there are other four epidemiological characteristics that have to be considered:

a) *G. truncatula* is a species that is closely related to the presence of livestock, so, its capacity to be passively transported by the animals is pronounce, thus there is a profound capacity for its geographical expansion.

b) *G. truncatula* was found in attitudinally high Andean region, which suggests an even greater transmission capacity due to the longevity of this snail in higher altitudes with respect to the snails in lower regions (MAS-COMA *et al.,* 2001).

c) The wide ecological features of *G. truncatula* do allow it to come close to human settings. This explains why this lymnaeid is in the background of many human infections. The peculiar ecology of this species brings it close to the human settlements

(MAS-COMA *et al.,* 1999a) and can even exist in urban settings, even in waters highly polluted by human activity (ESTEBAN *et al.,* 1999).

d) The adaptations of *F. hepatica* for its survival at high altitudes, which leads to an increase in the production of larvae (greater number of cercariae produced per individual snail) and to the increase in the duration of cercarial emission in each infected snail.

The presence of such a suitable vector for transmission may well be related to the high prevalences found in cattle in Mendoza. In one of the main slaughterhouses of the province a prevalence of 34.0% was found and in animals in a slaughterhouse which receives exclusively animals from Tupungato (an Andean valley region) a prevalence of 67.5% was found (MERA y SIERRA *et al.,* 2005a).

The existence of *G. truncatula* H3 poses a serious threat to public health due to its great capacity for transmission of fascioliasis en Mendoza, which alerts of the implications of its existence in other regions of Argentina. It is imperative to implement the use of molecular markers for the classification of lymnaeid snails due to the aforementioned difficulties to rely exclusively on morphological and anatomical criteria. The molecular markers, ITS-1 ad ITS-2 and particularly the latter is the best option for a definitive identification at species level. Future studies should be orientated towards an analysis of fascioliasis in humans and animals and its relation to the distribution of *G. truncatula*.

3.4.- GENETIC AND PHENETIC DESCRIPTION OF *LYMNAEA NEOTROPICA* N.SP.

In Latin America, although *P. columella* is a very peculiar species that can easily be identified by shell morphology and anatomy (PARAENSE, 1982a; MALEK, 1985), *L. cubensis* and *L. viator* belong to the morphologically and anatomically confusing *Galba/Fossaria* group. The division of *L. viator* into two varieties (A and B) dates right back to the description of the species (D'ORBIGNY, 1835). Their marked intraspecific morphological variability makes several 'sibling' species within this *Galba/Fossaria* group difficult or even impossible to separate without genetic analysis (BARGUES *et al.,* 2001; DURAND *et al.,* 2002), and the taxonomy of the whole group remains unclear (BARGUES *et al.,* 1997). The recent detection of lymnaeid vector species such as *G. truncatula* (BARGUES *et al*., 2006b, 2007), well known to be responsible for high prevalences and intensities of human fascioliasis in neighbouring and closely located countries such as Bolivia and Perú (BARGUES *et al*., 2012), adds concern to this question.

The aims of the present study, based on analyses of the sequences of the snails' 18S, ITS-1 and ITS-2 rDNA, and their cytochrome-c-oxidase-subunit-I (*cox*1) mitochondrial DNA (mtDNA), is to identify the members of the *Galba/Fossaria* group involved in transmission in Central and South America.

Species differentiation within this lymnaeid group is crucial given (i) their different disease transmission capacities to humans; (ii) the difficulties in species differentiation due to their pronounced morphological similarities; and (iii) the fact that there is a snail species of this group which, although usually present in high transmission areas, does not transmit (BARGUES *et al*., 2011c).

3.4.1.- GEOGRAPHICAL ORIGIN OF THE SPECIMENS STUDIED

In order to be systematically conclusive, specimens from the '*terra typica'* of each Latin American species of lymnaeid snail were used whenever possible. The exact type locality of *L. cubensis*, which was originally described from Cuba (PFEIFFER, 1839), is unknown but specimens from Vaqueria 21, Cuba, were selected for the present study. The original description of *L. viator* (D'ORBIGNY, 1835) includes two varieties: var. A ventricosa from three localities (Rio Negro area, Patagonia, Argentina, and two in Chile,

Santiago and Casablanca), and var. B elongata from Lima, Perú. European and South American specimens of *G. truncatula* were used for comparison.

The snail specimens investigated were of *L. cubensis* (from Vaqueria 21, La Habana, Cuba; 23º01'N, 82º32'W), *L. viator* var. A ventricosa (from Frias, Rio Negro, Argentina; 40º14'S, 64º10'W), *L. viator* var. B elongata (from Rio Rimac, Lima, Perú; 12º02'S, 76º56'-77º08'W), *L. viator* var. B elongata (from Rio Lurin, Lima, Perú; 12º03'S, 77º04'W), and *G. truncatula* (from Benicasim, Castellon, Spain; 40º06'N, 00º07'E), the latter just being used in the sequencing of *cox*1 mtDNA.

3.4.2.- MOLECULAR STUDY

3.4.2.1.- MOLECULAR MARKERS USED

The molecular characterisation of the snails has been made by DNA sequencing of the complete nuclear ribosomal 18S rRNA gene and the spacer markers ITS-2 and ITS-1 and a fragment of the mitochondrial mtDNA gene *cox*1. The usefulness of these markers has been already proven for invertebrates in general (MAS-COMA & BARGUES, 2009) and for the classification of the lymnaeid species and the comparative assessment of the intraspecific variability of their populations in many countries of Latin America (BARGUES & MAS-COMA, 2005, BARGUES *et al*., 20011,a,b), also including Argentina (BARGUES *et al*., 2006b, 2007, MERA Y SIERRA *et al*., 2009).

3.4.2.2.- DNA EXTRACTION

DNA was extracted from more than one specimen of a given population when this was deemed necessary for sequence verification. Only snails that appeared free of helminth infection were used in the molecular analyses. To reduce further the risk of contamination of DNA from helminths (which are more likely to be localized in other tissues), DNA was only isolated from the foot of each snail. Use of just the feet, rather than all the soft tissues, also prevented the development in the DNA pellets of the white flocculate substance (probably of polysaccharides) and melanic pigments that can

inhibit PCR and cause amplification of non-specific products (GASSER *et al.,* 1993; BARGUES *et al.,* 1997).

The phenol-chloroform method (SAMBROOK *et al.,* 1989) was used to extract total DNA from snail feet that had been fixed in 70% ethanol and stored at 4°C for several weeks (BARGUES and MAS-COMA, 1997), following the same procedure as previously described (see section 3.3.2)

3.4.2.3.- SEQUENCING OF rDNA AND mtDNA MARKERS

The complete rDNA small subunit (18SrRNA gene), the rDNA internal transcribed spacers (ITS-1 and ITS-2) and the *cox*1 mtDNA of each lymnaeid were amplified by PCR, using 4-6 μl of lymnaeid genomic DNA in each 50 μl reaction mixture (BARGUES & MAS-COMA, 1997; BARGUES *et al.,* 2001; MAS-COMA *et al.,* 2001). Eight conserved oligonucleotide primers were used for the amplification of five super imposed fragments of the 18S rRNA gene (BARGUES *et al.,* 1997), a 9600 thermocycler(Perkin Elmer, Waltham, MA) and a standard protocol (BARGUES *et al.,* 1995) being employed to amplify the specific 18S rDNA regions. The fragments corresponding to ITS-1 and ITS-2 were amplified using primers previously described (BARGUES *et al.,* 2001, 2006). A *cox*1 gene fragment was amplified using universal primers (FOLMER *et al.,* 1994).

PCR conditions were 30 cycles of 30 s at 94ºC, 30 s at 50ºC and 1 min at 72ºC, preceded by 30 s at 94ºC and followed by 7 min at 72ºCfor ITS-2 and ITS-1, and by 40 cycles of 30 s at 90ºC, 1 min at 48ºCand 1 min at 72ºC, preceded by 2.5 min at 94ºC and followed by10 min at 72ºC for *cox*1.

The products of each PCR (10 μl) were separated by electrophoresis in a gel of 1% Nusieve®GTG agarose (FMC Bioproducts, Rockland, ME) and visualized by staining with ethidium bromide and trans-illuminating with ultra-violet light. A set of molecular-weight markers (Marker VI; Boehringer Mannheim, Mannheim, Germany) containing 0.1 μg DNA/μl was run in each gel.

Primers and nucleotides were removed from the PCR products using a commercial DNA purification system (Wizard™ PCR Preps; Promega) before the products were resuspended in 50 μl 10 mMTE buffer (pH 7.6).The final DNA concentration was determined by measuring absorbances at 260 and 280 nm.

The sequencing, of the entire 18S rRNA gene, the complete ITS-1 and ITS-2 and a fragment of the *cox*1 gene was performed on both strands by the dideoxy chain-termination method (SANGER *et al.,* 1977). The Taq dye-terminator chemistry kit for the ABI 373A and ABI 3700 capillary system (Perkin Elmer) and the PCR primers were employed.

Sequences were aligned using version 1.8 of the CLUSTAL-W programme (THOMPSON *et al.,* 1994), with the sequences introduced in random order, to reduce bias (LAKE, 1991). Homologies were investigated using the BLAST programme, via the website of the United States National Center for Biotechnology Information (www.ncbi.nlm. nih.gov/BLAST). Genetic distances were estimated using parameters provided by version 4.0b10 of the PAUP programme (SWOFFORD, 2002). Alignments included not only the sequences determined in the present study but also relevant reference sequences stored in the GenBank databank. These reference sequences were those of the 18S rRNA genes of *G. truncatula* (accession Z73985; BARGUES & MAS-COMA, 1997) and *L. cubensis* (Z83831; BARGUES *et al.,* 1997), ITS-2, of haplotype H1, from *G. truncatula* from Spain, Portugal and Corsica, France (AJ296271), ITS-2, of haplotype H2, from *G. truncatula* from Spain, Portugal and Switzerland (AJ243017), ITS-2, of haplotype H3, from *G. truncatula* from Bolivia [= *L. viator* sensu Ueno *et al*. (1975) and *L. cubensis* sensu Ueno *et al*., (1975); accession AJ272051; BARGUES *et al.,* 2001; MAS-COMA *et al.,* 2001], ITS-1, of haplotype HA, from *G. truncatula* from Spain, Portugal, France and Switzerland (AJ243018), ITS-1, of haplotype HB, from *G. truncatula* from Morocco (AJ296270), and ITS-1, of haplotype HC, from *G. truncatula* from Bolivia (AJ272052;MAS-COMA *et al.,* 2001).

For haplotype nomenclature**,** the nomenclature proposed by BARGUES and MAS-COMA (2005) and BARGUES *et al*. (2006) for the ITS-1 and ITS-2 haplotypes of lymnaeid snails is used throughout. An 'H' followed by a lower-case letter (e.g. Ha) is used to identify the mtDNA *cox*1 haplotypes.

3.4.2.4.- REPRESENTATION OF THE 18S rRNA SECONDARY STRUCTURE

The previously published secondary structure prediction for *Limicolaria kambeul* 18S rRNA (WINNEPENNICKX *et al.,* 1992), which was based on the general eukaryote 18S-rRNA secondary structure (DE RIJK *et al.,* 1992), was used and extended to encompass the lymnaeid sequences. Version 2.54 of the DCSE programme (DE RIJK

& DE WACHTER, 1993) was used to examine potential secondary structures, with the RNA structure programme (MATHEWS *et al.,* 1999) employed to predict parts of the structure and the Rna Viz programme (DE RIJK & DE WACHTER, 1997) employed for the graphical representation.

3.4.2.5.- PHYLOGENETIC INFERENCE

Phylogenies were inferred, from ITS-1 and ITS-2 rDNA sequences, using maximum likelihood (ML) estimates with PAUP. Maximum-likelihood parameters such as model, base frequencies, transition/transversion ratio (ts/tv), the shape parameter for the gamma distribution, and the proportion of invariant sites, were optimised using the hierarchical likelihood ratio test (hLRT) and the Akaike information criterion (AKAIKE, 1974; POSADA & BUCKLEY, 2004), implemented in Modeltest 3.7 (POSADA & CRANDALL, 1998). Starting branch lengths were obtained using the least-squares method with ML distances. To provide an assessment of the reliability of the nodes of the trees, a quartet-puzzling analysis was employed, with 1000 puzzling steps.

Phylogenetic analyses were performed after adding reference sequences of ITS-2 and ITS-1 of lymnaeid rDNA stored in the GenBank databank. These sequences were of *L.(Stagnicola) palustris palustris*ITS-2 (AJ319620) and ITS-1 (AJ626849), *L. (S.) palustris turricola*ITS-2 (AJ319618) and ITS-1 (AJ626853), *L.(S.) fuscus*ITS-2 (AJ319621) and ITS-1 (AJ626856), *Catascopia occulta* ITS-2 (AJ319642) and ITS-1 (AJ626858), ITS-2 (AJ296271, AJ243017 and AJ272951, respectively) and ITS-1 (AJ243018, AJ272052 and AJ296270, respectively) of the H1, H2 and H3 haplotypes of *G. truncatula*, and the sequence from *P. columella* that includes both spacers (AY186751). The sequence (AY030361) including both spacers of a planorbid species, *Biomphalaria pfeifferi*, was used as outgroup.

3.4.3.- PHENOTYPIC STUDY

3.4.3.1.- SHELL MEASUREMENTS

Shells were measured, according to traditional malacological methods, under a calibrated stereomicroscope using a computerized image-analysis system (VALERO *et*

al., 2005). This system was based on a DXC-930P colour video camera (Sony, Tokyo) fitted to a stereomicroscope, and connected to a computer running image analysis software (ImagePro Plus 4.5; Media Cybernetics Inc., Silver Spring, MD). The morphometric study of the intraspecific variability of the snail shell included: shell length (SL), shell width (SW), last spire length (LSL), aperture length (AL), and aperture width (AW), as well as the ratios SL/SW, SL/AL and SL/LSL. Measurements were made following traditional malacological methods (OVIEDO *et al*., 1995; SAMADI *et al*., 2000).

3.4.3.2.- ANATOMICAL STUDIES

For anatomical studies, adult Peruvian *L. viator* var. B elongata were collected in the field and allowed to relax overnight in water containing menthol. They were then immersed for 40 s in hot water (70ºC) before transfer to water at room temperature. The soft parts were drawn from the shells with forceps applied to the cephalo-pedal mass, and fixed in slightly modified Railliet-Henry's fluid (930 ml distilled water, 6 g NaCl, 50 ml 40% formalin, and 20 ml glacial acetic acid). The fixed snails were then dissected under a stereomicroscope, so that drawings of the reproductive system could be made using a camera lucida (POINTIER *et al.,* 2006).

3.4.4.- MOLECULAR RESULTS

The results of the molecular analyses indicate that a new species, for which the name *L. neotropica* n. sp. (synomin of *L. viator* var. B elongata) is proposed and a diagnostic description provided, may be involved in the transmission of *F. hepatica* to humans and other mammals in Latin America.

3.4.4.1.- RESULTS OBTAINED IN THE STUDY OF rDNA MARKERS

3.4.4.1.1.- 18S rRNA GENE

The 18S rDNA sequence of *L. cubensis* from Cuba is 1860-bp long, with a GC content of 51.82%, and has base frequencies of 0.236 (A), 0.235 (G), 0.283 (C) and0.245 (T). This sequence is identical to that deposited in GenBank by BARGUES *et al*. (1997).

The sequence of the 18S rRNA gene of *L. viator* var. A ventricosa from Argentina is 1,860-bp long, shows a GC content of 51.82%, and has base frequencies of 0.236 (A), 0.236 (G), 0.282 (C) and 0.245 (T). The sequences of the 18S of *L. neotropica (=L. viator* var. B elongata*)* from the two localities in Lima, Perú, were identical, base to base, and did not differ, even by a single nucleotide, from the corresponding sequence of *L. viator* var. A ventricosa. This 18S sequence has been deposited in GenBank as accession AM412222.

The 18S sequence of *L. cubensis* differs from that of *L. viator* var. A ventricosa and *L. neotropica (= L. viator* var. B elongata*)* by a nucleotide divergence of only 0.32%, represented by six nucleotide differences [one transition (ts), three transversions (tv), and two insertions/deletions (indels)] in a 1,861-bp long alignment. According to the secondary structure, the four mutations appear to be located in helix E10-1 of the V2 variable area whereas the two indels appear to be scattered throughout the rest of the 18S sequence (Table 8).

The 18S sequence of *L. cubensis* differs much more from that of *G. truncatula*, with a nucleotide divergence of 1.50%, represented by 28 nucleotide differences (five ts, four tv and 19 indels) in a 1,861-bp-long alignment. Again, the secondary structure indicated that the differences were concentrated in E10-1 of V2 (Table 8).

Curiously, the 18S sequences indicate the same level of difference between *L. cubensis* and *G. truncatula* as between *L. viator* and *G. truncatula*, the latter comparison also showing a nucleotide divergence of 1.50%, represented by 28 nucleotide differences (six ts, three tv and 19 indels) in a 1,861-bp-long alignment. The majority of these differences also appear in helix E10-1 of V2 (Table 8).

The triple-comparison alignment, including the 18S sequences of *L. cubensis*, *L. viator* and *G. truncatula*, was 1,862-bp long and included 31 variable nucleotide positions, giving a nucleotide divergence of 1.66% (Table 8). The majority (24) of the

modified positions appeared to be grouped in the short sequence between positions 231 and 263, whereas the other seven modified positions appeared isolated and scattered throughout the rest of the 18S sequence (Table 8).

3.4.4.1.2.- ITS-2 rDNA

The three ITS-2 sequences of the four lymnaeid populations studied were deposited in GenBank as accessions AM412223 (*L. cubensis* haplotype H1 from Cuba), AM412224 (*L. viator* var. A ventricosa haplotype H1 from Argentina), and AM412225 (*L. neotropica* = *L. viator* var. B elongata haplotype H1 from Rio Rimac and Rio Lurin, Lima, Perú). The lengths and slightly GC-biased mean nucleotide compositions of these ITS-2 sequences were, respectively, 534 bp and 56.22% in *L. cubensis*, 452 bp and 54.20% in *L. viator* var. A ventricosa, and 417 bp and 56.83% in *L. neotropica*.

The numbers and percentages of nucleotide differences that appeared in the two sequence alignments of the ITS-2 sequences, and details of the ts, tv and indels, are shown in Table 9. The numbers of repeats of each microsatellite found in these comparisons are noted in Table 10. The ITS-2 sequence of *L. cubensis* was unusually long because of a tetra nucleotide microsatellite (CTTG) that appears to be repeated 25 times, consecutively, in positions 51-150.

The ITS-2 sequences of the specimens of *L. neotropica* from the populations of Rio Lurin and Rio Rimac were identical. They differed from those of *L. viator* var. A ventricosa at 53 positions, giving a relatively large nucleotide divergence of 11.67%. Eleven of the 39 indels found were not related to microsatellite repeats. The CTTG microsatellite seen in *L. cubensis* also appears in both *L. viator* var. A ventricosa and *L. neotropica* (= *L. viator* var. B elongata), together with other two tetra nucleotide repeats (TTCA and TGAA).

The number of variable positions (124) and level of nucleotide divergence (22.67%) were higher in the comparison between *L. cubensis* and *L. viator* var. A ventricosa. Amongst the numerous indels, most (76) were related to the CTTG repeat but other microsatellites, including the trinucleotide GGT and the tetranucleotides GCAA, TGAA and TCGA, were also present (Table 10).

Table 8.-Nucleotide differences found in comparisons of the complete 18S ribosomal DNA sequences of the lymnaeids, and their location in the secondary structure (BARGUES *et al*., 2007a)

Variable	Position, code numbers or sequences			
Variable area*	V V V V V V V V V V V V V V V V V V V V V V V V V V		V V V	V
	2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2		4 4 5	9
Helix*	E E E E E E E E E E E E E E E E E E E E E E E E E E	1	E E 2	3 3 4
	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	5	2 2 7	4 6 7
	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0		1 1	
	_ _		_ _	
	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1		1 7	
Position**			1	1 1 1
	2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2	4	7 8 1	2 2 7
	3 3 3 3 3 3 3 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 6	5	2 5 3	3 9 4
	1 2 3 4 5 6 7 2 3 5 6 7 8 9 0 1 2 3 4 5 6 7 8 3	9	1 2 5	0 9 7
L. cubensis Cuba	G T C G T G C G T C A A G C C G T G G T C G C C	C	G G _	_ T C
L. viator var. A ventricosa Argentina	G T G C C T C G T C A A G C C G T G G T C G C C	C	_ G _	C T C
L. viator var. B elongata Perú	G T C C C T C G T C A A G C C G T G G T C G C C	C	_ G_	C T C
G. truncatula Europe	_ _ C C T T T A G _ _ _ _ _ _ _ _ _ _ _ _ _ _ T	_	_ A C	_ G T
Difference number***	1 1 1 1 1 1 1 1 1 1 2 2 2 2 2	2	2 2 2	2 3 3
	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4	5	6 7 8	9 0 1

*Code numbers (to be read vertically) refer to the secondary structure proposed for the gastropod *Limicolaria kambeul* (Winnepenniekx *et al.,* 1992), which was based on the model and nomenclature for eukaryotes (De Rijk *et al.,* 1992).

** Numbers (to be read vertically) refer to positions obtained in the alignment made with CLUSTAL W.

*** To be read vertically.

In the comparison of *L. cubensis* with *L. neotropica* (= *L. viator* var. B elongata), the number of variable positions (143) and level of nucleotide divergence (26.78%) were even higher. Again, most of the indels detected (92) were related to the CTTG repeat. The microsatellites GGT, GCAA, TTCA, TGAA and TCGA, however, did not appear to be repeated in *L. neotropica* (=*L. viator* var. B elongata) (Table 10).

When the ITS-2 sequences of the *G. truncatula* haplotypes H1, H2 and H3 (which differed by only one or two mutations) were compared with those of the three taxa of American lymnaeids, a high number of nucleotide differences appeared in the alignments and a notable equidistance in the true mutations was observed (see Table 9). The results of the PAUP-based analysis of the pair-wise comparisons of ITS-2 sequences are shown in Table 11.

Table 9.-ITS-2 sequence differences detected in pair-wise comparisons between the lymnaeid species studied (BARGUES *et al*., 2007a).

Comparison	Alignment length (bp)	No. and (%) of nucleotide differences	No. and (%) of substitutions		No. and (%) of insertions/deletions
			Transitions	Transversions	
L. cubensis v. *L. viator* var. A ventricosa	547	124 (22.67)	8 (1.46)	8 (1.46)	108 (19.74)
L. cubensis v. *L. viator* var. B elongata	534	143 (26.78)	11 (2.05)	14 (2.05)	118 (22.09)
L. viator var. A ventricosa. *L. viator* var. B elongata	454	53 (11.67)	9 (1.98)	5 (1.10)	39 (8.59)
L. cubensis. *G. truncatula* (ITS-2 haplotypes 1, 2 and 3)	548	197-199 (35.94-36.31)	18-20 (3.28-3.65)	17-18 (3.10-3.28)	161 (29.38)
L. viator var. A ventricosa v. *G. truncatula* (ITS-2 haplotypes 1, 2 and 3)	465	113-114 (24.30-24.52)	11-12(2.36-2.58)	24-25 (5.16-5.38)	77 (16.56)
L. viator var. B elongata v. *G. truncatula* (ITS-2 haplotypes 1, 2 and 3)	438	93-94 (21.23-21.46)	13-14 (2.97-3.20)	21-22 (4.79-5.02)	58 (13.24)

Table 10.- The microsatellites in the ribosomal DNA ITS-2 sequences of the American lymnaeids studied, and number of times each one was repeated in pair-wise comparisons (BARGUES *et al*., 2007a).

Comparison	**Alignment length (bp)**	**Repeat**	**Start point (position no.)**	**No. of times each microsatellite appears**		
				Lymnaea cubensis	***L. viator* var. A** ventricosa	***L. viator* var. B** elongata
L. cubensis v. *L. viator* var. A ventricosa	547	CTTG	51	25	6	-
		GGT	328	3	1	-
		GCAA	431	2	1	-
		TGAA	487	2	4	-
		TCGA	529	2	1	-
			51	25	-	2
L. cubensis v. *L. viator* var. B elongata	540	CTTG	51	25	-	2
		GGT	328	3		0
		GCAA	431	2	-	1
		TTCA	445	2	-	1
		TGAA	487	2	-	1
		TCGA	529	2	-	1
L. viator var. A ventricosa v. *L. viator* var. B elongata	454	CTTG	51	-	6	2
		TTCA	352	-	2	1
		TGAA	398	-	3	1

Table 11.-Total character differences (above diagonal) and mean character differences adjusted for missing data (below diagonal), as determined using the PAUP programme, in pair-wise comparisons between the ITS-2 sequences of the lymnaeid species (BARGUES *et al*., 2007a).

	***G. truncatula* of ITS-2 haplotype:**					
	1	**2**	**3**	***L. viator* var. B** elongata	***L. viator* var. A** ventricosa	***L. cubensis***
G. truncatula						
ITS-2 haplotype 1	-	0.00249	0.08995	0.08995	0.09278	0.11340
ITS-2 haplotype 2	1	0.00499	0.08730	0.08730	0.09021	0.11082
ITS-2 haplotype 3	1	-	0.08730	0.08730	0.09021	0.11082
L. viator var. B elongata	34	33	33	-	0.03373	0.07090
L. *viator var*. A ventricosa	36	35	35	14	-	0.07289
L. cubensis	44	43	43	29	32	-

Table 12.- ITS-1 sequence differences detected in pair-wise comparisons between the lymnaeid species studied (BARGUES *et al*., 2007a).

Comparison	Alignment length (bp)	No. and (%) of nucleotide differences	No. and (%) of substitutions		No. and (%) of insertions/deletions
			Transitions	Transversions	
Lymnaea cubensis v. *L. viator* var. A ventricosa	569	86 (15.11)	18 (3.16)	22 (3.87)	46 (8.08)
L. cubensis v. *L. viator* var. B elongata	544	72 (13.23)	20 (3.67)	21 (3.87)	31 (5.69)
L. viator var. A ventricosa v. *L. viator* var. B elongata	570	98 (17.19)	30 (5.26)	29 (5.08)	39 (6.84)
L. cubensis v. *G. truncatula* (ITS-1 haplotypes A, B and C)	549	126-128 (22.95-24.78)	27-28 (4.92-5.10)	29-30 (5.28-5.46)	70 (12.75)
L. viator var. A ventricosa v. *G. truncatula* (ITS-1 haplotypes A, B and C)	569	139-141 (24.43-24.78)	34-35 (5.97-6.15)	39-40 (6.85-7.03)	66 (11.59)
L. viator var. B elongata v. *G. truncatula* (ITS-1 haplotypes A, B and C)	544	131-133 (24.08-24.45)	40-41 (7.35-7.54)	40-41 (7.35-7.54)	51 (9.37)

3.4.4.1.3.- ITS-1 rDNA

The three ITS-1 sequences of the four lymnaeid populations studied were deposited in GenBank as accessions AM412226 (*L. cubensis* haplotype HA from Cuba), AM412227 (*L. viator* var. A ventricosa haplotype HA from Argentina) and AM412228 (*L. neotropica* = *L. viator* var. B elongata haplotype HA from Rio Rimac and Rio Lurin, Lima, Perú). The lengths and slightly GC-biased mean nucleotide compositions of the ITS-1 sequences were, respectively, 524 bp and 56.29% in *L. cubensis*, 568 bp and 54.58% in *L. viator* var. A ventricosa, and 533 bp and 56.66% in *L. neotropica* (= *L. viator* var. B elongata).

The numbers and percentages of nucleotide differences that appeared in the two sequence alignments of the ITS-1 sequences, and details of the ts, tv and indels, are shown in Table 12.

The ITS-1 sequence of *L. cubensis* shows no microsatellite repeat. Those of the specimens of *L. neotropica* (= *L. viator* var. B elongata*)* from the populations of Rio Rimac and Rio Lurin in Lima, Perú, were identical but differed from the ITS-1 sequence of *L. viator* var. A ventricosa at 98 positions, giving a considerable nucleotide divergence of 17.19%. This divergence is markedly higher than that seen in ITS-2, with no microsatellite repeats found in the ITS-1 sequences.

Although the ITS-1 comparisons between *L. cubensis* and either *L. viator* var. A ventricosa or *L. neotropica* (= *L. viator* var. B elongata*)* showed numerous nucleotide differences, the levels of nucleotide divergence were less than those seen with ITS-2. A large number of nucleotide differences was also apparent in the alignments of the ITS-1 sequences of *G. truncatula* haplotypes HA, HB or HC (the sequences of which differed by only one, two or three mutations) with those of the three taxa of American lymnaeid (Table 12). The results of the PAUP-based analysis of the pair-wise comparisons of ITS-2 sequences are shown in Table 13.

Table 13.- Total character differences (above diagonal) and mean character differences adjusted for missing data (below diagonal), as determined using the PAUP programme, in pair-wise comparisons between the ITS-1 sequences of the lymnaeid species (BARGUES *et al.*, 2007a).

	***Galba truncatula* of ITS-1 haplotype**					
	A	**B**	**C**	***L. cubensis***	***L. viator* var. A** ventricosa	***L. viator* var. B** elongata
G. truncatula						
ITS-1 haplotype A	-	0.00198	0.00397	0.12343	0.15339	0.16057
ITS-1 haplotype B	1	-	0.00595	0.12552	0.15538	0.16260
ITS-1 haplotype C	2	3	-	0.12762	0.15737	0.16463
L. cubensis	59	60	61	-	0.076648	0.06126
L. viator var. A ventricosa	77	78	79	40	-	0.09943
L. viator var. B elongata	79	80	81	31	52	-

3.4.4.2.- RESULTS OBTAINED IN THE STUDY OF mtDNA MARKERS

3.4.4.2.1.-*cox*1 mtDNA

The three *cox*1 sequences of the four lymnaeid populations studied were deposited in GenBank as accessions AM494009 (*L. cubensis* haplotype Ha from Cuba), AM494010 (*L. viator* var. A ventricosa haplotype Ha from Argentina), AM494008 (*L. neotropica* = *L. viator* var. B elongata haplotype Ha from Rio Rimac and Rio Lurin, Lima, Perú), and AM494011 (*G. truncatula* corresponding to snails belonging to the rDNA ITS combined haplotype CH1A from Benicasim, Castellon, Spain). The lengths and highly AT-biased mean nucleotide compositions of the *cox1* sequence were, respectively, 672 bp and 68.45% in *L. cubensis*, 672 bp and 69.39% in *L. viator* var. A ventricosa, 672 bp and 69.93% in *L. neotropica* (= *L. viator* var. B elongata), and 672 bp and 68.45% in *G. truncatula* Ha.

The numbers and percentages of nucleotide differences that appeared in the two sequence alignments of the *cox1* sequences, and details of the ts, tv and indels, are shown in Table 14.

The *cox1* sequences of the specimens of *L. neotropica* (= *L. viator* var. B elongata) from the populations of Rio Rimac and Rio Lurin in Lima, Perú, were identical but differed from those of *L. viator* var. A ventricosa at 29 positions, giving a nucleotide divergence of 4.31%. In the *cox*1 comparisons of *L. cubensis* with either *L. viator* var. A ventricosa or L. neotropica (= *L. viator* var. B elongata*)*, numerous nucleotide differences were apparent although the levels of divergences were less than those seen in ITS-2 and ITS-1, mainly because of the absence of indels. Large numbers of nucleotide differences were also apparent in the alignments of the *cox*1 sequence of *G. truncatula* Ha with those of the three taxa of American lymnaeid (Table 14).

The results of the PAUP-based analysis of the pair-wise comparisons of *cox*1 sequences are shown in Table 15.

For each of the four lymnaeid taxa studied, the amino-acid sequence of the *cox*1 gene fragment that was obtained was 224 acids long. Pairs-wise comparison at the level of these *COX*1 amino-acid sequences indicated 100% identity between *L. cubensis* and *L. neotropica* (= *L. viator* var. B elongata), and only one amino-acid difference (isoleucine/ valine, at position 11) between them and *L. viator* var.

Table 14.- *cox*1 sequence differences detected in pair-wise comparisons between the lymnaeid species studied (BARGUES *et al*., 2007a).

Comparison	Alignment length (bp)	No. and (%) of nucleotide differences	No. and (%) of substitutions		No. and (%) of insertions/ deletions
			Transitions	Transversions	
L. cubensis v. *L. viator* var. A ventricosa	672	38 (5.65)	32 (4.76)	6 (0.89)	0 (0)
L. cubensis v. *L. viator* var. B elongata	672	14 (2.08)	14 (2.08)	0 (0)	0 (0)
L. viator var. A ventricosa v. *L. viator* var. elongata	672	29 (4.31)	23 (3.42)	6 (0.89)	0 (0)
L. cubensis v. *G. truncatula* (COI haplotype Ha)	672	75 (11.16)	43 (6.39)	32 (4.76)	0 (0)
L. viator var. A ventricosa v. *G. truncatula* (COI haplotype Ha)	672	68 (10.11)	38 (5.65)	30 (4.46)	0 (0)
L. viator var. B elongata v. *G. truncatula* (COI haplotype Ha)	672	67 (9.97)	35 (5.21)	32 (4.76)	0 (0)

Table 15.- Total character differences (above diagonal) and mean character differences adjusted for missing data (below diagonal), as determined using the PAUP programme, in pair-wise comparisons between the COI sequences of the lymnaeids species (BARGUES *et al*., 2007a).

	***L. viator* var. B** elongata	***L. cubensis***	***L. viator* var. A** ventricosa	***G. truncatula* (*cox*1 haplotype Ha)**
L. viator var. B elongata	-	0.02083	0.04315	0.09968
L. cubensis	14	-	0.05655	0.11158
L. viator var. A ventricosa	29	38	-	0.10117
G. truncatula (COI haplotype Ha)	67	75	68	-

A ventricosa. In terms of these amino-acid sequences, *G. truncatula* was identical to *L. cubensis* and *L. neotropica* (= *L. viator* var. B elongata).

3.4.4.3.- RESULTS OBTAINED IN THE PHYLOGENETIC ANALYSES

The combination of the two internal transcribed spacers in a single data set generated a robust tree, indicating phylogenetic accordance between the two spacers. The ML model best fitting this data-set was HKY85+G, using a ts/tv ratio of 1.12 (kappa=.2.2016), base frequencies for A, C, G and T of 0.3361, 0.1419, 0.1787 and 0.3433, respectively, a proportion of invariable sites=0, and a gamma-distribution shape parameter of 0.59. The resulting phylogeny (-Ln=6314.7671) was evaluated using a least-squares method with ML distances, with high puzzle values supporting the reliability of the nodes of the main branches.

In the tree obtained, *L. neotropica* (= *L. viator* var. B elongata) appears as sister species, with maximum support (puzzle value=100%), in the same clade as *L. cubensis* and *L. viator* var. A ventricosa, the latter two taxa also clustering with maximum support. The branch holding all three haplotypes of *G. truncatula* is grouped within the same *Galba/Fossaria* clade as *L. neotropica* (= *L. viator* var. B elongata), *L. cubensis* and *L. viator* var. A ventricosa. Stagnicolines are grouped in the other main clade. The European *Lymnaea* (*Stagnicola*) species are grouped together in a well supported branch, with the American *P. columella* and the Palearctic *C. occulta* appearing as basal species (Figure 7).

3.4.5.- PHENOTYPIC RESULTS

3.4.5.1.- SYSTEMATIC-TAXONOMIC CHARACTERISTICS OF *LYMNAEA NEOTROPICA* N.SP.

As a variety is not a taxonomic entity and a variety name cannot therefore be raised to level, the new species *Lymnaea neotropica* n. sp. is here proposed for the lymnaeid specimens from Lima previously known as *Lymnaea viator* var. B elongata. To avoid further confusion, the type locality for *L. viator* var. B elongata (D'ORBIGNY, 1835) the

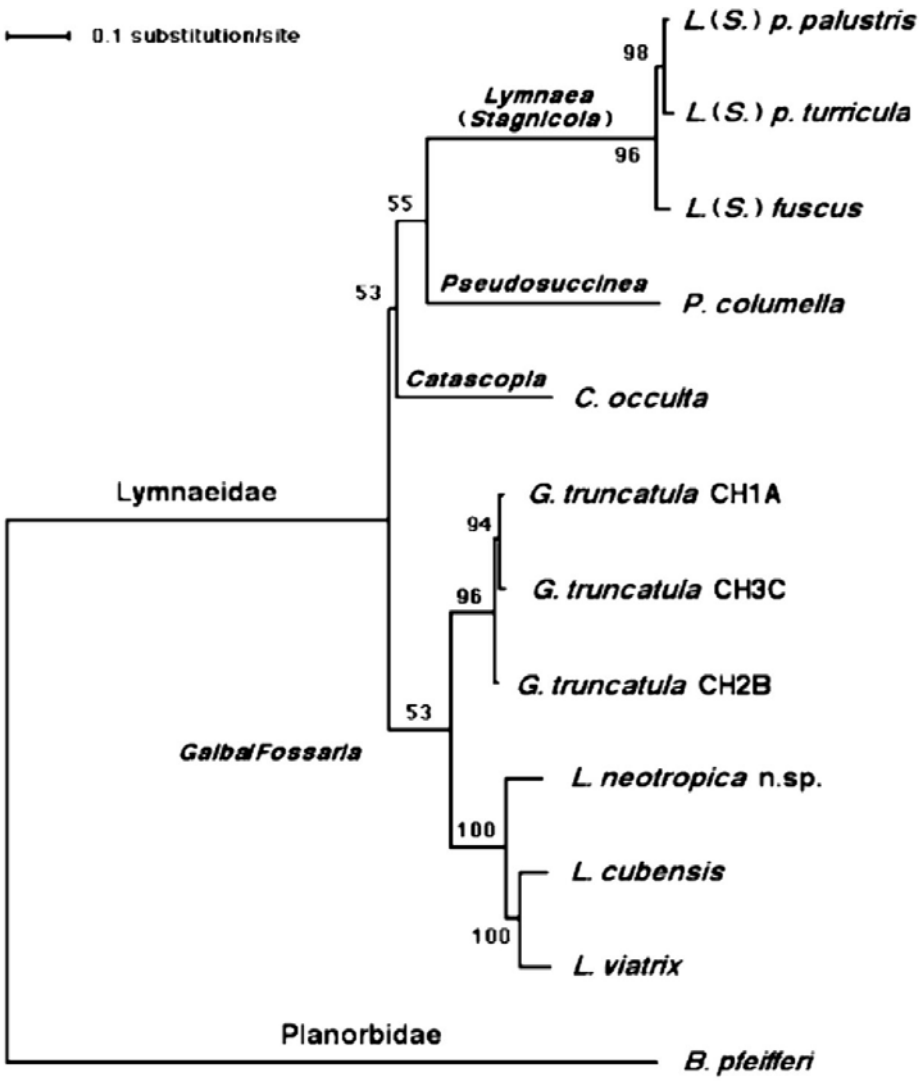


Fig. 7.- A phylogenetic tree of lymnaeid snails species based on ITS-1 and ITS-2 ribosomal sequences. The tree was derived from a maximum-likelihood model (HKY85+G), using a transition transversion ratio of 1.12, with a planorbid species *Biomphalaria pfeifferi* as an outgroup. The numbers on the nodes represent the percentage of 1000 puzzling replicates. (BARGUES *et al*., 2007a).

Rio Rimac at Lima, Perú is selected as type locality for the new species and *L. viator* var B. elongata becomes a synonym of *L. neotropica* n. sp. *Lymnaea viator* (= *L. viator* var. A ventricosa) is hence characterized by the features of the lymnaeids coming from Frias, Rio Negro, Argentina.

In summary the systematic-taxonomic characteristics of *L. neotropica* n. sp. are:

Synonym: *Lymnaea viator* var. B elongata D'ORBIGNY, 1835.

Type Locality: Irrigation canals running from the Rio Rimac, near Lima and Callao, Perú (12°02´S, 76°56´-77°08´W).

Other locality: Rio Lurin, Lima, Perú (12°03´S-77°04´W).

Type specimens: Holotype and a paratype deposited in the Muséum National d'Histoire Naturelle (MNHN) in Paris, France. Other voucher specimens deposited in the parasite collection of the Department of Parasitology, University of Valencia, Valencia, Spain.

3.4.5.2.- DESCRIPTION OF THE SHELL

The form of the shell is illustrated in Figure 8. Shell length in the specimens measured varied between 5.89 and 8.74 mm, with a mean of 7.13 and a standard deviation (SD) of 1.20 mm. Maximum width varied from 3.29 to 4.56 mm, with a mean of 3.95 mm, SD of 0.54 mm. The length/width ratio varied from 1.69 to 1.92, with a mean of 1.80, SD of 0.07. The shell is formed of 5.5 convex and deeply sutured whorls that gradually increase in diameter, with a shell spiral angle of 38.64° to 46.99° with a mean of 42.73° (SD=2.44). The last whorl is 4.47 to 6.78 mm long with a mean of 5.58mm, (SD=0.91), with a shell/last-whorl length ratio of 1.21 to 1.38, mean of 1.28, (SD=0.05). The spire is more-or-less pointed, or short with a rather obtuse apex. Growth lines and umbilicus are slightly pronounced. The spiral sculpture is very faint and not easily seen. The aperture is oval and almost round, with a thin peristome. The aperture measures 2.90 to 4.53 mm, mean 3.75mm (SD=0.64) in length and the width measures from 1.97 to 2.99 mm, mean 2.46 mm (SD=0.44). The length of the aperture is about half the shell length, with a shell/aperture length ratio of 1.76 to 2.03, mean 1.91 (SD=0.09).

Shells up to 10 mm long and 5.6 mm wide, with length/width ratios varying from 1.61 to 1.85 (with a mean of 1.75), a whorl increase ratio of 1.71 to 2.09 (with a mean of

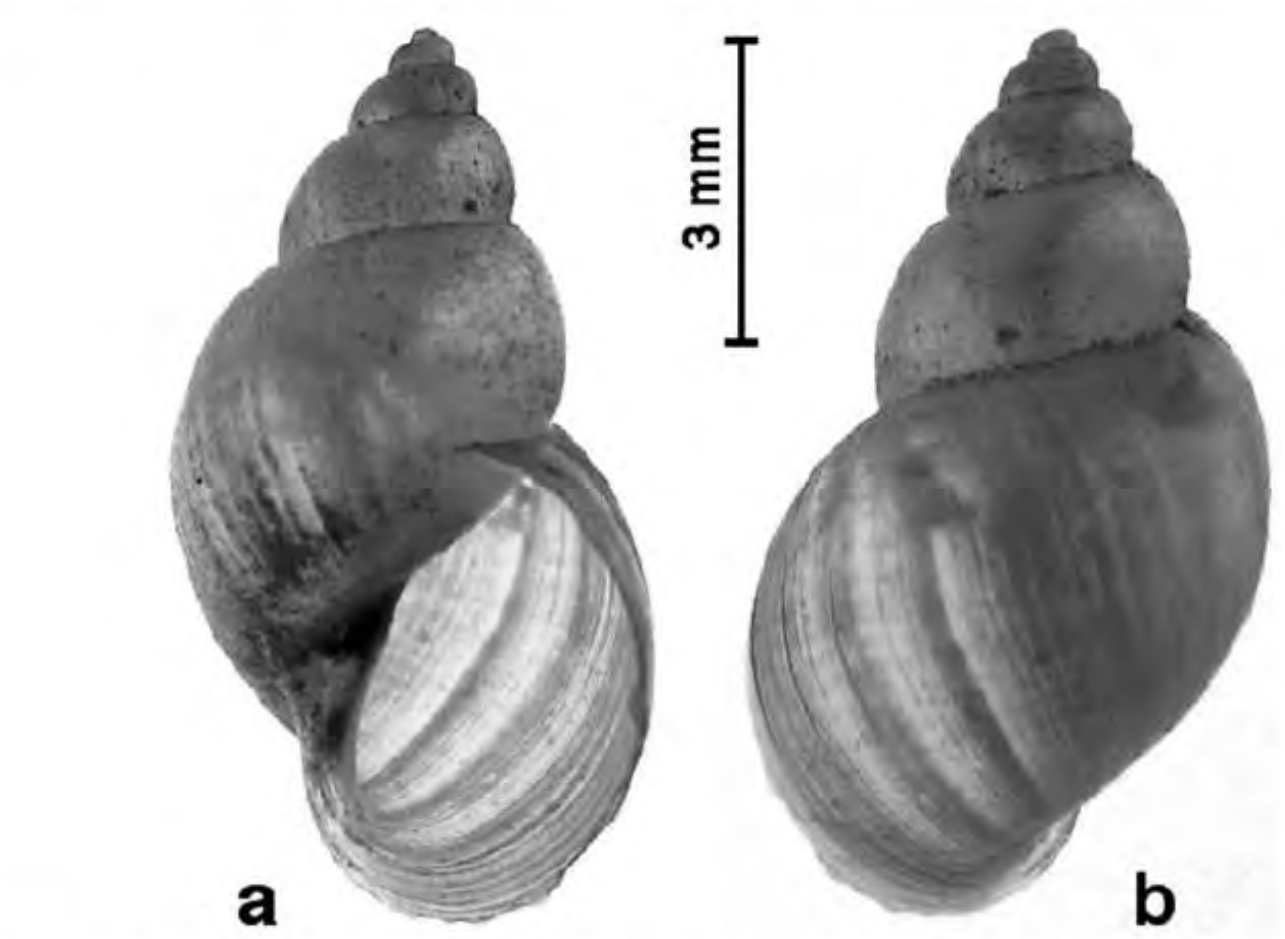


Fig. 8.- The shell of *Lymnaea neotropica* n. sp., showing ventral (a) and dorsal (b) views . (BARGUES *et al*., 2007a).

1.88), and shell/aperture length ratios of 1.81 to 2.02 (with a mean of 1.90) were recorded by PARAENSE (1976).

3.4.5.3.- DESCRIPTION OF THE ANATOMY

The morphology of the reproductive system is illustrated in Figure 9. The ovotestis, which has a lobulate surface formed by numerous acini, has a collecting canal that continues into the ovispermiduct. The latter, which appears to be a short and very thin tube, empties into the wider seminal vesicle, which has a bosselated surface and leads into an egg-shaped chamber which is located between the albumen gland, oviduct and spermiduct.

The voluminous albumen gland is roughly bean-shaped and has a funnel shaped duct that opens into the egg-shaped chamber. The oviduct is so markedly contorted that it nearly describes a complete circle around the spermiduct. It is in contact with the albumen gland, and, near to its cephalic end, has a wrinkle-walled, expanded pouch projecting from its right dorsal side. The final, thick-walled part of the oviduct is roughly cylindrical and continues into the nidamental gland. The nidamental gland is oblong, with rounded shoulders, and is initially wider than the cephalic portion of the oviduct before narrowing into a short uterus followed by a short vagina.

The spermatheca is round-ovoid, with a narrow and relatively long duct. Although the first expanded part of the spermiduct shows a granular surface, the duct becomes gradually thinner and smoother, as it runs across the ventral surface of the nidamental gland, before again widening to form a prostate with a granular surface. The prostate, which is generally ovoid but not infrequently elongate or pear- or spindle shaped, measures 580-1,120 µm, with a mean of 782 µm (S.D.=1.12) in length and is flattened dorsoventrally at its caudal end. The vas deferens emerges from the opposite part of the prostate, runs for short distance across the vagina and then forms a long caudal loop before emptying in a curved, cylindrical penis sheath. The penis is very slender, acicular, unarmed, with a terminal outlet, and about as long as the sheath. The penis sheath is frequently more-or-less invaginated into the preputium, from which it is separated, internally, by the sarcobelum, a perforated papilla mainly consisting of a gland-cell aggregate. The preputium is markedly wider and longer than the penis sheath, the latter appearing to be more than half as long as the preputium in around 40% of the specimens examined. The preputium/penis-sheath length ratio varied from

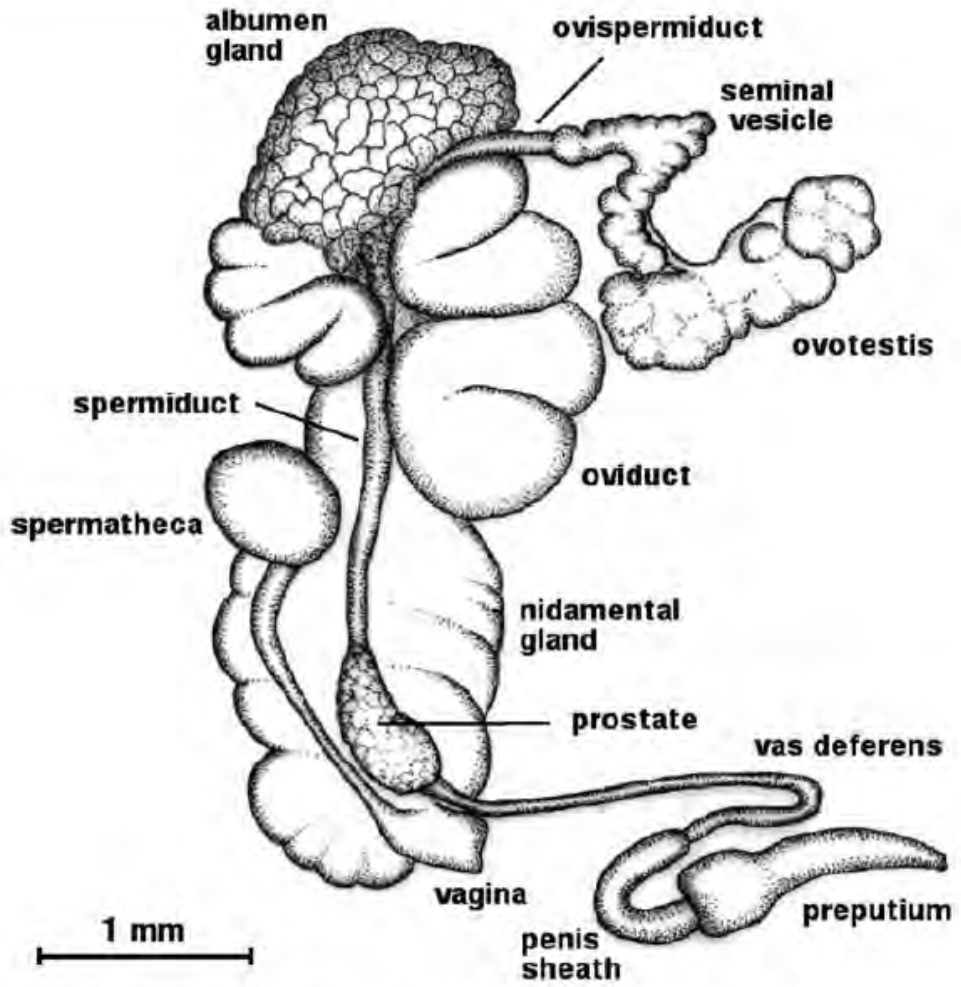


Fig. 9.- Anatomy of the reproductive system of *Lymnaea neotropica* (BARGUES *et al*., 2007a).

1.3-3.4, with a mean of 2.12 (SD=0.28) in the present study (and from 1.1-3.9, whit a mean of 2.7) in the study by PARAENSE (1976).

The central tooth of the radula is bicuspid, with a tiny cusp or protuberance in addition to the main cusp. The lateral teeth are usually bicuspid, although the first laterals may occasionally be tricuspid or very rarely quadricuspid. The intermediate teeth have the mesocone split into two parts, and the marginal teeth are multicuspidate. The teeth often appear irregularly arranged, groups of teeth with well divided cusps sometimes alternating with groups of teeth with relatively undivided cusps.

For additional information on the anatomy of the soft parts of this species see PARAENSE (1976).

3.4.6.- DISCUSSION

3.4.6.1.- EVALUATION OF MOLECULAR MARKERS USED IN LYMNAEIDS

For the Lymnaeidae in general, the four DNA markers used in the present study have the advantage of furnishing information at different levels. The 18S rRNA gene, for example, has a conserved, slowly evolving sequence that is useful mainly for supraspecific analyses. The ITS-2 andITS-1 sequences evolve relatively fast and are useful for species differentiation, although haplotyping also enables population characterisation within a species (BARGUES & MAS-COMA, 2005). *Cox*1 appears to evolve faster than 18S and ITS-2 but slower than ITS-1, its amino-acid sequence offering information at a similar or higher level than that furnished by the 18S rRNA gene.

The characteristics of the 18S rDNA sequences of *L. cubensis*, *L. viator* (= *L. viator* var. A ventricosa) and *L. neotropica* n. sp. (= *L. viator* var. B elongata) determined in the present study appear similar to those known for other lymnaeids, the localization of most nucleotide differences in the E10-1 helix of the variable area V2 being a common feature (BARGUES & MAS-COMA, 1997, 2005; BARGUES *et al.,* 1997). Interestingly, in spite of the morpho-anatomical similarity of the lymnaeid species studied, there are differences not only between *L. cubensis*, *L. viator* (= *L. viator* var. A ventricosa) and *L. neotropica* n. sp. (= *L. viator* var. B elongata), but also numerous differences between *G. truncatula* and the American lymnaeids, indicating that the lymnaeids investigated in detail in the present study may cover more than one supraspecific taxon.

The ITS-2 sequences of the lymnaeids investigated in the present study show a high number of microsatellite repeats. Microsatellites have already been detected in the ITS-2 of other lymnaeids (BARGUES *et al.,* 2001, 2003) but the details of their origin, mutation, evolution and function, if any, have yet to be elucidated (JARNE *et al.,* 1998). In theory at least, as microsatellite alleles exhibit extreme intraespecific variability, neutrality, Mendeleyan inheritance, co dominance and high mutation rates, they should form very good polymorphic molecular markers for the differentiation of populations within a given species (JARNE & LAGODA, 1996; ROOS *et al.,* 1998).

Microsatellites of ITS-2 may thus be useful markers for population differentiation in species of the *Galba/Fossaria* group, although their use must be handled with caution. When the ts, tv and indels not related to microsatellites are considered, the nucleotide differences and genetic distances observed, in the present study, between *L. viator* (= *L. viator* var. A ventricosa) and *L. neotropica* n. sp. (= *L.viator* var. B elongata) are similar to those known between some closely related but systematically distinct lymnaeid species and markedly greater than observed within some lymnaeid species that show high levels of intraespecific variability (BARGUES *et al.,* 2001, 2003). As no microsatellites were detected in ITS-1, all nucleotide differences seen in the sequence of this spacer may be considered for species differentiation. In all the pair wise comparisons in the present study, the differences seen in ITS-1 (Table 13) were greater than those in ITS-2 (Table 11). This was the expected result, as ITS-1 evolves faster than ITS-2 (BARGUES & MAS-COMA, 2005; BARGUES *et al.,* 2006a).

As with ITS-2, the ITS-1 sequences determined in the present study support species status for *L. cubensis*, *L. viator* var. A ventricosa, *L. neotropica* (= *L. viator* var. B elongata) and *G. truncatula* (BARGUES *et al.,* 2006a, 2007b).

3.4.6.2.- EVALUATION OF MOLECULAR AND MORPHOANATOMICAL STUDIES IN THE *GALBA/FOSSARIA* SPECIES

The results of the present DNA sequencing generally support those of recent phenotypic and morpho-anatomical studies performed on lymnaeid samples of the same species, varieties and type localities. In the phenotypic study by DURAND *et al*. (2002), for example, an analysis of variability based on 12 iso-enzyme loci revealed significant differences between *L. viator* var. B elongata (from the type locality in Lima) and *L. viator* var. A ventricosa (collected in Bahia Blanca, Argentina, about 350 km

north of the type locality of this variety, in Rio Negro), as well as between the *L. viator* var. B elongata and *L. cubensis* from Cuba. In their morpho-anatomical study, POINTIER *et al*. (2006) found that, in terms of the respective lengths of the penis sheath and preputium, *L. viator* var. A ventricosa was markedly different from *L. cubensis* or *L. viator* var. B elongata (although, none of the morpho-anatomical parameters that were studied allowed *L. cubensis* to be distinguished from *L. viator* var. B elongata).

The three species *L. cubensis*, *L. viator* and *L. neotropica* n. sp. are kept within the genus *Lymnaea* s.l. for the time being, awaiting a general review of the Lymnaeidae from Latin America which will include an appropriate systematic-taxonomic analysis of the taxa currently recognized within the *Galba/Fossaria* group. The relevant 18S rDNA sequences (BARGUES & MAS-COMA, 1997, 2005; BARGUES *et al*., 2007b) already indicate that even though these four species have similar shells (some old specimens of *L. cubensis* are larger than *G. truncatula*) and are difficult to distinguish anatomically (SAMADI *et al.,* 2000) *L. cubensis*, *L. viator*, *L. neotropica* n. sp. and *G. truncatula* should not be placed in the same supraspecific taxon.

The present results show that ITS-2, ITS- 1 and *cox1*are good markers not only for identifying *L. cubensis*, *L. viator*, *L. neotropica* and *G. truncatula*, in fascioliasis endemic areas in Central and South America, but also for the classification of samples of these species to haplotype level. This usefulness becomes crucial when considering that, anatomically, *L. cubensis* appears to be indistinguishable from *L. neotropica* (*L. viator* var. B elongata) and is distinguishable only with difficulty from *L. viator* (*L. viator* var. A ventricosa) (PARAENSE, 1982a; POINTIER *et al.,* 2006). Exhaustive ITS-2 and ITS-1 studies on single nucleotide polymorphisms (SNP) have already proved the value of both spacers for the distinction and identification of these lymnaeids (BARGUES *et al.,* 2001, 2003, 2005; BARGUES & MAS-COMA, 2005). Curiously, a single mutation in the ITS rDNA of *P. columella* has been associated with susceptibility to *F. hepatica* infection (GUTIERREZ *et al.,* 2003).

3.5.- IMPLICATIONS OF *LYMNAEA NEOTROPICA* IN FASCIOLIASIS TRANSMISSION IN ARGENTINA

In Argentina, production of mostly cattle, but also sheep and goats, is a main national economic activity, with wide areas mainly or totally dedicated to livestock management.

Therefore, animal fascioliasis has always been a target of major concern in this country, due to its high pathogenicity, losses it causes, and economic efforts which shall be made for its control (LOMBARDERO *et al*., 1979a; PIZZI *et al*., 1982; OLAECHEA, 1994). Moreover, human cases have been described in several provinces (see review in CHEN & MOTT, 1990) and the human infection situations in countries such as Chile and Bolivia are causes for concern in Argentina because of the close neighbourhood. Therefore, studies are being made since several years to assess the present situation of the disease throughout Argentina, taking into account the great climatic and physiographic heterogeneity of the different regions of this country.Special efforts are being made in Mendoza province where prevalences in cattle are very high (MERA Y SIERRA *et al*., 2005).

The aim of the present study is to describe the results obtained in a recreational farm, including liver fluke infection in different domestic animal species, classification of the vector and verification of natural transmission of fascioliasis by identification of the intramolluscan trematode larval stages found in a naturally infected snail. The classification of the lymnaeid species and the trematode larval stages was confirmed by molecular characterisation. Molecular markers were selected according to their usefulness for molluscs in general or lymnaeids in particular: small subunit or 18S rRNA gene (BARGUES & MAS-COMA, 1997) and the second and first internal transcribed spacers ITS-2 and ITS-1 (REMIGIO & BLAIR, 1997; MAS-COMA *et al*., 2001; BARGUES *et al*., 2001, 2003, 2006a, 2007a,b) within nuclear ribosomal DNA (rDNA), and the cytochrome c oxidase subunit I *cox*1 (REMIGIO & HEBERT, 2003; BARGUES *et al*., 2007b) within mitochondrial DNA (mtDNA).

3.5.1.- GEOGRAPHICAL ORIGIN OF SAMPLES STUDIED

The study area was a recreational farm studied situated in the locality of Perdriel (District Perdriel, Department of Lujan de Cuyo), at an altitude of 902 m, on Mendoza province, central-west Argentina. Climate data from this area was obtained using DIVA-GIS 5.2 software (WorldClim 1.4: climatic layers resolution 2.5 min) (HIJMANS *et al.*, 2005).

Lymnaeid snails were collected in the same area where definitive hosts showed liver fluke infection. Sampling of snails was carried out manually in December 2007, June 2008, July 2008 and August 2008, in water collections of an artifical pond and the

overflow originated from an artificial irrigation channel. Snail population dynamics was assessed by 15-minute manual collections by the same person within a reduced area of 10 m^2. Snails collected were transported alive to the laboratory for appropriate studies.

The morphometric study of the intraspecific variability of the snail shell included shell length (SL), shell width (SW), last spire length (LSL), aperture length (AL), and aperture width (AW), as well as the ratios SL/SW, SL/AL and SL/LSL. Measurements were made under a calibrated stereomicroscope following traditional malacological methods (OVIEDO *et al*., 1995; SAMADI *et al*., 2000; BARGUES *et al*., 2007b). Snail specimens for molecular analyses, as well as trematode larval stages found in one lymnaeid specimen, were fixed in 70% ethanol for DNA extraction procedures.

3.5.2.- MOLECULAR PROCEDURES

DNA extraction: the same procedure was performed for both lymnaeids and fluke larvae. Total DNA was isolated according to the phenol–chloroform extraction and ethanol precipitation method. The procedure steps were performed according to methods outlined previously (BARGUES & MAS-COMA, 1997; BARGUES *et al*., 2001, 2007a). The pellet was dried and resuspended in 30 µl sterile TE buffer (pH 8.0). This suspension was stored at −20º C unti use.

DNA sequence amplification of ribosomal and mtochondrial markers: for lymnaeids, the 18S rRNA gene, the two spacers ITS-1 and ITS-2 of the rDNA and a mitochondrial DNA *cox*1 gene fragment were amplified by PCR using the same primers and PCR conditions as described previously (BARGUES *et al*., 2001, 2006a, 2007a) (see details in section 3.4.2.3). The ITS-1 of the trematode rediae was amplified by PCR according to methods described in MAS-COMA *et al*. (2001).

Purification and quantification of PCR products: primers and nucleotides were removed from PCR products by purification on Wizard™ PCR Preps DNA Purification System (Promega, Madison, WI, USA), according to the manufacturer's protocol and resuspended in 50 µl of 10 mM TE buffer (pH 7.6). The final DNA concentration was determined by measuring the absorbance at 260 and 280 nm.

DNA sequencing: the sequencing of the complete 18S rRNA gene, the complete rDNA ITS-1 and ITS-2, and the fragment of the mtDNA *cox*1 gene was performed on both strands by the dideoxy chain-termination method (SANGER *et al*., 1977). It was

carried out with the Taq dye-terminator chemistry kit for ABI 373A and ABI 3700 capillary system (Perkin Elmer, Foster City, CA, USA), using PCR primers.

Software programs used for sequence alignment: sequences were aligned using CLUSTAL-W version 1.8 and MEGA 4.0 (TAMURA *et al.,* 2007), and assembly was made with the Staden Package (STADEN *et al*., 2001). Subsequently, minor corrections were manually introduced for a better fit of nucleotide correspondences in microsatellite sequence regions. Homologies were performed using the BLASTN programme from the National Center for Biotechnology information web site (http://www.ncbi.nlm.nih.gov/BLAST). Genetic distances were measured using parameters provided by PAUP v.4.0b10 (SWOFFORD, 2001).

3.5.3.- CHARACTERISATION OF *LYMNAEA NEOTROPICA* FROM ARGENTINA

3.5.3.1.- MORPHOLOGICAL AND MORPHOMETRIC RESULTS

Specimens of the lymnaeid population sampled in the farm present morphological and morphometric characteristics which indicate that there is only one lymnaeid species present. The form of the shell is illustrated in Figure 10 and its morphometric variability in Table 16. These characteristics fully agree with those of the species *L. neotropica* originally described from Perú. The morphometric shell comparison between *L. neotropica* from Argentina and *L. neotropica* from the type locality in Lima shows no significant differences in any of the parameters studied. The only small difference observed concerns shell length, which is indeed related to the age of the collected lymnaeids: specimens from the Peruvian type locality were described as presenting 5.5 deeply sutured whorls, whereas specimens from are smaller, with 4 whorls in 28 of the specimens collected (84.8%) and 5 whorls in the remaining 5 specimens (15.1%) (Table 16).

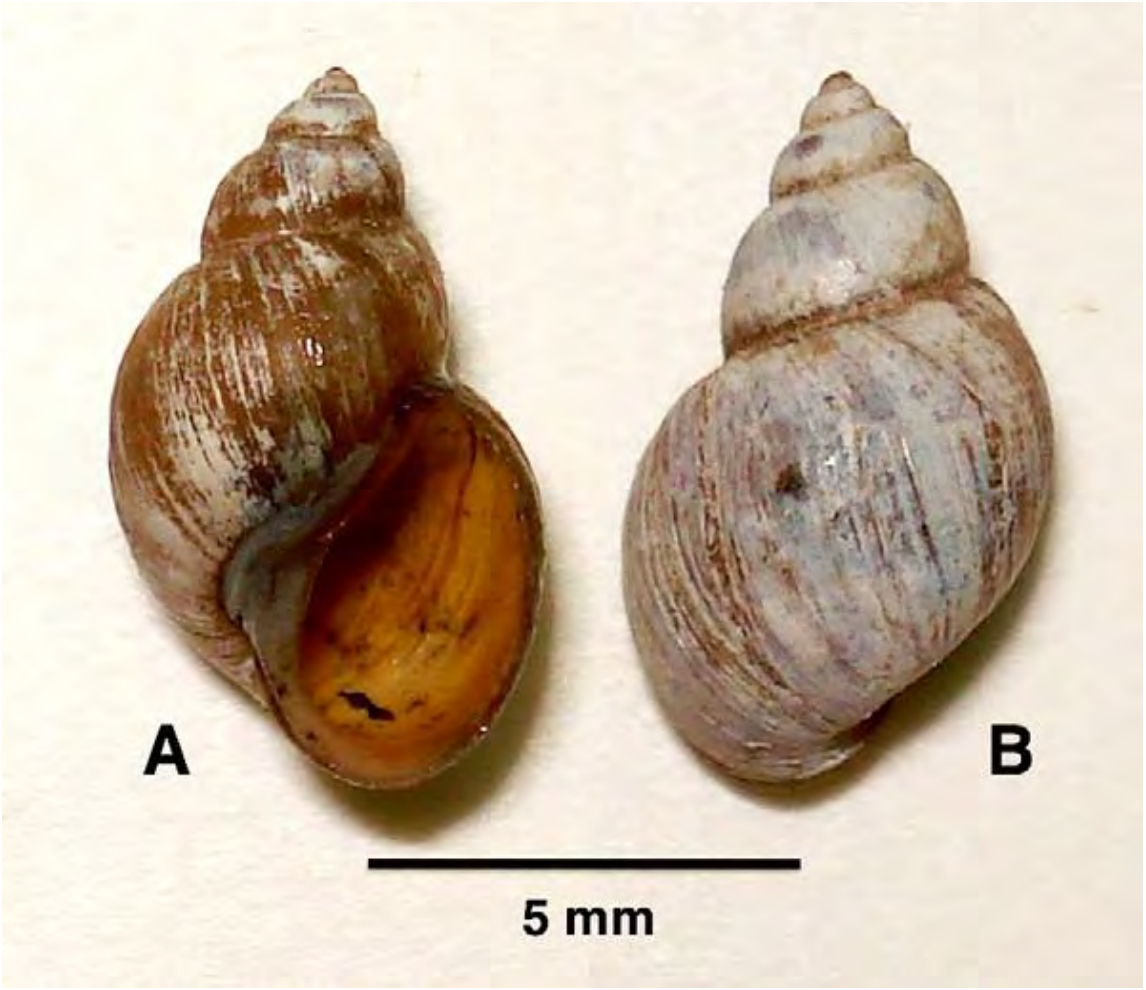


Fig. 10.-. Shell of *Lymnaea neotropica* from Argentina: (A) ventral view and (B) dorsal view (MERA Y SIERRA *et al*., 2009).

Table 16.- Lymnaeid shell measurement comparison between *Lymnaea neotropica* from Argentina and *L. neotropica* from the type locality in Perú. Range include minimum and maximum extremes; mean and standard deviation SD in parentheses. Measurements in mm (MERA Y SIERRA *et al*., 2009).

Shell parameters	***L. neotropica* Perdriel, Mendoza, Argentina (n = 33)**	***L. neotropica* Rio Rimac, Lima-Callao type locality, Perú (n = 33)**
Shell length (SL)	5.24-7.90 (6.28 T 0.67)	5.89-8.74 (7.13 T 1.20)
Shell width (SW)	2.95-4.76 (3.76 T 0.44)	3.29-4.56 (3.95 T 0.54)
Last spire length (LSL)	4.19-6.19 (5.02 T 0.54)	4.47-6.78 (5.58 T 0.91)
Aperture length (AL)	2.29-4.38 (3.19 T 0.50)	2.90-4.53 (3.75 T 0.64)
Aperture width (AW)	1.52-3.14 (2.16 T 0.36)	1.97-2.99 (2.46 T 0.44)
SL/SW ratio	1.50-1.86 (1.67 T 0.08)	1.69-1.92 (1.80 T 0.07)
SL/AL ratio	1.44-2.42 (1.99 T 0.20)	1.76-2.03 (1.91 T 0.09)
SL/LSL ratio	1.19-1.49 (1.25 T 0.05)	1.21-1.38 (1.28 T 0.05)

3.5.3.2.- MOLECULAR RESULTS

18S rRNA gene*:* Its sequence in the 10 lymnaeids analysed is 1,860 bp long, shows a GC content of 51.82%, and its base frequences are: A = 0.236; G = 0.236; C = 0.282; and T = 0.245. This sequence is identical to that of the same gene in the species *L. neotropica* and *L. viatrix,* already deposited in the GenBank under the Accession No. AM412222.

rDNA ITS-2*:* All specimens from Perdriel furnished the same sequence of 417 bp long and 56.83% of GC content. When compared to the ITS-2 of close lymnaeid species of the *Galba*/*Fossaria* group present in South America (*L. cubensis*, *L. viatrix*, *L. neotropica* and *G. truncatula*), no one nucleotide difference could be found with *L. neotropica* ITS-2 haplotype H1 (AM412225).

rDNA ITS-1*:* Lymnaeid specimens from Perdriel furnished one identical sequence of 533 bp long and 56.66% of GC content. When compared to the ITS-1 of close lymnaeid species of the *Galba*/*Fossaria* group present in South America, no one nucleotide difference could be found with *L. neotropica* ITS-1 haplotype HA (AM412228).

mtDNA *cox*1*:* Lymnaeids studied from Perdriel showed only one 672-bp-long sequence which has been deposited in the GenBank with the haplotype code *L. neotropica cox*1-b (this code is provisional because it only concerns a fragment and not the complete gene sequence). It differs by only 1 transition in position 468 from the *L. neotropica cox*1-a from the type locality of Rio Rimac, Lima, Perú (AM494008). This mutation (C/T in Ha/Hb, respectively) gives rise to no difference in the amino acid sequence between both haplotypes. Genetic pairwaise distances at the level of *cox*1 between these *L. neotropica* haplotypes and other lymnaeids of the *Galba*/*Fossaria* group are noted in Table 17.

3.5.4.- MOLECULAR CHARACTERISATION OF TREMATODE LARVAL STAGES IN *LYMNAEA NEOTROPICA*

The complete sequence of the rDNA ITS-1obtainedin the trematode rediae found in a *L. neotropica* specimen from Perdriel is 432 bp long and with a 51.85% GC content.

This sequence was compared with rDNA ITS-1 of: *F. hepatica* from Spain, France, Poland, Ireland, Iran, Japan, Korea, Vietnam, Autralia, Egypt, Bolivia, Perú, Uruguay, Argentina, Chile, Venezuela, Ecuador and Mexico (AB207139, AB207140, AB207141,

Table 17.- Pairwise distances between mtDNA *COX*1 sequences of the lymnaeid species analysed according to PAUP. Below diagonal = total character differences; above diagonal = mean character differences (adjusted for missing data) (MERA Y SIERRA *et al*., 2009).

		1	2	3	4	5	6
1 *L. neotropica cox*1-b	ARGENTINA	_	0.00149	0.02232	0.04464	0.10119	0.10833
2 *L. neotropica cox*1-a	PERU	1	_	0.02083	0.04315	0.09970	0.10667
3 *L. cubensis cox*1-a	CUBA	15	14	_	0.05655	0.11161	0.12000
4 *L. viator cox*1-a	ARGENTINA	30	29	38	_	0.10119	0.10500
5 *G. truncatula cox*1-a	SPAIN	68	67	75	68	_	0.00333
6 *G. truncatula*	GERMANY	65	64	72	63	2	_

AB207145, AB211236, AB385611, AJ243016, EF612468, EF612469) (MAS-COMA *et al*., 2001, 2009a).

Not a single nucleotide difference was found when compared this ITS-1 sequence with that spacer in *F. hepatica* from the Northern Bolivian Altiplano and Spain (AJ243016), and thus corresponds to the haplotype code of *F. hepatica*: Fh ITS1-HA.

3.5.5.- REPERCUSSIONS OF THE FINDING OF *LYMNAEA NEOTROPICA* IN ARGENTINA

Sequences of rDNA 18S, ITS-2 and ITS-1 and mtDNA *cox*1 prove that the lymnaeids present in the farm belong to the combined haplotype 1A of the species *L. neotropica* (BARGUES *et al*., 2007b). This is the first finding of this lymnaeid vector species in Argentina (MERA y SIERRA *et al*., 2009). The morphological characteristics of the shell of the Argentinean specimens (Table 16) agree with those of this species in the type locality of Perú (BARGUES *et al*., 2007b). Up to the present, only six or seven lymnaeid species were considered valid amongst the numerous species described in Argentina: *L. columella*, *L. viatrix*, *L. diaphana*, *L. peculiaris*, *L. pictonica* and *L. plicata* (HUBENDICK, 1951; PARAENSE, 1976, 1982a, b, 1983, 1984; CASTELLANOS & LANDONI, 1981). MALECK (1985), in the most recent general review on South American lymnaeids, listed the same species mentioned by HUBENDICK (1951), but accepting *L. patagonica* as valid and not including *L. peculiaris*.

The report in Argentina of an European stagnicoline species as *L. palustris* by N.J. Evans of the British Natural History Museum (LOMBARDERO *et al*., 1979b) is a little bit surprising, as it would indeed be the only stagnicoline known in the Neotropical Region. However, this report should be confirmed, as it is well known that lymnaeids are able to be transported from one continent to another (MAS-COMA *et al*., 2005). Similarly, the quotation of the South American species *L. cousini* by OLAECHEA (1994) should also be confirmed. Anyway, there may be a misunderstanding on this species because, contrary to what is noted in OLAECHEA (1994), no report of the presence of *L. cousini* in Argentina is included in the review of CASTELLANOS & LANDONI (1981).

More recently, the finding of *G. truncatula* in the province of Mendoza (MERA Y SIERRA, 2001), has represented both a new lymnaeid species for the Argentinean fauna and an additional concern from the point of view of the disease. Sequencing of the rDNA ITS-2 and ITS-1 proved that Argentinean specimens belong to *G. truncatula*

combined haplotype G.tru-3C, the same as the one responsible for the fascioliasis transmission in the human hyperendemic area with the highest human prevalences and intensities known, the Northern Bolivian Altiplano (BARGUES *et al*., 2006b, 2007b). No additional lymnaeid species was added in the most recent review of freshwater molluscs in Argentina (RUMI *et al*., 2008).

The discovery of *L. neotropica* in Argentina represents moreover the first finding of this vector species in countries of the Southern Cone. Up to the present, this species was only known from its type locality at the Rimac river near the cities of Lima and Callao in Perú (BARGUES *et al*., 2007b). The total absence of nucleotide differences between the sequences of specimens from Argentina and the specimens from the Peruvian type locality at the levels of rDNA 18S, ITS-2 and ITS-1, and the only one mutation at the mtDNA *cox*1 gene suggest that such a great geographical separation between the two localities in Perú and Argentina is the consequence of a very recent spread of *L. neotropica*. The ecological characteristics of this lymnaeid, living in small, superficial water collections frequented by livestock, suggest that it may be carried from one place to another by remaining in dried mud stuck to the feet of transported animals, similarly as *G. truncatula* (MAS-COMA *et al*., 2009x). This passive spreading capacity suggests, moreover, that this lymnaeid species may be more widespread, not only in Argentina but also in other South American countries.

The ITS-1 sequence proves that the trematode rediae found in one *L. neotropica* specimen was *F. hepatica*. The sequence from the Argentinean farm does not show any nucleotide difference with the ITS-1 sequence of the liver fluke, which appears to be identical throughout the world and hence an excellent species marker (MAS-COMA *et al*., 2009x).

The presence of *L. neotropica* in Argentina adds pronounced complexity to the transmission and epidemiology of fascioliasis in this country. The problem in Argentina does not only lie on the different lymnaeid vector species which are present, such as *L. viatrix*, *L. diaphana*, *P. columella*, *G. truncatula* and now *L. neotropica*, but also to the fact that three of them belong to the controversial *Galba*/*Fossaria* group of species. Thus, *L. viatrix*, *G. truncatula* and *L. neotropica* are lymnaeids which are very similar from the phenotypic point of view, including (i) shell characteristics and snail anatomy (SAMADI *et al*., 2000; POINTIER *et al*., 2006) and (ii) ecological characteristics (EUZEBY, 1971; MERA Y SIERRA, 2001; CIOCCO & SCHEIBLE, 2008). The close systematic relationships between these lymnaeid vector species has recently been confirmed by DNA marker sequences and corresponding phylogenetic reconstructions

(BARGUES *et al*., 2007b). This similarity poses a great problem, as there is no way to distinguish between one and other species of these three lymnaeids when working on the field (even expert malacologists may misclassify specimens) and it is evident that the need to obtain DNA sequences each time a lymnaeid vector population is found does not appear to be feasible.

Moreover, this problem also concerns several molecular techniques which do not show sufficient accuracy. For instance, molecular techniques which rely on the 18S rRNA gene or parts of it, as the helix E10-1 of the variable region V2 which is useful for the differentiation of European lymnaeids (BARGUES & MAS-COMA, 1997), are evidently not useful in Argentina because of the existence of species as *L. viatrix* and *L. neotropica* which present identical 18S sequence (BARGUES *et al*., 2007b). Consequently, the recently proposed, 18S-based, real-time PCR strategy for the rapid discrimination amongst main lymnaeid species from Argentina (DUFFY *et al*., 2009) cannot be applied because it does not distinguish between these two vector species.

All suggests that vector misclassifications may probably be included in several of the numerous fascioliasis studies made in Argentina in which *L. viatrix* was the species noted to be involved in disease transmission. Many of these studies probably included *L. neotropica* and/or even *G. truncatula*. Therefore, appropriate molecular studies are needed to assess the geographical distribution of each one of these three lymnaeid vector species in Argentina, for which ITS-2, ITS-1 and *cox*1 appear to be the most useful markers.

3.5.6.- CURRENT HAPLOTYPE DISTRIBUTION OF *LYMNAEA NETROPICA* IN SOUTH AMERICA

After the first citation of *L. neotropica* in Argentina, in Perdriel, Departamento de Lujan de Cuyo, Mendoza (MERA Y SIERRA *et al*., 2009), a new report for this species was done in two geographical areas of the Province of Catamarca, Argentina (BARGUES *et al*., 2016). The extreme desertic-arid environmental characteristics surrounding locality A (Tatón-Rio Grande) and the slightly less extreme conditions of semiaridity-aridity of those surrounding locality B (Ipizca), as well as the very low yearly precipitation in both localities, are surprising and very different from the typical environmental characteristics surrounding transmission foci of fascioliasis.

The new ITS-1 (L.neo-HB) haplotype present in Catamarca is worth noting, given the uniformity so far detected at the level of the nuclear rDNA spacers in this species throughout. The only previously known ITS-1haplotype (L.neo-HA) has been reported from Lima (type locality) (BARGUES *et al*., 2007b) and Cajabamba (BARGUES *et al*., 2012), both in Perú, and also in Perdriel, Mendoza, Argentina (MERA Y SIERRA *et al*., 2009).

The other new haplotype in a mtDNA marker (L.neo-*cox*1e) differs from the *cox*1 haplotypes described in *L. neotropica* in other countries such as Perú, Venezuela and also Argentina. In the latter country, this new L.neo-*cox*1e haplotype only shows identity with the *cox*1 sequence of the isolate NtC2 (GenBank: JN872453) previously reported from the same lymnaeid species in Mendoza (STANDLEY *et al*., 2013), although unfortunately the fragment available from that isolate is shorter (655 bp vs 672 bp).

The haplotypes found in the other two markers in *L. neotropica*, one in rDNA ITS-2 (L.neo-H1) and the other in mtDNA 16S (L.neo-16SA), were already reported from Mendoza, Argentina (MERA Y SIERRA *et al*., 2009). These two ITS-2 and 16S haplotypes were also detected in Perú, in the type-locality besides Lima (BARGUES *et al*., 2007b) and also in Cajamarca (BARGUES *et al*., 2012).

3.6.- GENETIC ANALYSYS OF OTHER LYMNAEID SPECIES GEOGRAPHYCALLY OR TAXONOMICALLY RELATED

Within the several human fascioliasis hotspot regions known, South America is characterized by the so-called Andean transmission pattern, including the Altiplano subpattern and Valley subpattern. Both subpatterns are characterized by high altitude endemic areas, including high prevalences and intensities in humans caused by *F. hepatica*, such as in Bolivia (HILLYER *et al*., 1992; ESTEBAN *et al*., 1997a, b, 1999) and Perú (ESTEBAN *et al*., 2002, GONZALEZ *et al.,* 2011).

In Argentina the human fascioliasis situation, although underestimated, also shows a link to altitude areas (MERA Y SIERRA *et al*., 2011). However, human infection in South America has also been described to be relatively frequent in given low altitude areas, such as in Arequipa region, Perú and southern Chile, where the species *L. diaphana* King, 1830 has been noted to be directly involved in the transmission (CORDOVA *et al.,* 1961; TANTALEAN *et al*., 1974; LARREA *et al*., 1994) or known to

be present in the transmission area (SIELFELD, 2001; VALDOVINOS 2006), respectively.

Additionally, *L. diaphana* is known to inhabit the southernmost areas of South America (HUBENDICK, 1951) where animal fascioliasis has been described, in both Chile (ALCAINO & APT, 1989, MORALES *et al*., 2000) and Argentina (OLAECHEA, 1994).

Here we describe a multigenic sequence analyses of *L. diaphana* thanks to the attainment of complete sequences of the 18S gene and the first and second internal transcribed spacers, ITS-1 and ITS-2, of the rDNA and partial sequences of the 16S gene and cytochrome c oxidase subunit I (*cox*1) coding gene. To avoid any possible doubt, the molecular characterisation is based only on specimens collected in the type locality of this species. These sequences are analysed in full detail, by means of pairwise comparisons and phylogenetic methods, with those of the same markers in (i) other American lymnaeid species of the "fossarine" or *Galba*/*Fossaria* group, most of them represented by specimens from the respective type localities of the species, (ii) the main fascioliasis vector species throughout the world *G. truncatula*, which also belongs to the *Galba*/*Fossaria* group and (iii) morphologically close Nearctic and Palearctic species of the "stagnicoline" group. Finally, the study is also used for the analysis of the lymnaeid genus *Pectinidens*, which was proposed with *L. diaphana* as type species long ago (PILSBRY, 1911). *Pectinidens* has also been used at subgenus level to even include other *Galba*/*Fossaria* vector species such as *L. viatrix* (ALCAINO & APT, 1989).

3.6.1.- MULTIGENIC SEQUENCE ANALYSYS OF *LYMNAEA DIAPHANA*

Nuclear rDNA 18S, ITS-2 and ITS-1 and mtDNA 16S and *cox*1 nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DNA Data Bank of Japan databases under the accessions noted in Table 18.

The 18S sequence of *L. diaphana* (1,848 bp) is slightly longer than that of *G. truncatula* (1,843 bp) (BARGUES *et al*., 1997), equally long than that of *L. humilis* (1,848 bp) (BARGUES *et al*., 2011c), similar to that of the European stagnicolines *L. stagnalis*, *O. glabra* and *L*. (*S*.) *palustris*, the radicines *R. auricularia* and *R. balthica,* as well as to *P. columella* (ranging between 1,849-1,852 bp) (BARGUES *et al*., 1997, 2011a), but pronouncedly shorter than that of *L. cubensis*, *L. viatrix* and *L. neotropica*

(all three 1,860-bp long) (BARGUES *et al*. 2007b). This suggests that *L. diaphana* may be considered an old species within the family Lymnaeidae, according to their

Table 18.- Nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) bar code haplotype identification and respective GenBank accessions for the lymnaeid species *Lymnaea diaphana* from its type locality (BARGUES *et al*., 20012).

DNA marker	**Haplotype code**	**Accession**
18S rRNA	L.dia 18S-H1	JF909497
rDNA ITS-2	L.dia ITS2-H1	JF909498
rDNA ITS-1	L.dia ITS2-HA	JF909499
mtDNA 16S	L.dia 16S-HA*a*	JF909500
mtDNA *cox*1	L.dia *cox*1-Ha*a*	JF909501

phylogeny in which the oldest lymnaeid fossil known is *Galba* from the Jurassic (ZILCH, 1959-1960; INABA, 1969), a shorter sequence would be the plesiomorphic condition and an increase in sequence length would have occurred during lymnaeid evolution (BARGUES *et al*., 2001).

With regard to the ITS-2, *L. diaphana* presents a sequence whose 495-bp length fits within the group of lymnaeids having the longest ITS-2 sequences (BARGUES *et al*., 2001). This might be interpreted as a species having long time derived from the old form suggested by the 18S. In this context, *L. diaphana* shows evolutionary characteristics similar to the Nearctic species *L. humilis* (BARGUES *et al*., 2011c). Additionally, the very high number of nucleotide differences it shows when compared to all other lymnaeid species (Table 19) and groups (Table 20) is surprising. Moreover, contrary to what was expected, the distances regarding Nearctic stagnicolines appear to be lower than those regarding Holarctic *Galba*/*Fossaria* species (Table 20). This indicates that *L. diaphana* should not be included in the *Galba*/*Fossaria* group as its shell and anatomic characteristics suggest (PARAENSE, 1984).

Such a relationship with American stagnicolines does, however, not appear so clear at ITS-1 level, a marker in which the lower *L. diaphana* differences appear with Old World originary *G. truncatula* (73) and North American *H. caperata* (76) (Table 21). Interestingly, the length of ITS-1 in *L. diaphana* is the shortest hitherto known in Lymnaeidae (520 bp), only surpassed by *G. truncatula* (504 bp).

Particular aspects of the results obtained in mtDNA 16S sequences should be highlighted: (i) AT composition appears to be pronouncedly biased, which should be taken into account when analyzing the significance of the information this marker offers, (ii) variable positions do not appear regularly distributed throughout the sequence, but concentrated in hot spot regions which indicates that the information furnished by the fragment may not appropriately reflect whole gene evolution, as already seen in other organisms (MAS-COMA & BARGUES, 2009), and (iii) nucleotide differences appear to be less in number than those logically expected from mtDNA (Table 22), which suggests a low mutation rate indeed only apparent, as a consequence of an evolutionary parallelism of its rRNA gene function inside the mitochondrial genome with fast evolving mtDNA coding genes giving rise to position saturation, as already seen in lymnaeids and other freshwater molluscs (BARGUES *et al.,* 2011a). Thus, the somewhat closer 16S sequence of *L. diaphana* to that of American stagnicolines, represented by *S. bonnevillensis*, may be considered with great caution.

Table 19.- Pairwise distances between ribosomal DNA internal transcribed spacer nucleotide-2 sequences according to Phylogenetic Analysis Using Parsimony including the lymnaeid species studied, together with other proximal lymnaeid species available in GenBank. (BARGUES *et al*., 2012).

	1	2	3	4	5	6	7	8	9	10	11	12	13
Lymnaea (Stagnicola) palustris-H1	-	0.02998	0.24737	0.29398	0.28362	0.25693	0.23200	0.23174	0.22922	0.21782	0.22879	0.25899	0.2046
2 *Lymnaea (Stagnicola)*fuscus-H2	14	-	0.25397	0.28467	0.28889	0.26209	0.23720	0.22251	0.21995	0.21197	0.22798	0.25547	0.20308
3 *Galba truncatula*-H1	94	96	-	0.14767	0.09819	0.07752	0.07937	0.16992	0.16992	0.17080	0.18732	0.20968	0.20000
4 *Lymnaea humilis*-H1	122	117	57	-	0.20920	0.18313	0.16203	0.23940	0.24190	0.25776	0.26799	0.27751	0.27632
5 *Lymnaea cubensis*-H2	116	117	38	91	-	0.04556	0.04380	0.21120	0.21628	0.20603	0.21558	0.24010	0.25000
6 *Lymnaea viatrix*-H1	102	103	30	76	20	-	0.02651	0.17016	0.17016	0.17708	0.17935	0.21429	0.22841
7 *Lymnaea neotropica*-H1	87	88	30	64	18	11	-	0.15342	0.15342	0.16120	0.17280	0.18329	0.20178
8 *Catascopia catascopium*	92	87	61	96	83	65	56	-	0.00676	0.05301	0.10579	0.15603	0.17297
9 *Stagnicola elodes*	91	86	61	97	85	65	56	3	-	0.05301	0.10327	0.15603	0.17838
10 *Catascopia occulta*-H1	88	85	62	108	82	68	59	22	22	-	0.13382	0.18476	0.18684
11 *Hinkleyia caperata*	89	88	65	108	83	66	61	42	41	55	-	0.17831	0.17204
12 *Lymnaea diaphana*-H1	108	105	78	116	97	84	68	66	66	80	74	-	0.20876
13 *Pseudosuccinea columella*-H1	80	79	67	105	93	82	68	64	66	71	64	81	-

Below diagonal: total character differences; above diagonal: mean character differences (adjusted for missing data).

Table 20.- Total character differences (extreme and average values of nucleotide differences) at ribosomal DNA internal transcribed spacer (ITS)-2 and ITS-1 sequences according to Phylogenetic Analysis Using Parsimony in the pairwise distance comparisons between *Lymnaea diaphana* and the different lymnaeid groups studied. Data set contains 736 and 765 characters for ITS-2 and ITS-1, respectively (BARGUES *et al*., 2012).

DNA marker	***Galba*/*Fossaria* group**	**European stagnicolines**	**American stagnicolines**	***Pseudosuccinea columella***
ITS-2	68-116 (88.6)	105-108 (106.5)	66-80 (71.5)	81 (81)
ITS-1	73-95 (85.6)	100-101 (100.5)	76-93 (86.7)	105 (105)

Table 21.- Pairwise distances between ribosomal DNA internal transcribed spacer (ITS)-1 nucleotide sequences according to Phylogenetic Analysis Using Parsimony including the lymnaeid species studied, together with other proximal lymnaeid species available in GenBank (BARGUES *et al*., 2012).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1 *Lymnaea (Stagnicola) palustris*-HA	-	0.01512	0.21258	0.21849	0.20211	0.21268	0.21118	0.23409	0.24280	0.23984	0.23320	0.20739	0.25319
2 *Lymnaea (Stagnicola)fuscus*-HA	8	-	0.20950	0.21757	0.19874	0.21341	0.21237	0.23770	0.24486	0.24646	0.23123	0.20534	0.25319
3 *Galba truncatula*-HA	98	97	-	0.15208	0.12000	0.14286	0.14639	0.22009	0.22698	0.21097	0.18298	0.16115	0.24890
4 *Lymnaea humilis*-HA	104	104	73	-	0.14868	0.15551	0.15569	0.25859	0.26316	0.26000	0.23846	0.20085	0.25652
5 *Lymnaea cubensis*-HA	96	95	57	73	-	0.04864	0.05952	0.23061	0.23950	0.21946	0.18672	0.17570	0.25217
6 *Lymnaea viatrix*-HA	104	105	71	79	25	-	0.05642	0.23374	0.24033	0.22088	0.20160	0.18737	0.25934
7 *Lymnaea neotropica*-HA	102	103	71	78	30	29	-	0.24746	0.25000	0.23695	0.19960	0.19108	0.24034
8 *Catascopia catascopium*	114	116	103	128	110	115	122	-	0.00741	0.07129	0.22543	0.18699	0.25684
9 *Stagnicola elodes*	118	119	106	130	114	118	123	4	-	0.07910	0.22929	0.18941	0.25367
10 *Catascopia occulta*-HA	118	122	100	130	106	110	118	38	42	-	0.20992	0.17304	0.25156
11 *Hinkleyia caperata*	118	117	86	124	90	101	99	117	119	110	-	0.14729	0.23701
12 *Lymnaea diaphana*-HA	101	100	73	95	81	89	90	92	93	86	76	-	0.22532
13 *Pseudosuccinea columella*-HA	119	119	113	118	116	125	112	122	121	121	114	105	-

Below diagonal: total character differences; above diagonal: mean character differences (adjusted for missing data).

Table 22.- Pairwise distances between mitochondrial DNA 16S ribosomal DNA gene data set nucleotide sequences according to Phylogenetic Analysis Using Parsimony including the lymnaeid species studied together with other proximal lymnaeid species available in GenBank (BARGUES *et al*., 2012).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1 *Lymnaea diaphana*	-	0.09762	0.09739	0.10072	0.09785	0.09927	0.09716	0.11005	0.07329
2 *Lymnaea humilis*	41	-	0.04276	0.10526	0.00000	0.00241	0.04976	0.08115	0.08789
3 *Lymnaea cubensis*	41	18	-	0.10048	0.04265	0.04589	0.01174	0.07619	0.08706
4 *Pseudosuccinea columella*	42	44	42	-	0.10526	0.10706	0.10263	0.10287	0.10476
5 *Stagnicola elodes*	41	0	18	44	-	0.00242	0.04965	0.07889	0.08768
6 *Fossaria obrussa*	41	1	19	44	1	-	0.05301	0.08495	0.09179
7 *Fossaria bulimoides*[a]	41	21	5	43	21	22	-	0.07601	0.08216
8 *Fossaria bulimoides*[b]	46	34	32	43	34	35	32	-	0.10214
9 *Stagnicola bonnevillensis*	31	37	37	44	37	38	35	43	-

a: a F485657; *b*: EU038315. Below diagonal: total character differences; above diagonal: mean character differences (adjusted for missing data).

Table 23.- Pairwise distances between mitochondrial DNA cytochrome c oxidase (*cox*)1 nucleotide sequences according to Phylogenetic Analysis Using Parsimony including the lymnaeid species studied together with other proximal lymnaeid species available in GenBank (BARGUES *et al*., 2012).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 *L. diaphana cox*1-a	-	0.11737	0.13889	0.13426	0.13542	0.13889	0.13839	0.14333	0.11905	0.12054	0.11161	0.11012	0.11756	0.13542	0.13690	0.13839	0.15696
2 *F. bulimoides*	75	-	0.14867	0.13302	0.14867	0.14867	0.10798	0.10829	0.03443	0.03286	0.02191	0.02347	0.05008	0.10955	0.10955	0.11111	0.14950
3 *S. elodes*	90	95	-	0.13889	0.14815	0.14815	0.15432	0.16000	0.14660	0.14815	0.14815	0.14969	0.14043	0.13735	0.13735	0.13889	0.16694
4 *A. tomentosa*	87	85	90	-	0.14352	0.14352	0.13117	0.13833	0.13426	0.13580	0.12500	0.12654	0.13117	0.13735	0.13889	0.14043	0.14894
5 *P. columella*[a]	91	95	96	93	-	0.00000	0.14286	0.15000	0.14583	0.14435	0.14435	0.14583	0.14881	0.13542	0.13542	0.13690	0.14725
6 *P.columella*[b]	90	95	96	93	0	-	0.14815	0.15000	0.15123	0.14969	0.14969	0.15123	0.15278	0.14043	0.14043	0.14198	0.14894
7 *G. truncatula*[c]	93	69	100	85	96	96	-	0.00333	0.11161	0.11012	0.09970	0.10119	0.10119	0.08929	0.08780	0.08929	0.15372
8 *G. truncatula*[d]	86	64	96	83	90	90	2	-	0.12000	0.11833	0.10667	0.10833	0.10500	0.09667	0.09500	0.09667	0.15878
9 *L. cubensis cox*1-a	80	22	95	87	98	98	75	72	-	0.00149	0.02083	0.02232	0.05655	0.10714	0.10714	0.10565	0.15372
10 *L. cubensis cox*1-b	81	21	96	88	97	97	74	71	1	-	0.01935	0.02083	0.05804	0.10565	0.10565	0.10417	0.15210
11 *L. neotropica cox*1-a	75	14	96	81	97	97	67	64	14	13	-	0.00149	0.04315	0.10119	0.09821	0.09970	0.14725
12 *L. neotropica cox*1-b	74	15	97	82	98	98	68	65	15	14	1	-	0.04464	0.10268	0.09970	0.10119	0.14887
13 *L. viatrix cox*1-a	79	32	91	85	100	99	68	63	38	39	29	30	-	0.10714	0.11012	0.11161	0.15372
14 *L. humilis cox*1-a	91	70	89	89	91	91	60	58	72	71	68	69	72	-	0.00744	0.00595	0.14401
15 *L. humilis cox*1-b	92	70	89	90	91	91	59	57	72	71	66	67	74	5	-	0.00149	0.14563
16 *L. humilis cox*1-c	93	71	90	91	92	92	60	58	71	70	67	68	75	4	1	-	0.14401
17 *R. rubiginosa*	97	90	102	91	91	91	95	94	95	94	91	92	95	89	90	89	-

a: FN598165; *b*: AY227366; *c*: AM494011; *d*: EU818799. Haplotype codes only provisional due to incomplete sequences of the gene. Below diagonal: total character differences; above diagonal: mean character differences (adjusted for missing data).

Table 24.- Cytochrome c oxidase (*cox*)1 amino acid sequence differences detected in pairwise comparisons between haplotypes of *Lymnaea diaphana* and other proximal lymnaeid species available in GenBank (BARGUES *et al*., 2012) .

Nucleotidic haplotype *cox*1	**GenBank accession**	**Country**	**Variable position**											
										1	1	1	2	
				1	3	9	9	9	0	2	6	7	0	
			8	1	2	6	8	9	0	5	0	6	4	
L.dia-*cox*1-a	JF909501	Chile	0	I	T	I	L	L	C	S	P	V	S	
F. bulimoides	AY227367	Canada	.	.	.	.	.	.	.	G	.	.	.	
S. elodes	AY227368	Canada	.	V	L	V	.	.	.	G	.	.	.	
A. tomentosa	AY227365	Australia	.	V	M	.	.	.	.	?	S	I	T	
P. columella	FN598165	Venezuela	V	V	M	.	.	.	.	G	.	.	T	
P. columella	AY227366	Australia	V	V	M	.	.	.	.	G	.	.	T	
G.tru-*cox*1-a	AM494011	Spain	.	.	.	.	.	.	.	G	.	.	.	
G. truncatula	EU818799	Germany	-	-	.	.	.	.	.	G	.	.	.	
L.cub-*cox*1-a	AM494009	Cuba	.	.	.	.	.	.	.	G	.	.	.	
L.cub-*cox*1-b	FN182205	USA	.	.	.	.	.	.	.	G	.	.	.	
L.neo-*cox*1-a	AM494008	Perú	.	.	.	.	.	.	.	G	.	.	.	
L.neo-*cox*1-b	FN356741	Argentina	.	.	.	.	.	.	.	.	.	.	.	
L.via-*cox*1-a	AM494010	Argentina	.	V	.	.	.	.	.	G	.	.	.	
L.hum-*cox*1-a	FN182197	USA	.	V	.	.	.	.	.	G	.	.	.	
L.hum-*cox*1-b	FN182198	USA	.	V	.	.	.	.	.	G	.	.	.	
L.hum-*cox*1-c	FN182199	USA	.	V	.	.	.	.	.	G	.	.	.	
R. rubiginosa	GU451737	Thailand	.	V	S	.	F	F	V	G	.	.	.	

Nucleotide saturation and biased composition may also pose a significance question mark on the potential relationships of *L. diaphana* with the different lymnaeid groups, as suggested by the high number of nucleotide differences in the mtDNA *cox*1 gene (Table 23). This mtDNA saturation problem has already been highlighted in lymnaeids very recently (BARGUES *et al.,* 2011a). Moreover, the very few amino acid differences in the protein sequence (Table 24) indicate that most of the nucleotide differences are silent. Additionally, there is unfortunately only one stagnicoline from which the *cox*1 fragment in question is available (*S. elodes*), so that no conclusions may be obtained from that comparison.

Summing up, the following conclusions may be obtained from the sequence analyses of nuclear rDNA and mtDNA markers: (i) *L. diaphana* shows sequences very different from all hitherto lymnaeid sequences available at the level of both nuclear rDNA and mtDNA markers, (ii) each one of the five markers analysed, including rDNA 18S, ITS-2 and ITS-1, as well as mtDNA 16S and *cox*1, allow the differentiation of *L. diaphana* specimens from all other lymnaeids; only the *COX*1 amino acid sequence does not, (iii) nuclear rDNA suggest that *L. diaphana* is not a fossarine lymnaeid, but rather a relict form related to ancestral stagnicolines and (iv) mtDNA markers do not furnish genetic distance information useful for the analysis of the relationships of *L. diaphana* with the different lymnaeid groups.

3.6.2.- PHYLOGENETIC RELATIONSHIPS WITH OTHER *GALBA/FOSSARIA* AND OTHER LYMNAEID REPRESENTING DIFFERENT GROUPS OF INTEREST

Phylogenetic analysis of ITS-1 and ITS-2 combined haplotypes of *L. diaphana* and other New and Old World *Galba/Fossaria,* Palearctic stagnicolines, Nearctic stagnicolines, Old World *Radix and Pseudosuccinea* was firstly performed with a maximum likelihood (ML) approach using Phylogenetic Analysis Using Parsimony (PAUP) version 4.0b10 (SWOFFORD, 2002) and the PhyML program version 3.0 aLRT. ML parameters and the evolutionary model best fitting our dataset and the methods used to provide an assessment of the reliability of the nodes in the ML tree are detailed in BARGUES *et al*., (2012).

The combination of the two ITS in a single data-set generated a robust tree, indicating phylogenetic accordance between the two spacers. The ML model best fitting this data-set was HKY85+G+I, using a ts/tv ratio of 1.32 (kappa = 2.5975285), base

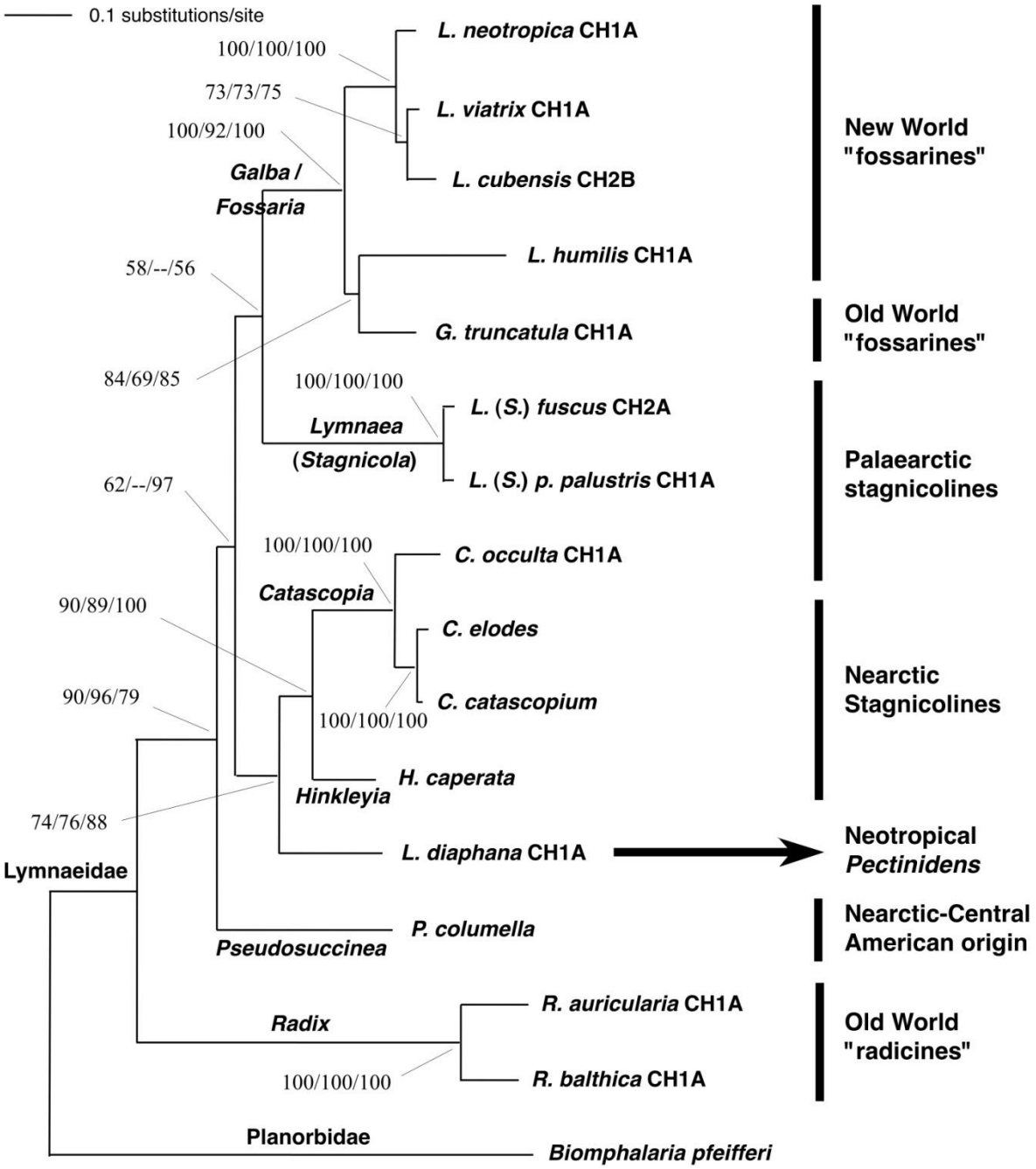


Fig. 11.- Phylogenetic tree of lymnaeid species studied obtained using the planorbid *Biomphalaria pfeifferi* as outgroup, based on maximum-likelihood (ML) estimates. CH: internal transcribed spacers composite haplotype. Scale bar indicates the number of substitutions per sequence position. Support for nodes a/b/c: *a*: bootstrap with neighbour-joining reconstruction using Phylogenetic Analysis Using Parsimony (PAUP) with ML distance and 1,000 replicates; *b*: bootstrap with ML reconstruction using PAUP with 1,000 heuristic replicates; *c*: Bayesian posterior probability with ML model using MrBayes (BARGUES *et al*., 2012) .

frequencies for A, C, G and T of 0.22830, 0.26280, 0.22550 and 0.28340, respectively, gamma (continuous) with shape parameter alpha of 1.07 and a proportion of invariable sites equal to 0.121 (-ln L 9275.68103).

In the ML tree obtained (Figure 11), the species of the *Radix* group appear clearly independent from all other lymnaeids, this independence always showing the highest support values. Worth emphasizing is also the presence of *P. columella* located basal to the branch, including species of both the *Galba*/*Fossaria* group and the stagnicolines, although such a location is not supported in the Heuristic analysis in which *P. columella* appears in a paraphyly regarding the fossarines and all stagnicolines nor in the Bayesian phylogeny reconstruction in which it appears basal to the *Galba*/*Fossaria* group (trees not shown).

In all trees obtained, European stagnicolines appear clustering together with the *Galba*/*Fossaria* group, whereas American stagnicolines (*Hinkleyia* and *Catascopia*, the latter also including the Palearctic species *C. occulta*) appear separately, grouped within the same branch.

L. diaphana does not cluster together with other morphologically similar fossarine lymnaeids as the New World *L. cubensis*, *L. viatrix* and *L. neotropica*, the Nearctic *L. humilis*, or the Old World *G. truncatula*. Contrary to what would be phenotypically expected, *L. diaphana* appears basal to the Nearctic stagnicolines (Figure 11), with a bootstrap support of 74%, 76% and 88% with NJ, Heuristic and BPP algorithms, respectively. However, in the ML tree obtained with PhyML (tree not shown), reliability for this *L. diaphana* - American stagnicolines cluster appear with a lower support of only 45% when applying the aLRT test.

Our results based on phylogenetic analysis and large genetic distances of *L. diaphana* with New and Old World *Galba/Fossaria*, Palearctic stagnicolines, Nearctic stagnicolines, Old World *Radix* and *Pseudosuccinea* allowed to support the genus *Pectinidens* as the first stagnicoline representative in the southern hemisphere, including colonization of extreme world regions, as most southern Patagonia, long time ago. The phylogenetic link of *L. diaphana* with the stagnicoline group may give light to the aforementioned peculiar low altitude epidemiological scenario of fascioliasis.

The participation of *L. diaphana* in disease transmission in the southernmost fascioliasis endemic areas of South America, in both Chile (ALCAINO & APT, 1989, MORALES *et al*., 2000) and Argentina (OLAECHEA, 1994), whether as only vector or coexisting with other lymnaeid vector species (e.g., *L. viator*) (KACZORKIEWICK, 1983, RUBEL *et al*,. 2005, KLEIMAN *et al*., 2007) should be assessed.

CHAPTER IV
ANIMAL FASCIOLIASIS IN ANDEAN REGIONS

4.- ANIMAL FASCIOLIASIS IN ANDEAN REGIONS

4.1.- BACKGROUND

Fascioliasis produces a serious economic impact in the livestock industry (reduction in milk and meat production, liver condemnation, reproductive failure and mortality) (KAPLAN, 2001; SCHWEIZER *et al*., 2005). In Argentina there are published reports of animal fascioliasis since the 19th century (SAVOYAT, 1867; WERNICKE, 1888) and, since it has to be reported at the slaughterhouses, there are official numbers relating to the condemnation of the livers (SENASA, 2006). Currently, Argentina presents a very widely distributed veterinary problem of fascioliasis in livestock (OLAECHEA 2007). Animal fascioliasis has been reported in all provinces except in the southern end of Patagonia in the island of Tierra del Fuego; high prevalences have been reported in the mesopotamic provinces and in the Central West region mainly in cattle (DWINGER *et al.,* 1982; MORIENA *et al.,* 2001, 2002, 2004, 2008).

In Andean regions, the studies are scant and are not oriented toward species that are predominant in many of these remote places, such as the goat or wild animals. They usually lack information to relate the presence in animals to the presence of the populations of lymnaeid snails. Very high prevalences have been described in cattle of Patagonia, even exceeding 90% upon inspection at the slaughterhouse in the provinces of Rio Negro and Neuquén (KACZORKIEWICZ, 1983). In the Patagonian province of Chubut, in coprological studies in cattle from Andean valleys, a prevalence of over 50% was described (RUBEL *et al*., 2005).

Though scarcely considered, it is known that wildlife species may play a significant role as reservoirs of fascioliasis (MAS-COMA *et al.,* 1988; DASZAK *et al.,* 2000; BENGIS *et al.,* 2004; KRUSE *et al.,* 2004; POLLEY, 2005; GAYO *et al.,* 2011; MEZO *et al.,* 2013). Introduced non-indigenous species (NIS) are widely recognized as a source of disease (DASZAK *et al.,* 2000; KELLY *et al.,* 2009). The importance and consequences of the introduction of NIS in fascioliasis has been the subject of several analyses, concerning both lymnaeids (MAS-COMA *et al.,* 2003, 2005, 2009a; BARGUES & MAS-COMA, 2005) and animal reservoirs (MAS-COMA *et al.,* 2009a).

In Argentina, most cases of human fascioliasis have been reported in Andean and other mountainous regions with over 600 cases published (MERA Y SIERRA *et al*., 2011). At least at the nationwide level, considering official slaughterhouse reports, there appears to be little relationship between cattle fascioliasis and human fascioliasis in

Argentina. In the province of Mendoza, traversed by the Andes Mountains, 28 human cases have been reported, and they have all been from mountainous areas. (MERA y SIERRA *et al.,* 2011). At slaughterhouses, discriminating on the origin of the animals, analyzing exclusively cattle coming from Mendoza prevalences of 67.5% and 34% have been reported (MERA Y SIERRA *et al*., 2005a; GONZÁLEZ *et al*., 2006). Yet the distribution of fascioliasis in cattle in the province is not known nor is it known if it distributed in altitude areas such as the reported cases of human fascioliasis. In human fascioliasis, different transmission patterns have been described in different endemic areas of the world. The very high altitude pattern has been described in Andean regions with *G. truncatula* as its vector which can be subdivided in two sub-patterns, the altiplanic pattern with transmission year-round (at altitudes that can exceed 4,000 m.a.s.l.) and the valley pattern, with seasonality's, prevalences and intensities related to altitude.

In the Andean provinces, goats are very important to local economies. Goat ranching is an example of a sustainable production that is fully integrated within the local rural development, being the domestic livestock species with the most significant population growth worldwide in recent years, mainly in developing countries (MORAND-FEHR *et al*., 2004; BOYAZOGLU *et al*., 2005). Its current success appears to be related with two characteristics: i) goats are efficient converters of low-quality forages into quality products, and ii) constitute a source of high quality protein; both of them high valuable properties in farming systems with limited resources (LEBBIE, 2004; BOYAZOGLU *et al*., 2005), such as those present in most developing countries (LEBBIE, 2004). These characteristics may explain the frequent labeling of goats as "the cow of the poor", highlighting its importance in small farming systems (BOYAZOGLU *et al*., 2005; HOSTE *et al*., 2010).

Gastrointestinal parasitism constitutes one of the main constraints to the outdoor and extensive breeding of goats in temperate and tropical countries (HOSTE *et al*., 2005, 2008; COELHO *et al*., 2012). For years, it has been considered that data obtained on parasite infections in sheep could be directly transferred to goats (HOSTE *et al*., 2010), and thus disregarding specific studies on goat parasitism and its consequences. However, in recent years, accumulated information have underlined the fact that gastrointestinal parasitism in goats differed in many aspects to that in sheep (HOSTE *et al*., 2008), and thus studies in the area are encouraged (SAHLU & GOETSCH, 2005).

The goat population in Argentina reaches about 4 million heads, mainly distributed in the harsh and dry environments of the central and western regions. The dominant flocks

are of Creole goats, derived from introductions during the Spanish colonial times, and mainly owned by small holders, usually belonging to the most marginalized sector of the population. Animals are raised extensively, grazing rangelands during the day and housed during the nights in rustic corrals near the households (DE GEA *et al*., 2005).

Also important in many Andean regions of Argentina are South American camelids which are valuable resource for the production of wool, leather and meat with a high socioeconomic value for rural populations in remote areas (MONTES *et al*. 2006). The guanaco, (*Lama guanicoe*) is the bigger of the south American camelids in Argentina and is currently distributed mainly in Andean regions, with an estimated population of 550,000 animals. There are few reports of *F. hepatica* in guanacos and llamas in Argentina. In guanacos, the presence of other gastrointestinal parasites has also been reported (*Nematodirus sp, Eimeria sp, Marshallagia, Trichuris sp*) (CAFRUNE *et al*., 1996; BELDOMENICO *et al*., 2003; ISSIA *et al*., 2009).

4.2.- OBJECTIVES

The present study aims to characterize animal fascioliasis in Andean regions of Argentina, describe its relation to epidemiological factors and clarify its potential role as reservoirs of *F. hepatica*.

The specific aims of the present study are:

a) To study the morphological characteristics of parasite adults and eggs in a population of *L. europaeus* in the northernmost part of Patagonia region (Argentina), to assess liver fluke prevalence and to analyse the potential reservoir role of this wild lagomorph in an area where fascioliasis is known to occur in livestock.

b) To describe the prevalence of cattle fascioliasis in Mendoza in relation to the altitude were the animals live.

c) To determine i) the prevalence rates of gastrointestinal parasites, ii) single and multiple infections, and iii) statistical associations, with special emphasis on liver fluke *F. hepatica*, in Creole goats from the plateau and Andean regions of western Argentina.

d) To present cases of fascioliasis in two species of captive camelids in the province of Mendoza

e) To describe cases of animal fascioliasis in livestock in relation to naturally infected *L. neotropica*

4.3- *FASCIOLA HEPATICA* NATURALLY INFECTING INTRODUCED EUROPEAN BROWNHARE (*LEPUS EUROPAEUS*) IN NORTHERN PATAGONIA: PHENOTYPE, PREVALENCE AND POTENTIAL RISK

Introduced into South America at the end of 19th century, the European brown hare (*Lepus europaeus*) represents one of the most widespread species of mammals (BONINO *et al.,* 2010). The species has invaded almost all the extension of Argentina, Chile and Uruguay, and southern regions of Perú, Bolivia, Paraguay, and Brazil (BONINO *et al.,* 2010). Despite old reports of *F. hepatica* in lagomorphs in general (ARRU *et al.,* 1967) and specifically infecting the hare in its original home range (TROPILO, 1964; KUTZER & FREY, 1976; NICKEL & GOTTWALD, 1979; SHIMALOV, 2001; ZIEGE *et al.,* 2009; WALKER *et al.,* 2011), the latter has been rarely considered in the epidemiology of the disease, particularly with regard to South American introduced populations. Additionally, phenotypic descriptions of adults and eggs of *F. hepatica* infecting natural populations of *L. europaeus* are lacking in the Neotropical region and even scarce worldwide.

4.3.1.- SAMPLES ANALYSED

Specimens of the European brown hare were obtained from the outskirts of Malargüe city (Mendoza province, Argentina), within the northernmost unit (Payunia district) of the Central Patagonia biogeographic province (MORRONE, 2006). Animals were captured by local hunters between August and September 2010, in an area of a mean altitude around 1,500 m.a.s.l. A total of 35 refrigerated intestinal tracts and 27 livers were received for parasitological examination.

4.3.2.- PARASITOLOGICAL TECHNIQUES

Refrigerated intestinal tracts were immediately inspected for helminthes, while faeces and livers were preserved in 4% formaldehyde for later examination. The content from the gastrointestinal tracts from each hare were thoroughly examined following standard methods (EGERTON *et al.,* 1979). Previously preserved faecal samples were analysed by means of two methods: Sheather's sucrose flotation technique (SHEATHER, 1923)

and Lumbreras' rapid sedimentation technique (LUMBRERAS *et al.,* 1962). Sediment obtained from Lumbreras´ technique was subsequently passed through a 140 µm sieve. Both techniques were performed with three grams of material. Slides from Sheather's technique and filtered sediment from Lumbreras´ technique were microscopically examined. Fecal counts (eggs per gram = epg; oocysts per gram = opg) were determined in every sample. Liver fluke adults were recovered from preserved livers, while eggs were concentrated by means of sedimentation and filtration from the remaining fecal material previously found 'positive'. Adult worms were stained with Grenacher's borax carmine and mounted in Canada balsam between slide and cover glass but without cover glass pressure (VALERO *et al.,* 2005, 2012).

4.3.3- MEASUREMENT TECHNIQUES AND DATA ANALYSIS

Egg characteristics studied were egg length (EL) and egg width (EW) in µm. The product of these 2 dimensions was used as a measure of egg size (EL × EW = ES μm^2), and the ratio as a measure of egg shape (EL/EW = ER) (POULIN, 1997; ABROUS *et al.,* 1998; VALERO *et al.,* 1998, 2001a, 2002). For egg classification, egg size was considered according to recent updates on this characteristic (VALERO *et al.,* 2009; MAS-COMA *et al.,* 2014), and by taking into account the influence of the host species (VALERO *et al.,* 1998, 2001a, 2002). For adult fasciolids, the following standardized measurements were taken (VALERO *et al.,* 2005; PERIAGO *et al.,* 2006) (Figure 12): (i) lineal biometric characters (mm): body length (BL), maximum body width (BW), body width at ovary level (BWOv), body perimeter (BP), body roundness (BR), cone length (CL), cone width (CW), maximum diameter of oral sucker (OS max), minimum diameter of oral sucker (OS min), maximum diameter of ventral sucker (VS max), minimum diameter of ventral sucker (VS min), distance between the anterior end of the body and ventral sucker (A-VS), distance between the oral sucker and ventral sucker (OS-VS), distance between the oral sucker and the union of the vitelline glands (VS-Vit), distance between the union of the vitelline glands and the posterior end of the body (Vit-P), distance between the ventral sucker and the posterior end of the body (VS-P),pharynx length (PhL), pharynx width (PhW), testicular space (taking both testes together) length (TL), testicular space width (TW), testicular space perimeter (TP); (ii) areas (mm2):body area (BA), oral sucker area (OSA), ventral sucker area(VSA), pharynx area (PhA), testicular space area (taking both testes together, TA); (iii) ratios:

body length over body width (BL/BW), body width at ovary level over cone width (BWOv/CW), oral sucker area over ventral sucker area (OSA/VSA), and body length over the distance between the ventral sucker and the posterior end of the body (BL/VS-P). Morphometric measurements used for *F. hepatica* adults follow a logistic growth model with respect to time (VALERO *et al.,* 2001a,b, 2005) (Figure 12). This implies that the morphometric development of the fasciolid adult is not limited but 'damped' and does not exceed certain characteristic maximum (VALERO *et al.,* 1998, 2006b). Since the morphometric maximum values are characteristic for each population, they are considered the comparative base of this study (Table 25).

4.3.4.- COPROLOGICAL RESULTS

Five faecal samples were detected positive to *F. hepatica* (14.28%, 2.7-25.8% CI 0.95), while 33 showed *Eimeria* spp. oocysts (94.28%, 86.61-100% CI 0.95). No nematode and cestode eggs or adults were observed. Faecal counts showed between 1 and 3 epg for liver fluke (mean 2.08 epg, ± 1.25), and 91.73 mean opg (± 155.84) for *Eimeria* sp.

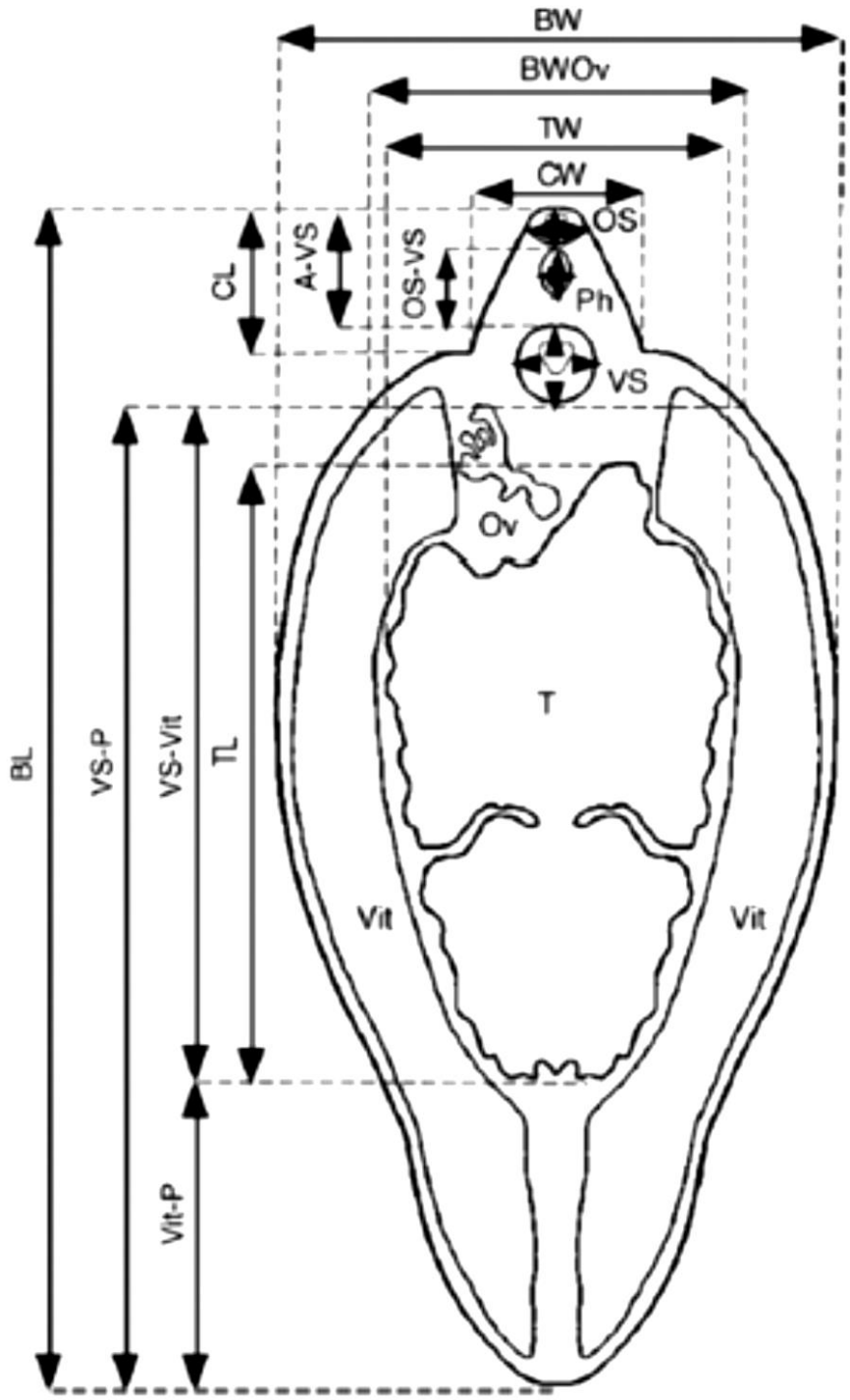


Fig.12.-Standardised measurements in gravid adults of *F. hepatica* (CUERVO *et al*., 2015)

Table 25.-Comparative morphometrics of 6 adult liver flukes in European brown hare (*Lepus europaeus*) (after PERIAGO *et al.,* 2006). Data from liver flukes naturally obtained in domestic species by a) Valero *et al*. (2001a). Data from liver flukes experimentally obtained in Black rat (*Rattus rattus*) and Wistar rat (*Rattus norvegicus*) by a) Valero *et al*. (2001a), and b) Valero *et al*. (1998). All values are shown as range, with the mean and standard deviation (SD) in parentheses. (CUERVO *et al*., 2013)

	Natural Infection				**Experimental infection**	
	European brown hare	**Sheep[a]**	**Cattle[a]**	**Pig[a]**	**Wistar rat[a]**	**Black rat (50d.p.i)[b]**
BL	7.46-11.35 (9.13±1.54)	4.90-31.11 (16.10±4.8)	8.92-28.74 (19.07±3.45)	10.03-24.87 (16.91±3.00)	4.26-23.37 (14.24±4.59)	5.7-13.1 (9.4)
BW	3.23-4.53 (4.01±0.47)	1.58-12.55 (7.11±2.27)	3.16-1295 (8.39±1.51)	5.21-11.45 (8.50±1.55)	1.90-12.95 (8.11±3.08)	2.0-5.1 (3.5)
BWOv	2.18-3.52 (2.94±0.58)	-	-	-	-	-
BP	19.64-24.79 (22.37±2.09)	12.39-68.35 (43.13±12.54)	23.60-67.73 (52.03±8.33)	30.23-66.39 (47.32±7.79)	12.28-62.91 (40.33±13.12)	-
BR	1.35-2.19 (1.70±0.34)	-	-	-	-	-
CL	0.85-1.49 (1.06±0.26)	0.80-3.04 (2.06±0.31)	1.38-3.06 (2.11±0.31)	1.60-3.38 (2.33±0.33)	0.85-3.08 (1.95±0.42)	1.1.2.3 (1.7)
CW	1.23-2.32 (1.59±0.39)	1.20-2.09 (2.46±0.43)	1.48-3.75 (2.76±0.41)	2.03-4.47 (3.18±0.47)	1.03-3.73 (2.60±0.72)	-
OSmax	0.61-0.69 (0.63±0.04)	0.37-1.06 (0.72±0.10)	0.54-0.92 (0.73±0.07)	0.57-1.15 (0.84±0.10)	0.32-0.86 (0.63±0.14)	-
OSmin	0.50-0.56 (0.52±0.02)	-	-	-	-	-
VSmax	0.32-0.49 (0.41±0.07)	0.43-1.25 (0.94±0.14)	0.69-1.88 (1.02±0.11)	0.75-1.29 (1.07±0.10)	0.40-1.20 (0.88±0.20)	0.5-0.8 (0.6)
VSmin	0.17-0.24 (0.20±0.03)	-	-	-	-	-

Tabla 25 Continuation

	Natural Infection					Experimental infection
	European brown hare	**Sheep[a]**	**Cattle[a]**	**Pig[a]**	**Wistar rat[a]**	**Black rat (50d.p.i)[b]**
OS-VS	0.33-0.80 (0.65±0.24)	0.06-2.56 (1.51±0.31)	0.86-2.29 (1.63±0.27)	1.03-2.55 (1.70±0.33)	0.60-2.26 (1.51±0.39)	0.60-1.4 (1.0)
VS-Vit	4.22-6.55 (5.69±1.05)	-	-	-	-	-
Vit-P	1.20-2.74 (2.17±0.55)	-	-	-	-	-
VS-P	6.73-9.26 (7.86±0.94)	3.16-27.39 (13.03±4.45)	6.63-24.95 (15.81±3.19)	7.58-20.18 (13.52±2.64)	2.76-19.58 (11.41±4.03)	4.2-9.8 (7.0)
PhL	0.48-0.68 (0.55±0.12)	-	-	-	-	-
PhW	0.30-0.32 (0.31±0.01)	-	-	-	-	-
TL	2.85-4.08 (3.56±0.57)	-	-	-	-	-
TW	1.87-2.72 (2.09±0.36)	-	-	-	-	-
TP	12.16-7.96 (10.84±1.69)	-	-	-	-	-
VSA	0.6-0.7 (0.6±0.05)	0.15-1.23 (0.71±0.19)	0.37-1.70 (0.80±0.15)	0.45-1.25 (0.88±0.17)	0.12-1.06 (0.63±0.26)	-
PhA	0.11-0.16 (0.13±0.03)	-	-	-	-	-
TA	5.57-6.78 (6.24±0.49)	-	-	-	-	-

Table 25 Continuation

	Natural Infection					Experimental infection
	European brown hare	**Sheep[a]**	**Cattle[a]**	**Pig[a]**	**Wistar rat[a]**	**Black rat (50d.p.i)[b]**
BL/BW	1.68-3.22 (2.33±0.63)	1.33-4.17 (2.33±0.44)	1.40-3.48 (2.30±0.37)	1.50-2.68 (2.02±0.27)	1.18-2.99 (1.85±0.36)	-
BWOv/CW	1.39-2.79 (1.92±0.52)	-	-	-	-	-
OSA/VSA	O.43-0.57 (0.50±0.05)	0.25-0.70 (0.49±0.08)	0.22-0.74 (0.47±0.08)	0.30-0.70 (0.49±0.08)	0.19-0.71 (0.42±0.09)	-
BL/VS-P	1.32-1.07 (1.16±0.09)	1.13-1.55 (1.25±0.06)	1.00-1.36 (1.21±0.04)	1.18-1.37 (1.26±0.03)	1.06-1.60 (1.27±0.08)	-

Abbreviations: BL: body length; BW: body width; BWOv: body width at ovary level; BP: body perimeter; BR: body roundness; CL: Cone length; CW: cone width; OSmax: maximum diameter of the oral sucker; OSmin: minimum diameter of the oral sucker; VSmax: maximum diameter of the ventral sucker; OS-VS: distance between the oral sucker and the ventral sucker; VS-Vit: distance between the ventral sucker and the union of the vitelline glands; Vit-P: distance between the union of the vitelline glands and the posterior end of the body; VS-P: distance between the ventral sucker and the posterior end of the body; PhL: pharynx length; PhW: pharynx width; TP: testicular perimeter; VSA: ventral sucker area; PhA: pharynx area; TA: testicular area; BL/BW: body length/body width ratio; BWOv/CW: body width at ovary level/cone width ratio; OSA/VSA: oral sucker area/ventral sucker area ratio; BL/VS-P: body length/ distance between the ventral sucker and the posterior end of the body ratio.

Table 26.- Measurements taken from 280 eggs of *F. hepatica*, recovered from faeces of European brown hare (*Lepus europaeus*). All values are shown as range, with the mean and standard deviation (SD) in parentheses. Data from rodents and other domestic species by a) Valero *et al.,* (2002), and b) Valero *et al.,* (2001a).(CUERVO *et al*., 2013).

Host	**Location**	**EL**(µm)	**EW**(µm)	**EA**(µm^2)	**ER**
Lepus europaeus n.i.	NPA	90.5-143.7 (120.0±8.9)	56.6-86.2 (68.9±4.9)	6,142.4-1,1408.7 (8,275.1±919.3)	1.3-2.3 (1.7±0.2)
Mus musculus n.i.	Corsica	117-122 (119±2)	60-83 (74±7)	7,158-9,887 (8,836±809)	-
Rattus rattus n.i.	Corsica	122-148 (133±8)	60-74 (67±3)	7,148-10,344 (9011±685)	-
Bos Taurus n.i.	Corsica	125-149 (136±9)	68-83 (74±6)	9,129-11,300 (10,114±801)	-
Bos Taurus n.i.	NBA	105.3-155.9 (132.0±10.5)	61.7-82.5 (71.1±4.4)	5,286.5-9,676.8 (7,170.2±802.5)	1.6-2.3 (1.9±0.2)
Ovis aries n.i.	NBA	114.8-151.2 (130.8±7.1)	65.5-81.4 (72.6±3.9)	5,998.2-8,608.5 (7,238.0±532.8)	1.5-2.2 (1.8±0.1)
Sus scrofa domestica n.i.	NBA	73.8-148.6 (125.4±8.3)	58.1-82.5 (71.8±4.4)	3,988.7-8,626.9 (6,836.0±820.4)	1.1-2.1 (1.7±0.2)
Equus asinus n.i.	NBA	96.4-140.8 (125.4±8.3)	63.3-84.7 (75±3.7)	5,562.6-8,686.2 (7,177.4±646.1)	1.3-2.0 (1.7±0.1)
Rattus norvegicus e.i.	Corsica	122-148 (134±6)	63-80 (70±4)	7,681-11,841 (9,376±866)	-
Rattus norvegicus e.i.	NBA	98.1-144.2 (124.6±7.8)	56.9-80.8 (67.6±3.4)	4,836.2-7,982.3 (6,380.1±510.8)	1.4-2.2(1.9±0.2)

Abbreviations: EL: egg length; EW: egg width; EA: egg area; ER: egg ratio; n.i.: natural infection; e.i.: experimental infection; NPA: Northern Patagonia, Argentina; NBA: Northern Bolivian Altiplano.

4.3.5.- MORPHOMETRIC RESULTS

Twenty-two liver fluke adults were recovered from a single liver, but only six of them could be measured (Table 25), while a total of 280 eggs were recovered from faeces and measured (Table 26).

4.3.6.- ANALYSIS OF MORPHOMETRIC CHARACTERISTICS OF *FASCIOLA HEPATICA* IN *LEPUS EUROPAEUS*

Each trematode species has its own adult and egg phenotype, generally within a specific range (VALERO *et al.,* 2009). However, small host body mass offers limited microhabitat (e.g. liver) and places a physical constraint upon the trematodes body size and number of flukes that can fit in (POULIN, 1997; VALERO *et al.,* 2001a, 2005); while it has been associated with diminished egg size (VALERO *et al.,* 2002). Consequently, the final host species decisively influences the size of adults and eggs of *F. hepatica* (VALERO *et al.,* 2001a, b, 2005, 2009). Measures of *F. hepatica* found in *L. europaeus* from Malargüe department proved to be amongst the smaller described in adults and eggs recovered from naturally and experimentally infected murid rodents, lagomorphs and domestic species (Table 25, 26) (ABROUS *et al.,* 1998; VALERO *et al.,*1998, 2001a, 2002). With regard to adult liver flukes, it shall be considered that samples were preserved in 4% formaldehyde during, at least, two months, which might have slightly decreased measures. Size of the fasciolids from the European brown hare appears similar to fasciolids of 50 days of age experimentally obtained in the Black rat (VALERO *et al.,* 1998) (Table 25). However, the size of *F. hepatica* eggs found in faecal samples of the hares fully overlap not only with those of natural and experimental infections in murid rodents, but also with those of natural infections in cattle and other domestic animals (VALERO *et al.,* 2001a, 2002). All in all, the data obtained indicates that the liver fluke is able to fully develop in wild hares and to shed normal eggs through their faeces. Additionally, the heavy parasite burden observed (22 liver flukes in a single liver) and the small adult size described strongly suggest an effect of crowding, a phenomenon reflected in a decreased adult development when the number of flukes is high (VALERO *et al.,* 2006). Meanwhile, due to experimental evidence of a direct relation between uterus size and the numbers of eggs shed per gram of faeces

(VALERO *et al.,* 2001b, 2011), the reduced uterus development as consequence of smaller adults (POULIN, 1997) may explain the low epg observed.

4.3.7.- IMPORTANCE OF *LEPUS EUROPAEUS* AS *FASCIOLA HEPATICA* RESERVOIR

Although *F. hepatica* infection in wild *L. europaeus* has-been detected before in its original European range (CUERVO *et al*., 2016), to the best of our knowledge only one report deals with that aspect in South America (KLEINMAN *et al.,* 2004). Unfortunately, the information provided is only restricted to the local prevalence found. The high prevalence found in our study (14.28%, 2.7-25.8% CI 0.95) strongly contrasts with the very low one registered (<1‰) in the aforementioned study (KLEIMAN *et al.,* 2004). Our results suggest an even more important role in the transmission cycle than previously considered, at least in given areas. Considering a daily defecation rate of 410 faecal pellets per hare (NOVARO *et al.,*1992), a pellet weight between 1 and 1.4 gr (KLEIMAN *et al.,*2004), and the epg here obtained, each hare could shed to the environment a daily rate of 410-1,722 eggs of *F. hepatica*. This result is close to the lower limit obtained for pigs (2,000-19,5000 eggs/individual/day), a domestic animal whose epidemiological importance in endemic areas has already been highlighted (MAS-COMA *et al.,*1997, 2005). Parasites tend to have threshold levels of host populations size below which they are unable to persist (TOMPKINS & POULIN, 2006). The population dynamics of the European brown hare, as a competent host for liver fluke (i.e. hosts in which the parasites can develop normally), may allow parasite spillback by amplifying the total number of infective stages and increasing the infection burdens in populations of other susceptible hosts (wild or domestic) (KELLY *et al.,*2009; POULIN *et al.,*2011).

This situation set the stage for the European brown hare, a NIS, to alter local parasite dynamics in ways that could lead to disease emergence and an outbreak (RACHOWICZ *et al.,* 2005; THIELTGES *et al.,* 2009; POULIN *et al.,* 2011).The results obtained do not only remark the extraordinary plasticity and adaptability of this trematode species to different host species, but also highlight the role of the European brown hare, and other NIS, as reservoirs capable for parasite spillback to domestic and native cycle, representing a potentially important, but hitherto neglected, cause of disease emergence. The present finding of *F. hepatica* in hares indicates that the

geography of the populations of this lagomorph will be in need to be considered when analyzing the distribution and extent of fascioliasis infection risk areas (FUENTES *et al.,* 1999, 2001; AFSHAN *et al.,* 2014) in Argentina and also in other South American endemic countries where the European hare has been introduced.

4.4.- ALTITUDINAL DISTRIBUTION OF *FASCIOLA HEPATICA* IN CATTLE OF THE PROVINCE OF MENDOZA

In the province of Mendoza, There are 404,710 heads of cattle (SENASA, 2006), and after goats, cattle is the most numerous species of livestock and it is distributed practically in all its territory. According to the official records of slaughterhouses, a prevalence of 5,68%. Was found (MERA Y SIERRA, 2001) In other reports, when only animals that came from ranches from the province of Mendoza, a much higher prevalence was found (MERA Y SIERRA *et al*., 2005a; GONZALEZ *et al*., 2006) , yet there is no information regarding the distribution of the cases and whether they follow or not an altitudinal gradient. Due to the different species of lymnaeids present in Mendoza, province, it is important to know the distribution of fascioliasis to investigate if it follows a determined epidemiological pattern related with altitude.

4.4.1.- GEOGRAPHICAL ORIGIN OF THE SAMPLES STUDIED

A total 325 bovines from 18 different livestock ranches dedicated to cattle production as well as to other species (sheep and goats) were sampled. In each site, altitude was recorded with a GPS (GARMIN Etrex Vista®).

The ranches were grouped according to altitude: low altitude (<800 m.a.s.l.), intermediate altitude (between 800-1,500 m.a.s.l.) and ranches at high altitude (>1,500 m.a.s.l.). Four ranches (47 animals) below 800 m.a.s.l. were sampled in the departments of Lavalle and La Paz. Eight ranches (127 animals) were sampled between 800-1,500 m.a.s.l. in the departments of Lujan de Cuyo, Tunuyán and Tupungato. Six ranches (151 animals) above 1,500 m.a.s.l. were sampled from the departments of Malargüe, Tupungato, San Carlos and Las Heras. The samples were collected directly from the rectum and transported refrigerated in plastic bags to the laboratory and maintained at 4ºC until processed (FIEL *et al.,* 2011). The diagnosis of fascioliasis was done by means of the rapid sedimentation technique (LUMBRERAS *et al.,* 1962). The proportion of animals with fascioliasis in each altitudinal range were compared by means of the χ^2 test. Data processing was done with Infostat® software. Results were considered statistically significant when $p< 0.05$. All procedures with animals were approved by the animal ethics committee of Universidad Juan Agustin Maza (CICUALID).

4.4.2.- COPROLOGICAL RESULTS

In 66 (20.31%) animals *F. hepatica* eggs were observed at the coprological exam. According to the altitude range, in the lower altitude, below 800 m.a.s.l., 2 (4.44%) animals were positive, between 800 and 1,500 m.a.s.l., 10 (7.87%) animals were positive and above 1,500 m.a.s.l., 54 (35.76%) animals were positive (Table 27). There is an ascending gradient as altitude increases (Figure 13). The differences between the groups were significative, $p<0.0001$, yet no statistically significative differences were found between the low altitude and intermediate altitude group ($p=0.4377$). Positive animals were found in all the departments except in La Paz (Table 28).

Table 27.- Prevalence of *F. hepatica* in cattle of Mendoza diagnosed by coprological according to altitude (MERA Y SIERRA *et al*., 2016).

Altitude m.a.s.l.	Total animals sampled	Positive *to F. hepatica*	Prevalence
<800	47	2	4.44%
800-1,500	127	10	7.87%
>1,500	151	54	35.76%
Total	325	66	20.31%

Table 28.- Prevalence of *F. hepatica* in coprological exams in cattle of Mendoza according to department (MERA Y SIERRA *et al*., 2016).

Department	Total animals sampled	Positive to *F. hepatica*	Prevalence
Tunuyán	92	6	6.52%
San Carlos	76	17	22.37%
Lavalle	15	2	13.33%
Lujan de Cuyo	4	4	100.00%
Malargüe	24	4	16.67%
Tupungato	36	1	2.78%
La Paz	32	0	0.00%
Las Heras	46	32	69.56%
Total	325	66	20.31%

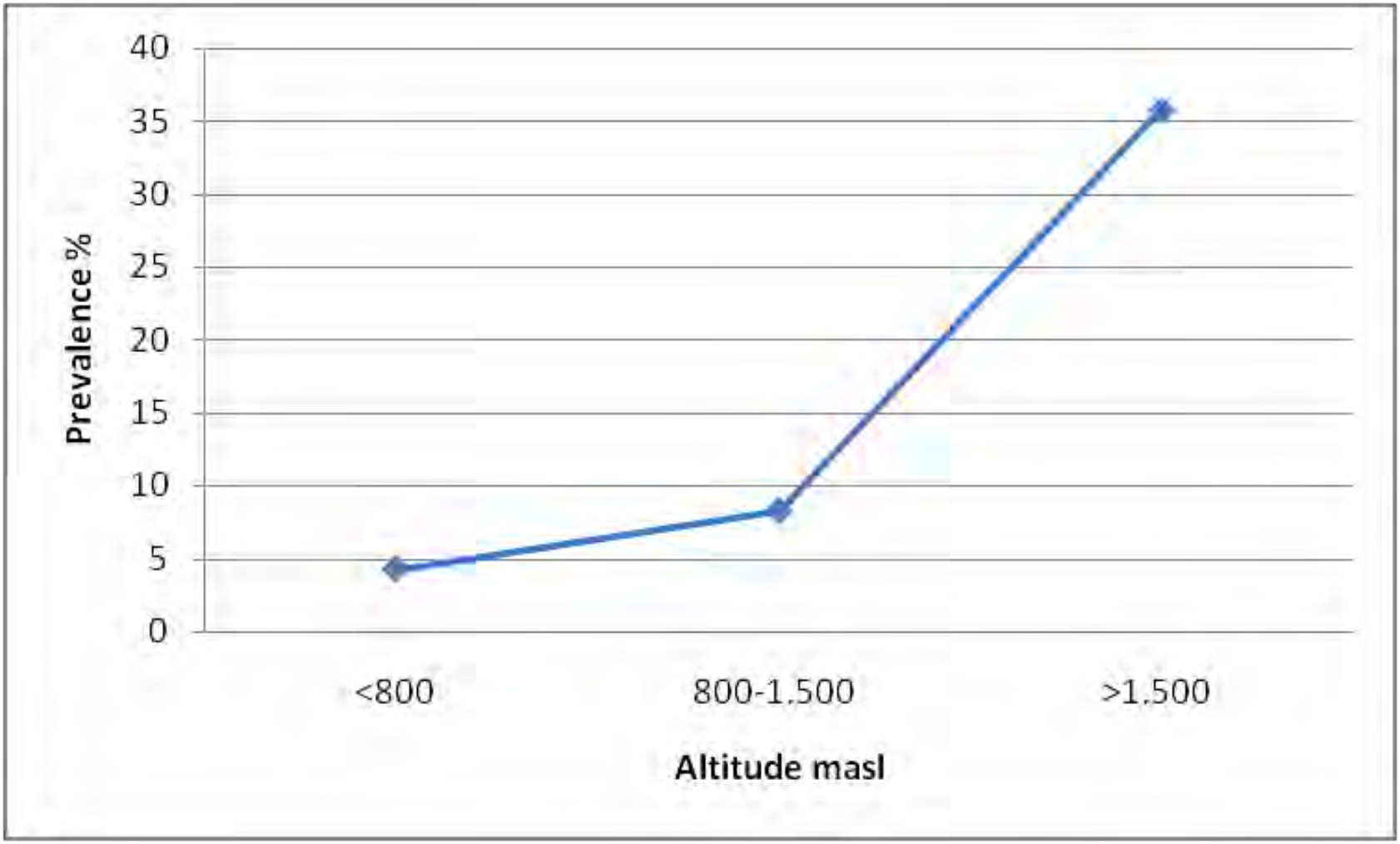


Fig. 13.-Altitudinal distribution of cattle fascioliasis in the province of Mendoza, diagnosed by coprological exams (MERA Y SIERRA *et al*., 2016).

4.4.3.- RELATIONSHIPS OF *FASCIOLA HEPATICA* PREVALENCE IN CATTLE, ALTITUDE RANGE, AND TRANSMISSION PATTERN

According to this study, prevalence of bovine fascioliasis is significatively greater in the mountainous regions. If we take into account that in the rest of the country, there are endemic zones of fascioliasis in cattle at very low altitudes, such as in the mesopotamic region or coastal plains in the pampas, altitude *per se* does not explain this (OLAECHEA, 1994; MORIENA *et al.,* 2001, 2002, 2004). In Andean regions of Perú and Bolivia, where there are highly endemic regions, the presence of *G. truncatula*, which has adapted very well to altitude, could explain this distribution (MAS-COMA *et al.,* 2001). A previous study, which included the main river basins of the province, (Rio Mendoza, Rio Tunuyán, Rio Diamante, Rio Atuel and Llancanelo), found lymnaeids only in an altitudinal range between 1,526 and 2,638 m.a.s.l. (MERA Y SIERRA, 2001; MERA Y SIERRA *et al.,* 2005b). No lymnaeid snails were found below 1,500 m.a.s.l. Molecular studies identified the presence of *G. truncatula* in mountainous regions of the province (BARGUES *et al.,* 2006b).

In great part of the livestock ranches in the Andean regions of the provinces of Mendoza and Neuquen, trashumance is practiced in which the animals go to much higher grounds, above 2,500 m.a.s.l. during the summer, seeking good pastures (DAYENOFF, 2001). In the winter, escaping extreme temperatures and snow covered ground, they are taken to lower regions. So even though the prevalence at inferior altitudes is low, the real situation could be even lower since some of these anials could have acquired fascioliasis during their stay at higher altitudes.

Thus, the distribution of *G. truncatula* could very well explain the bovine fascioliasis distribution found. Very recently, *L. neotropica* has been identified in the department of Lujan de Cuyo at a much lower altitude, 902 m.a.s.l., at the foot of the Andes associated with fascioliasis in cattle, sheep, goats, donkeys and horses (MERA Y SIERRA *et al.,* 2009). This could be associated to a recent introduction of *L. neotropica* which would be conforming isolated transmission foci, situation that has been described for *L. neotropica* in the Argentinean province of Catamarca, were *L. neotropica* was described at the very similar altitude of 994 m.a.s.l. (BARGUES *et al.,* 2016).

The high prevalence found at higher altitudes is similar to the Andean transmission pattern described for Perú and Bolivia were *G. truncatula* is the vector. Yet, it is not possible to determine to what sub-pattern, if any, is similar what is occurring in Mendoza since to the present the seasonality of the transmission has not been determined. This should be done so more efficient control methods can be applied.

4.5.- *FASCIOLA HEPATICA* INFECTION AND ASSOCIATION WITH GASTRO-INTESTINAL PARASITES IN CREOLE GOATS FROM WESTERN ARGENTINA

Despite the mentioned economic impact and an emergent, potentially important, goat production in South America (DUBEUF *et al*., 2004), goat fascioliasis is mainly neglected in the sub-continent. In Argentina, according to official slaughterhouse records, animal fascioliasis covers the whole country (MERA Y SIERRA *et al*., 2011), but this information only comprises cattle and sheep, and rarely involves goats. Despite goat fascioliasis in the country not being even considered (ROSSANIGO, 2007), there are few, usually opportunistic, reports of infection, but demonstrating high local prevalence rates (AGUIRRE *et al*., 2005; RUBEL *et al*., 2005; ISSIA *et al*., 2009).

4.5.1.-FAECES COLLECTION AND LABORATORY PROCEDURES

Six hundred sixty-three (663) Creole goats born in the plateau and Andean regions of Mendoza (623), San Juan (16) and La Rioja (24) provinces (western Argentina) were surveyed between June 2006 and December 2011. Samples were sent by private practitioners, who requested coproparasitological analysis. Individuals were selected by convenience and fecal samples were directlycollected from the rectum. The collected samples were labeled, refrigerated and transported to the laboratory for examination. Coproparasitological exams were systematically performed by means of three methods: Sheater's flotation technique (SHEATHER, 1923), Ritchie's formol-ether concentration technique (RITCHIE, 1948) and Lumbreras technique (LUMBRERAS *et al*., 1962). Faecal culture and larvae identification were performed when sufficient material from positive samples was available (NIEC, 1968).

4.5.2.- STATISTICAL ANALYSIS

Statistix® for Windows 7.0 and SPSS® for Windows 17.0 software programs were used for statistical analysis, comparison of categorical variables and chi-square test. Chi-square test was applied for comparison between observed and expected prevalence.

Associations between *F. hepatica* and other parasite types were investigated by 2 × 2 contingency tables, from which the chi-square was calculated. First, each parasite type occurrence was compared to each other; and then, *F. hepatica* occurrence was compared with pairs of parasite types (in each threesome, all combinations possible were tested). Due to small N and loss of statistical power, no further comparisons were investigated. Values of $p < 0.05$ were taken as significant. Odds Ratio (OR), OR confidence interval (95%) and Relative Risk (RR) were calculated in cases where significant positive association was detected. Considering that, unlike OR, the calculation of RR is influenced by the position of the variable in the 2 × 2 contingency table, both combinations were tested.

4.5.3.- COPROLOGICAL RESULTS

Out of the total 663 goats examined, 84.8% (562) were found to host one or more parasite types (as described in Table 29). Amongst the infected animals, 46% hosted mixed infections. 64.7% of the animals were infected with a protozoan species and 63.49% with at least one helminth species. Two hundred and seventeen (32.9%) of the examined animals (659) were positive for *F. hepatica*, 344 (51.88%) were positive for nematodes, while 422 (64.72%) were positive for *Eimeria* sp. oocysts (Table 29).

Following the few reports available (AGUIRRE *et al*., 2005; RUBEL *et al*., 2005; ISSIA *et al*., 2009), expected prevalence could only be determined for *F. hepatica* infection (94.7%), being the observed prevalence (32.9%) significantly lower than expected ($p<0.05$).

The prevalence rates of single and multiple parasite type infections are shown in Table 30. Considering presence of parasite types, single presence (38.61%) was the most common, with *Eimeria* sp. as the predominant type (21.27%), followed bydouble presence (31.82%). The most frequent combinations were *Eimeria* sp. + *Nematodirus* sp. (15.38%) and *F. hepatica* + *Eimeria* sp. (11.01%).

F. hepatica occurred mainly as a co -infection with anotherparasite, while only 22.12% (48) of the total cases of fascioliasis (217) occurred as monoparasitism (Table 30). It was most frequently combined with *Eimeria* sp. (11.01%), followed by the duet *F. hepatica* + *Nematodirus* sp. (2.11%), and lastly combined with Strongyle eggs (0.90%).

Table 29.- Observed prevalence rates of parasite infection amongst 663 Creole goats in the plateau and Andean regions, Argentina (overall and per province surveyed). (CUERVO *et al*., 2013)

Parasites	N	% Infection (CI 95)	Mza (%)	S. Juan (%)	La Rioja (%)
All	663	84.8 (82.2-87.3)	623	16	24
Strongyle eggs	652	9 (6.9-11)	42 (6.74)	0	17 (70.83)
Nematodirus sp.	663	40.6 (37.1-44.1)	265 (42.54)	2 (12.5)	2 (8.33)
T. ovis	663	2.3 (1.2-3.4)	15 (5.7)	0	0
F. hepatica	659	32.9 (29.6-36.2)	208 (33.39)	0	9 (37.5)
Eimeria sp.	652	64.7 (61.3-68.1)	414 (66.45)	1 (6.25)	7 (29.17)

Mza: Mendoza province; S. Juan: San Juan province.

Table 30.- Combination of infecting parasites amongst 663 Creole goatsin the plateau and Andean regions, Argentina (combinations with less than 0.5% are not shown) (CUERVO *et al*., 2013).

Parasites of combination	**Nº**	**(%)**
Any single type	256	(38.61)
Strongyle eggs	8	(1.21)
Nematodirus sp.	59	(8.90)
F. hepatica	48	(7.24)
Eimeria sp.	141	(21.27)
Double combination	211	(31.82)
F. hepatica + *Eimeria* sp.	73	(11.01)
F. hepatica + Strongyle eggs	6	(0.90)
F. hepatica + *Nematodirus* sp.	14	(2.11)
Eimeria sp. + Strongyle eggs	11	(1.66)
Eimeria sp. + *Nematodirus* sp.	102	(15.38)
Triple combination	77	(11.61)
F. hepatica + *Eimeria* sp. + *Nematodirus* sp.	53	(7.99)
Eimeria sp. + Strongyle eggs + *Nematodirus* sp.	10	(1.51)
Eimeria sp. + *Nematodirus* sp. + *T. ovis*	8	(1.21)
Quadruple combination	17	(2.56)
F. hepatica + *Eimeria* sp. + Strongyle eggs + *Nematodirus* sp.	16	(2.41)

Table 31.- Associations between gastrointestinal parasite amongst 663 Creole goats in the plateau and Andean regions, Argentina. (CUERVO *et al*., 2013)

Parasite sp.	χ^2	*p*-value	OR (CI 95%)
*F. hepatica** Strongyle eggs	6.11	0.0134	1.96 (1.14-3.36)
Eimeria sp.* *Nematodirus* sp.	7.91	0.0049	1.61 (1.15-2.25)
Nematodirus sp.* *T. ovis*	9.89	0.0017	6.09 (1.70-21.78)
Eimeria sp.* *F. hepatica* + *Nematodirus* sp.	12.54	0.0004	2.70 (1.53-4.78)
Nematodirus sp.* *F. hepatica* + *Eimeria* sp.	4.68	0.0306	1.50 (1.04-2.17)
*F. hepatica** *Eimeria* sp. + Strongyle eggs	4.38	0.0363	1.94 (1.03-3.63)
Strongyle eggs* *F. hepatica* + *Eimeria* sp.	4.79	0.0287	1.88 (1.06-3.34)
*F. hepatica** Strongyle eggs + *Nematodirus* sp.	13.2	0.0003	3.76 (1.76-8.05)
Strongyle eggs* *F. hepatica* + *Nematodirus* sp.	19.9	0.0000	3.67 (2.01-6.69)
Nematodirus sp.* *F. hepatica* + Strongyle eggs	9.03	0.0027	3.25 (1.45-7.30)

* Significant associations.

4.5.4.- ANALYSIS OF *FASCIOLA HEPATICA* AND GASTROINTESTINAL PARASITES PREVALENCE IN GOATS IN WESTERN ARGENTINA

4.5.4.1.- GASTROINTESTINAL PARASITES

To the authors' knowledge, this study is the first to report precise goat parasites prevalence rates in western Argentina (CUERVO *et al*., 2013). As usual, and in accordance with previous world tendencies, goat parasitism in the country has been frequently disregarded, and such valuable information not informed due to general disinterest. Most of the available local studies only informed about seasonality of gastrointestinal parasites fecal counts and treatment results, but gave no information about local prevalence that allowed further epidemiological comparison.

This study reveals a high overall prevalence of gastrointestinal parasites, reaching almost 85% - maximum for *Eimeria* sp. (64.7%) and minimum for *Trichuris ovis* (2.3%). Meanwhile, the high prevalence (32.9%) of *F. hepatica* is remarkable.

Due to the mentioned scarcity of local data regarding prevalence of goat gastrointestinal parasitism, observed and expected values could only be compared in the case of infection by *F. hepatica*, which was significantly lower than expected ($p< 0.05$) (Table 29). In the few national reports where *F. hepatica* infection in goats was described (AGUIRRE *et al*., 2005; RUBEL *et al*., 2005; ISSIA *et al*., 2009), studies were developed in response to outbreaks, sample numbers were small, and individuals sampled belonged to the same herd, thus explaining the very high local prevalence and the consequent difference between observed and expected values.

4.5.4.2.- TYPES OF PARASITIC ASSOCIATIONS

The status of polyparasitism using coproscopy as the method indicated that almost half (46.1%) of these animals harbored 2-5 different parasite eggs. Despite the numerous parasite combinations observed (4.6), significant positive associations ($p< 0.05$) were detected only between *F. hepatica* + Strongyle eggs,

Eimeria sp. + *Nematodirus* sp. and *Nematodirus* sp. + *T. ovis* (Table 31).In the first case, *F. hepatica* + Strongyle eggs, the association could be non-causal (confounding factor), possibly due to favourable environmental characteristics for the development of larvae and infection in the same places where infection with *F. hepatica* occurs (e.g.

swamps and waterlogged areas). On the other hand, the OR and RR analysis suggests that infection by *F. hepatica* may act as a contributing factor for Strongyle infection, enhancing almost twice (1.96 and 1.83 respectively) the odds, probably due to the host immunosuppression attributed to the trematode (CERVI *et al*., 1996; GIRONES *et al*., 2007; VALERO *et al*., 2017) or just due to a more vulnerable host (a host's weakened state) (BEGON *et al*, 2006). Considering that most of the larvae identified belonged to the family Trichostrongylidae, association could also be attributed to the arrested larvae released by the suggested immunosuppression (ANDERSON, 2000).

The significant association observed between *Eimeria* sp. + *Nematodirus* sp. and *Nematodirus* sp. + *T. ovis* couldbe a consequence of infective stages (oocysts and eggs) resistant to extreme environmental conditions or related to housing conditions, with animals returning to corrals every day and manure being accumulated during months.

When complex associations were evaluated, significant positive associations were detected in three sets of combinations: *F. hepatica* + *Eimeria* sp. + *Nematodirus* sp., *F. hepatica* + *Eimeria* sp. + Strongyle eggs, and *F. hepatica* + Strongyle eggs + *Nematodirus* sp. (Table 31). In the first two threesomes, the association observed is attributed to the significant associations detected when analyzing by pairs (*Eimeria* sp. + *Nematodirus* sp. and *F. hepatica* + Strongyle eggs), even more when the third parasite type (*F. hepatica* and *Eimeria* sp., respectively) is tested against the associated pair and no significant association is detected. It is worth noting the last threesome, where, following the previous criteria, the presence of eggs of *Nematodirus* sp. is significantly associated with the combination *F. hepatica* + Strongyle eggs ($\chi^2 = 9.03$, $p = 0.0027$).

Further studies are required to define whether these associations are causal or not, and their relevance in the epidemiology of the parasite types implicated. Future studies should also analyse the economic losses generated by goat gastrointestinal multiparasitism, especially considering its impact on local extensive small farming systems.

As highlighted before, *F. hepatica* is rarely considered as a parasite of goats in the country. Consequently, not only the recognized economic losses are not analysed, but also this ruminant is neglected as an important reservoir of this zoonoses. Furthermore, the stunning 33% prevalence detected poses an interrogation and alerts about the role goats play on the transmission and dissemination of this zoonotic trematode

4.6.- *FASCIOLA HEPATICA* IN LLAMAS (*LAMA GLAMA*) AND GUANACOS (*LAMA GUANICOE*) IN THE PROVINCE OF MENDOZA

In Mendoza province, guanacos are distributed practically all along the Andes and are particularly abundant in wildlife reserves. Its population in Mendoza is estimated in 16,000 animals. Due to the great expanse of livestock ranches in Andean regions, it is very frequent for them to have guanaco populations that live side to side with cattle sheep and goats (BAIGUN *et al*., 2008). There are many projects orientated towards a sustainable use of this species, particularly for the use of its valuable fiber. Most of the domestic camelids, llama and alpaca, are in the Northwest of the country, with a total population of approximately 160,000 animals (SENASA, 2002). In Mendoza province, there are very few llamas yet there are animals used for ornamental purposes or in private collections. Fascioliasis can not only affect the health of the camelids but can be a potential reservoir of the parasite for domestic livestock and humans.

4.6.1.- GEOGRAPHICAL ORIGIN OF SAMPLES AND COPROLOGICAL TECHNIQUES USED

The animals were from a ranch in southern Mendoza, department of Malargüe, dedicated to the rehabilitation of guanacos. The veterinarian consults because of the death of a one year old animal following a bout of diarrhea. The farm had 38 guanacos and 6 llamas. The animals fed on natural pasture and alfalfa (*Medicago sativa*) and drank water from an artificial irrigation channel; there were no natural superficial water in the premises. Faeces were recollected directly from the rectum from four guanacos and two llamas, and were sent refrigerated to the laboratory were the following techniques were implemented: Sheather's sucrose flotation technique (SHEATHER, 1923) and Lumbreras' rapid sedimentation technique (LUMBRERAS *et al.,* 1962). Sediment obtained from Lumbreras´ technique was subsequently passed through a 140 µm sieve. Both techniques were performed with three grams of material. Slides from Sheather's technique and filtered sediment from Lumbreras´ technique were microscopically examined. Faecal counts (eggs per gram = epg; oocysts per gram = opg) were determined in every sample. Necropsy was performed on the dead guanaco and samples were sent in 10% Formaline for histopathological studies.

4.6.2.- COPROLOGICAL RESULTS

In the guanaco that died the coprological exam was positive. *F. hepatica* eggs were found (1 epg) and *Eimeria* spp. oocysts were also found (4 opg). Histopathology showed mild hepatosis, suppurative peritonitis, suppurative inflammation of the spleen and renal venous congestion. The other three guanacos were negative at the coprological examinations. Both llamas were positive for *F. hepatica* with 6 and 38 epg respectively. One of the llamas also had eggs of *Nematodirus* sp. (2 epg).

4.6.3.- REPERCUSSIONS OF THE FINDING OF *FASCOLA HEPATICA* IN SOUTH AMERICAN CAMELIDS

Even though the death of the guanaco cannot be attributed to *F. hepatica*, it has been described as a cause of serious disease and death in the other South American camelids, llama, vicuña and alpaca. (LEGUIA, 1997, CAFRUNE *et al*., 1996a, 1996b, 2004); the other parasite found, *Eimeria* sp. can produce important clinical conditions but are usually associated with very high parasitic burdens. Finding *F. hepatica* in a guanaco associated with its death highlights the possible pathogenic role it could have in this species (MERA Y SIERRA *et al*., 2015). If we take into account the very high prevalence of *F. hepatica* in livestock of the region and the impact it could produce in the health and productivity of guanacos, fascioliasis could very well be a determining factor in the conservation and sustainable use of this species. Emerging diseases have demonstrated that can be a threat to wild populations and *F. hepatica* could very well be a menace to wildlife of the American continent were the disease was absent before the European colonization (CUERVO *et al*., 2008). The threat of fascioliasis even to the existence of a population should not be underestimated, as it has been stated that fascioliasis could have been one of the factors associated with the extinction of the Persian onager, (*Equus hemionus onager*) in Syria (ASKARI *et al*., 2018)

The fact that both llamas tested positive for *F. hepatica* alerts not only of the effect it can have on this species but also of its role as reservoir of the disease for other animals, both domestic and wild, and humans.

The absence of natural water bodies in the site implies that the lymnaeids are well adapted to artificial irrigation systems, which reflects their adaptability to different environments. This has already been demonstrated in other parts of the world

(ESTEBAN *et al*., 2002; AFSHAN *et al*., 2014). The conjunction of various species of lymnaeid snails in the region with the capacity to adapt to diverse environments, with high endemicity in domestic livestock and the contact of these with wild autochthonous species can suscitate a very problematic situation that should be addressed.

4.7.- *FASCIOLA HEPATICA* IN LIVESTOCK ASSOCIATED WITH THE PRESENCE OF *LYMNAEA NEOTROPICA*

Three species of lymnaeid vectors have been described in Andean regions of Argentina: *L. viator, L. neotropica* and *G. truncatula* (BARGUES *et al*., 2006a; MERA Y SIERRA *et al.,* 2009, BARGUES *et al*., 2016). It is known that *G. truncatula* is very well adapted to altitude in South America and its main vector of *F. hepatica* in human hyper-endemic regions. It has been identified in Argentina in high altitude regions where there are reports of human cases (MERA Y SIERRA *et al*., 2011) and associated with a high prevalence in cattle (MERA Y SIERRA *et al*., 2016). The recent finding of *L. neotropica* infected with *F. hepatica* at the relatively low altitude of 902 m.a.s.l. (MERA Y SIERRA *et al*., 2009) gives rise to the question of what may be the situation in the exposed animals.

4.7.1.- MAMMAL HOST SAMPLED AND STUDY AREA

The recreational farm studied is situated in the locality of Perdriel (District Perdriel, Department of Lujan de Cuyo), in the province of Mendoza at an altitude of 902 m.a.s.l. The region is known mainly for its vineyards and it is not a region dedicated to livestock production, yet there are small farms that may harbor few animals either for local consumption or horses, mules or donkeys for transport and as draft animals. It is at the foot of the mountains and it is a region in which plants and animals use water provided by irrigation systems that divert water from Río Mendoza. In this farm *L. neotropica* was identified for the first time in Argentina and a snail was found to be infected by *F. hepatica* by molecular methods (see Chapter III). Climate data from this area was obtained using DIVA-GIS 5.2 software (World- Clim 1.4: climatic layers resolution 2.5 min) (HIJMANS *et al.,*2005). Sampling of snails was carried out manually

Fig. 14.- Recollecting faeces from the rectum of a donkey for coprological studies at a farm in Perdriel (33°03´18.76´´S-68°50´18.43´W), province of Mendoza.

During the month of June 2008 faeces were recollected directly from the rectum in 4 cows, 4 goats, 7 horses, 4 donkeys, and 1 llama (Figure 4.3). The animals were fed alfalfa hay and supplemented with grains and had access to pasture on the premises. They drank water directly from an artificial pond and irrigation channels were *L. neotropica* specimens were identified. The sample was sent refrigerated to the laboratory were a rapid sedimentation technique was performed (LUMBRERAS *et al*., 1962). Samples were considered positive if *F. hepatica* eggs were observed.

4.7.2.- COPROLOGICAL RESULTS

Coprological studies showed that all mammal species of the farm were affected by *F. hepatica* infection: 3 infected amongst 4 cattle analysed (75%), 3 amongst 4 goats (75%), 3 amongst 7 horses (42.8%), 4 amongst 4 donkeys (100%), and 1 out of 1 llama (100%). A total of 5 lymnaeid specimens were collected in December 2007, and 50 and 12 specimens, respectively June and August 2008. In July 2008 only 1 living snail was found in the pond, when the artificial irrigation channel was dried for reparation. A month later, with overflow present, snails were there again and the population seemed to have quickly recovered. Except in cases of such artificial modifications, the fluctuations of the lymnaeid snail number appear to show a parallelism with the oscillation of temperature and rainfall, both with maximums in the November-March period. The main climatic characteristics of the endemic locality in question appear to be adequate for both lymnaeid vector population dynamics and *F. hepatica* intramolluscan larval development: annual mean temperature (15.0ºC), maximum temperature in the warmest month (30.9°C), minimum temperature in the coldest month (0.1ºC), mean temperature of wettest and driest quarters (21.0 and 7.8ºC), precipitation in wettest and driest quarters (103 and 23 mm), and precipitation in warmest and coldest quarters (98 and 23 mm).

4.7.3.- ANALYSIS OF PREVALENCE OF INFECTION OF *FASCIOLA HEPATICA*

Studies performed demonstrate that the high prevalences of infection by *F. hepatica* in the animals inhabiting the recreational farm of Perdriel are related to the presence of a snail population of only one lymnaeid species with vectorial capacity. Climatic

conditions appear to be appropriate for fascioliasis transmission, with temperatures surpassing the *F. hepatica* minimum threshold of 10ºC (ROSS & MCKAY, 1929; MAS-COMA & BARGUES, 1997; MALONE *et al.,* 1998) throughout most of the months. However, temperatures and precipitation rates in the May-August period are so low that fascioliasis transmission cannot occur. Hence, in this area the disease could l follow a seasonality, similarly as in the Northern Hemisphere (MAS-COMA & BARGUES, 1997). Yet the presence of irrigation channels may well alter this seasonality and the presence or absence of lymnaeids and infective metacercariae may depend not on the pluviometry but on the availability of irrigation water that is determined more by the watering regimen of the farms depending on the agricultural necessities. A similar situation was recently observed in the province of Catamarca, also with the presence of *L. neotropica* (BARGUES *et al*., 2016). The prevalence found in donkeys and horses, 42.8% and 100% respectively puts the focus on the role equines may have as reservoirs of fascioliasis in the region, since studies in these species are extremely few.

The high prevalence found in livestock and the colonization of *L. neotropica* in manmade irrigation systems at an altitude were fascioliasis is not usually important in Andean regions highlights the adaptability of *F. hepatica* and its vectors (BARGUES *et al.,* 2009).

CHAPTER V
CONCLUSIONS

5.- CONCLUSIONS

The conclusions obtained from the present work are numerous and due to the diverse aspects addressed, to simplify their presentation, they will be listed according to the different objectives.

A) Human fascioliasis in Argentina.

- Regarding the distribution, the majority of cases are in mountainous regions. This coincides with the altitudinal range were lymnaeids snails are found and also coincides with the altitudinal distribution of cattle fascioliasis. What is noteworthy, that, in non Andean, low altitude regions, were animal fascioliasis is endemic, such as in the mesopotamic provinces, the almost total lack of human cases. Even if we consider the possibility of under diagnosis and under reporting, the difference with the mountainous regions is quite surprising. The species of lymnaeids reported for those regions, *Pseudosuccinea columella* and *Lymnaea viator*, would imply that the human cases are related to the presence of *Galba truncatula* and *Lymnaea neotropica* as compared to the animal cases
- The amount of cases found (619) in the bibliographical revision is more than seven times the amount found in a previous revision (85). If we consider that human fascioliasis is not a disease that has to be officially reported and the cases found were exclusively the ones that were published in the scientific literature, the real number of human fascioliasis cases is probably much greater. Judging from the time elapsed between the onset of symptoms and the diagnosis of fascioliasis, which ,was more than three years, it is clear that the medical community in Argentina has difficulties in diagnosing human fascioliasis. So, to underreporting we should add under diagnosis. The real situation of human fascioliasis is quite probably currently underestimated and surveys should be done to know the real situation.
- Regarding the relationship with climatic factors such as temperature and precipitation, no correlation was found between annual precipitation and annual incidence. Yet, since we can only take into consideration the cases published in the literature and the total lack of official reports, care must be taken in the interpretation of these results. Yet, a strong correlation was found between the human cases and the month of the year, with a 3-4 month lag in relation with the monthly precipitation. This could be initially explained by the relationship

between the lymnaeid populations and precipitation, yet, since the analysis was done in a highly touristic area were the affluence of people coincides with the favourable conditions for lymnaeids, the proliferation of cases could be due to the presence of persons in the endemic regions rather than a greater possibility or probability of infection in those months. To clarify this, studies should be done in the field to evaluate the seasonal elimination of infective stages of *F. hepatica*.

B) Species of lymnaeids in Andean regions of Argentina.

- In the endemic regions of fascioliasis in the Andes of Argentina, there are three species of lymnaeids involved: *Galba truncatula, Lymnaea viator* and *Lymnaea neotropica*. Previous to these studies, when only morphological characteristics were used to identify species of lymnaeids, *Lymnaea viator* was the only species implied.
- This emphasizes on the necessity and difficulty in the identification of these species in which is imperative the use of molecular techniques which are obviously very difficult if not impossible to implement in field conditions. Even so, molecular techniques have to be used with adequate criteria since great care must be exercised when choosing the genes.
- *Galba truncatula*, of the same combined haplotype as the one found in the Bolivian Altiplano, is present in Andean valleys of the province of Mendoza. This haplotype is the one responsible of fascioliasis in an endemic area with the highest prevalences and intensities known. The climatic characteristics and the closeness of the lymnaeid populations to human dwellings and in livestock areas, imply a high transmission and dispersal capacity.
- The genetic differences and phylogenetic analysis between *Lymnaea viator* var. B elongata and *Lymnaea viator* var. A ventricosa originally from Lima, Perú and Rio Negro, Argentina, respectively, allow to distinguish them as two different species: *Lymnaea viator* var. B elongata was proposed as *Lymnaea neotropica*, n. sp.and *Lymnaea viator* var. A ventricosa would remain as *Lymnaea viator*. *Lymnaea neotropica* is clearly differentiated from the aforementioned *Lymnaea viator, L. cubensis* and *Galba truncatula*. Even though these four species have similar shells and are difficult to distinguish anatomically, the relevant 18S rDNA sequences already indicate that *L. cubensis*, *Lymnaea viator*, *Lymnaea neotropica* n. sp. and *Galba truncatula* should not be placed in the same supraspecific taxon.

- ITS-2, ITS-1 and COI are good markers not only for identifying *L. cubensis*, *Lymnaea viator*, *Lymnaea neotropica* and *Galba truncatula*, in fascioliasis endemic areas in Central and South America, but also for the classification of samples of these species to haplotype level. Exhaustive ITS-2 and ITS-1 studies on single nucleotide polymorphisms (SNP) have already proved the value of both spacers for the distinction and identification of these lymnaeids.
- Care must be taken when performing and interpreting genetic studies since several molecular techniques do not show sufficient accuracy. For instance, molecular techniques which rely on the 18S rRNA gene or parts of it, as the helix E10-1 of the variable region V2 which is useful for the differentiation of European lymnaeids, are not useful in Argentina because of the existence of species as *Lymnaea viator* and *Lymnaea neotropica* which present identical 18S sequence.
- *Lymnaea neotropica* infected by *F. hepatica* was found at a lower altitude than *Galba truncatula* and associated to very high prevalences in livestock. It is an important vector for animal fascioliasis and the risk of transmission to humans has to be considered.

C) Animal fascioliasis in Andean regions of Argentina.

- The prevalence of fascioliasis in cattle is high at altitude above 1500 m.a.s.l., and diminishes with altitude, with much lower prevalence at lower altitude. This is related to the distribution of the lymnaeid populations, particularly *Galba truncatula* that was found at high altitudes.
- At a lower altitude than where the high prevalence was found in cattle (>1,500 m.a.s.l.) *Lymnaea neotropica* has been identified in the department of Lujan de Cuyo at 902 m.a.s.l., at the foot of the Andes associated with very high fascioliasis prevalence in cattle, sheep, goats, llama, donkeys and horses. This could be associated to a recent introduction of *Lymnaea neotropica* which would be conforming isolated transmission foci.
- A high prevalence of *F. hepatica* was found in the European brown hare, which suggests it may have a more important role in the transmission cycle than previously considered adding the possibility of spillback to the domestic cycle.
- Fascioliasis was diagnosed in an autochthonous camelids, the guanaco (*Lama guanicoe*) and was even associated to the death of an animal, alerting of the role *F. hepatica* may have in the conservation and sustainable use of this species.

- *F. hepatica* was detected in goats in different Andean provinces, yet, it is rarely considered as goat parasite in the country, but a 33% prevalence poses an interrogation on the role goats play on the transmission and dissemination of this zoonotic trematode. Significant positive associations between *F. hepatica* + Strongyle eggs, *Eimeria* sp. + *Nematodirus* sp. and *Nematodirus* sp. + *Trichuris ovis* were detected. Further studies are required to define the causality of these associations and their relevance in epidemiology.

CHAPTER VI
BIBLIOGRAPHY

6.- BIBLIOGRAPHY

ABROUS (M.), COMES (A.M.), GASNIER (N.), RONDELAUD (D.), DREYFUSS (G.), CHAUVIN (A.), MÉNARD (A.), AGOULON (A.) & CABARET, (J.), 1998.- Morphological variability in *Fasciola hepatica* eggs in ruminants, rodents and lagomorphs. *Journal of Helminthology*, 72 (4): 313-317.

ACOSTA (D.), CARDOZO (H.), NARI (A.), SOLARI (M.), 1989.- Ecología y dinámica de población de *Limnaea viatrix* D'Orbigny (1835) en un nicho ecológico del sur de Uruguay. XVII Jornadas Uruguayas de Buiatría, Centro Médico Veterinario de Paysandú, Uruguay. Libro de Resúmenes.

AFSHAN (K.), FORTES-LIMA (C.A.), ARTIGAS (P.), VALERO (M.A.), QAYYUM (M.) & MAS-COMA (S.), 2014.- Impact of climate change and man-made irrigation systems on the transmission risk, long-term trend and seasonality of human and animal fascioliasis in Pakistan.*Geospatial Health*, 8 (2): 317-34.

AGUILAR (M.J.) & OLAECHEA (F.V.), 2014.- Situación de *Fasciola hepatica* al sur del paralelo 48º S. *Veterinaria Argentina*, 21 (311): 2-11.

AGUIRRE (D.H.), CAFRUNE (M.M.), SALATIN (A.O.), ABEYÁ (A.A.), 2005.- *Fasciolosis clínica en cabras* de Metán (Salta, Argentina). *Revista FAVE Ciencias Veterinarias,* 4 (1‑2): 17-21.

AHUALLI (A.) & ARIAS (E.), 1961.- *Fasciola hepatica*. *Revista de la Facultad de Medicina de Tucumán*, 3: 105-118.

AKAIKE (H.), 1974.- A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, 19 (6): 716-723.

ALAGGIA (L.E.), 1981.- Patología hepatobiliar por distomatosis. In: *XXIII Congreso Cuyano de Cirugía* (San Luis, Argentina, 1981): unpublished (meeting communication cited in ANDRADA *et al.,* 1984).

ALBRECHT (C.), WOLF (C.), GLÖER (P.) & WILKE (T.), 2008.- Concurrent evolution of ancient sister lakes and sister species: the freshwater gastropod genus *Radix* in lakes Ohrid and Prespa. *Hydrobiologia*, 615 (1): 157-167.

ALE (D.), MERCIAI (G.) & GIL ECHEVERRIA (M.), 2000.- *Fasciola hepatica,* intensificación de su búsqueda en pacientes con eosinofilia y epidemiología positiva. In: III Congreso Argentino de Parasitología (Mar del Plata, Argentina, 1-4 Nov. 2000), Conference Abstracts: No. 421.

ANDERSON (R.C.), 2000.- Nematode parasites of vertebrates: Their development and transmission. 2nd ed. CAB International, United Kingdom.

ANDRADA (J.G.), AVALOS (E.), EGUEZ (A.) & BOSCH ANDRADA (J.P.), 1983.- Aspectos quirúrgicos de la distomatosis biliar. *Revista Argentina de Cirugía*, 46 (1/2): 44-49.

ANDREWS (S.J.), 1999.-The life cycle of *Fasciola hepatica* In: Fasciolosis (J.P. Dalton Ed.), CAB International Publishing, Wallingford, Oxon, United Kingdom, pp. 435-447.

APT (W.), AGUILERA (X.), VEGA (F.), ZULANTAY (I.), RETAMAL (C.), APT (P.) & SANDOVAL (J.), 1992.- Fascioliasis en la población rural de las provincias de Curico, Talca y Linares. *Revista Médica de Chile*, 120: 621-626.

APT (W.), AGUILERA (X.), VEGA (F.), ALCAINO (H.), ZULANTAY (I.), APT (P.), GONZALEZ (V.), RETAMAL (C.), RODRIGUEZ (J.) & SANDOVAL (J.), 1993.- Prevalencia de fascioliasis en humanos, caballos, cerdos y conejos silvestres en tres provincias de Chile. *Boletín de la Oficina Sanitaria Panamericana*, 115 (5): 405-414.

ARRU (E.), DEIANA (S.), NUVOLE (A.), 1967.- Distomatosi epatica nei leporidi selvatici in Sardegna. *Atti della Societa Italiana delle Scienze Veterinaria,* 21: 762-766.

ARTIGAS (P.), MERA Y SIERRA (R.), CORREA (A.P.), MORALES (J.R.), ZURIAGA (M.A.), JIMÉNEZ (M.), KHOUBBANE (M.), BARGUES (M.D.) & MAS-COMA (S.), 2005.- Comparison of the conchiologic morphology of lymnaeid molluscs from eastern and western Andean slopes of the endemic zones of fasciolosis in Argentina and Chile. *Acta Parasitologica Portuguesa*, 12 (1-2): 243-244.

ARTIGAS (P.), MERA Y SIERRA (R.), CUERVO (P.), BARGUES (M.D.) & MAS- COMA (S.), 2009.- Scolopacidae and other aquatic migratory birds as possible dispersal agents of lymnaeid snails, in Mendoza province, Argentina. In: 6th International Symposium on Limnology and Aquatic Birds: Monitoring, Modelling and Management (Huesca, Spain, 27-30 October 2009), International Society of Limnology (SIL), Pyrenean Institute of Ecology (CSIC), Proceedings: 80-82.

ARTIGAS (P.), BARGUES (M.D.), MERA Y SIERRA (R.L.), AGRAMUNT (V.H.) & MAS-COMA (S.), 2011.- Characterisation of fascioliasis lymnaeid intermediate hosts from Chile by DNA sequencing, with emphasis on *Lymnaea viator* and *Galba truncatula*. *Acta Tropica*, 120 (3): 245-257.

ASHRAFI (K.), VALERO (M.A.), MASSOUD (J.), SOBHANI (A.R.), SOLAYMANI-MOHAMMADI (S.), CONDE (P.), KHOUBBANE (M.), BARGUES (M.D.) & MAS-COMA (S.), 2006.- Plant-borne human contamination by fascioliasis. *American Journal of Tropical Medicine and Hygiene*, 75 (2): 295-302.

ASHRAFI (K.), BARGUES (M.D.), O'NEILL (S.) & MAS-COMA (S), 2014.- Fascioliasis: a worldwide parasitic disease of importance in travel medicine. *Travel Medicine and Infectious Disease*. 12 (6): 636-49.

ASKARI (Z.), MAS-COMA (S.), BOUWMAN (A.S.), BOENKE (N.), STÖLLNER (T.), AALI (A.), REZAIIAN (M.), MOWLAVI (G.), 2018.- *Fasciola hepatica* eggs in paleofaeces of the Persian onager *Equus hemionus onager*, a donkey from Chehrabad archaeological site, dating back to the Sassanid Empire (224-651 AD), in ancient Iran. *Infection, Genetics and Evolution*, 62: 233-243.

BACIGALUPO (J.), 1932.- Hallazgo en la ciudad de Buenos Aires de *Limnaea viator* Dorb infectada espontáneamente con cercarias de *Fasciola hepatica*. *Revista Sociedad Argentina de Biología*, 8: 511-513.

BACIGALUPO (J.), 1942.- *Fasciola hepatica*, su ciclo evolutivo en la República Argentina. *Anales de la Facultad de Veterinaria de Montevideo*, 4 (1): 7-134.

BACIGALUPO (J.), BENGOLEA (J.) & VELAZCO SUAREZ (G.), 1930.- Algunas consideraciones biológicas sobre distomatosis por *Fasciola hepatica* en el hombre. A propósito de dos casos encontrados en la Argentina. *Archivos de la Sociedad de Biología de Montevideo*, 5: 1211.

BACIGALUPO (J.), 1932.- Hallazgo en la ciudad de Buenos Aires de *Limnaea viatrix* Dorb infectada espontáneamente con cercarias de *Fasciola hepatica*. *Revista Sociedad Argentina de Biología*, 8: 511-513.

BACIGALUPO (J.), ZAVALETA (D.E.) & LAURITO (J.O.), 1943.- *Fasciola hepatica* en la Argentina. *La Semana Médica*, 4(1): 5-9.

BAIGÚN (R.J.), BOLKOVIC (M.L.), AVED (M.B.), PUMA (M.C), SCANDALO (R.P.), 2008.- Manejo de fauna silvestre en la Argentina. Primer censo nacional de camélidos silvestres al norte del río Colorado. Secretaría de Ambiente y Desarrollo Sustentable de la Nación. 1st ed. Buenos Aires, Argentina.

BALDOMENICO (P.M.), UHART (M.), BONO (M.F,), MARULL (C.), BALDI (R.), PERALTA (J.L.), 2003.- Internal parasites of free-ranging guanacos from Patagonia. *Veterinary Parasitology*, 118 (1-2): 7177

BALLARD (J.W.) & RAND (D.M.), 2005.- The population biology of mitochondrial DNA and its phylogenetic implications. *Annual Review of Ecology, Evolution and Systematics*, 36: 621-642.

BARCAT (J.A.), 2005.- El berro y otras comidas peligrosas. *Medicina, Buenos Aires*, 65 (3): 277-79.

BARGUES (M.D.) & MAS-COMA (S.), 1997.- Phylogenetic analysis of lymnaeid snails based on 18S rDNA sequences. *Molecular Biology and Evolution*, 14 (5): 569-577.

BARGUES (M.D.) & MAS-COMA (S.), 2005.- Reviewing lymnaeid vectors of fascioliasis by ribosomal DNA sequence analyses. *Journal of Helminthology*, 79 (3): 257-267.

BARGUES (M.D.), MANGOLD (A.J.), MUÑOZ-ANTOLI (C.), POINTIER (J.P.) & MAS-COMA (S.), 1997.- SSU rDNA characterization of lymnaeid snails transmitting human fascioliasis in South and Central America. *Journal of Parasitology*, 83 (6): 1086-1092.

BARGUES (M.D.), MARCILLA (A.), RAMSEY, (J.M.), DUJARDIN (J.P.), SCHOFIELD (C.J.) MAS-COMA (S.), 2000.- Nuclear rDNA-based molecular clock of the evolution of Triatominae (Hemiptera: reduviidae), vectors of Chagas disease. *Memórias do Instituto Oswaldo Cruz*, 95 (4): 567-573.

BARGUES (M.D.), VIGO (M.), HORAK (P.), DVORAK (J.), PATZNER (R.A.), POINTIER (J.P.), JACKIEWICZ (M.), MEIER-BROOK (C.) & MAS-COMA (S.), 2001.- European Lymnaeidae (Mollusca: Gastropoda), intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS-2 sequences. *Infection, Genetics and Evolution*, 1 (2): 85-107.

BARGUES (M.D.), HORAK (P.), PATZNER (R.A.), POINTIER (J.P.), JACKIEWICZ (M.), MEIER-BROOK (C.) & MAS-COMA (S.), 2003.- Insights into the relationships of Palaearctic and Nearctic lymnaeids (Mollusca: Gastropoda) by rDNA ITS-2 sequencing and phylogeny of stagnicoline intermediate host species of *Fasciola hepatica*. *Parasite*, 10 (3): 243-255.

BARGUES (M.D.), ARTIGAS (P.), JACKIEWICZ (M.), POINTIER (J.P.) & MAS-COMA (S.), 2006a.- Ribosomal DNA ITS-1 sequence analysis of European stagnicoline Lymnaeidae (Gastropoda). *Heldia (Münchner Malakologische Mitteilungen), München*, 6 (1/2): 29-40.

BARGUES (M.D.), MERA Y SIERRA (R.), GOMEZ (H.G.), ARTIGAS (P.) & MAS-COMA (S.), 2006b.- Ribosomal DNA ITS-1 sequencing of *Galba truncatula* (Gastropoda: Lymnaeidae) and its potential impact on fascioliasis transmission in Mendoza, Argentina. *Animal Biodiversity and Conservation*, 29 (2): 191-194.

BARGUES (M.D.), ARTIGAS (P.), MERA Y SIERRA (R.L.), POINTIER (J.P.) & MAS-COMA (S.), 2007a.-Characterisation of *Lymnaea cubensis*, *L. viator* and *L. neotropica* n. sp., the main vectors of *Fasciola hepatica* in Latin America, by analysis of their ribosomal and mitochondrial DNA. *Annals of Tropical Medicine and Parasitology*, 101 (7): 621-641.

BARGUES (M.D.), MERA Y SIERRA (R.), GOMEZ (H.G.), ARTIGAS (P.) & MAS-COMA (S.), 2007b.- Caracterización molecular de *Galba truncatula*, vector principal de la Fascioliasis, en Argentina. Implicaciones en salud pública. *Enfermedades Emergentes, Barcelona*, 9 (2): 77-82.

BARGUES (M.D.), 2010.- Snail vectors of trematodiases: evaluation of the usefulness of nuclear ribosomal DNA and mitochondrial DNA markers for lymnaeid vectors. XII Congress ICOPA, 16-20 August 2010 Melbourne. CD-ROM.

BARGUES (M.D.), GONZALEZ (C.), ARTIGAS (P.) & MAS-COMA (S.), 2011a.- A new baseline for fascioliasis in Venezuela: lymnaeid vectors ascertained by DNA sequencing and analysis of their relationships with human and animal infection. *Parasites & Vectors*, 14 (4): 200.

BARGUES (M.D.), ARTIGAS (P.), KHOUBBANE (M.) & MAS-COMA (S.), 2011b.- DNA sequence characterisation and phylogeography of *Lymnaea cousini* and related species, vectors of fascioliasis in northern Andean countries, with description of *L. meridensis* n. sp. (Gastropoda: Lymnaeidae). *Parasites & Vectors*, 12 (4): 132.

BARGUES (M.D.), ARTIGAS (P.), KHOUBBANE (M.), FLORES (R.), GLÖER (P.), ROJAS-GARCIA (R.), ASHRAFI (K.), FALKNER (G.) & MAS-COMA (S.), 2011c.- *Lymnaea schirazensis*, an overlooked snail distorting fascioliasis data: genotype, phenotype, ecology, worldwide spread, susceptibility, applicability. *PLoS ONE*, 6 (9): e24567.

BARGUES (M.D.), MERA Y SIERRA (R.L.), ARTIGAS (P.) & MAS-COMA (S.), 2012.- DNA multigene sequencing of topotypic specimens of the fascioliasis vector *Lymnaea diaphana* and phylogenetic analysis of the genus *Pectinidens* (Gastropoda). *Memórias do Instituto Oswaldo Cruz,* 107 (1): 111-124.

BARGUES (M.D.), MALANDRINI (J.B.), ARTIGAS (P.), SORIA (C.C.), VELAZQUEZ (J.N.), CARNEVALE (S.), MATEO (L.) KHOUBBANE (M.) & MAS-COMA (S.), 2016.- Human fascioliasis endemic areas in Argentina: multigene characterisation of the lymnaeid vectors and climaticenvironmental assessment of the transmission pattern. *Parasites & Vectors* 9:306.

BARGUES (M.D.),GAYO (V.), SANCHIS (J.), ARTIGAS (P.), KHOUBBANE (M.), BIRRIEL (S.), & MAS-COMA (S.), 2017.- DNA multigene characterization of *Fasciola hepatica* and *Lymnaea ne*otropica and its fascioliasis transmission capacity in Uruguay, with historical correlation, human report review and infection risk analysis. *PLOS Neglected Tropical Disease*s, 11(2): e0005352

BARRIOS (U.), DRAKE (C.T.), GONZALEZ (G.), CASAS (A.), 2001.- Evaluación de la excreción de huevos de *Fasciola hepatica* por tres esquemas terapéuticos. *Revista Archivo Médico de Camagüey,* 5 (4): 4.

BEAVER (P.C.), JUNG (R.C.) & CUPP (E.W.), 1986.- Anfistomas y distomas. In: Parasitología Clínica. 2nd ed (Beaver PC, Jung RC, Cupp EW eds.). Barcelona: Salvat Editores, pp.485-525.

BEGON (M.), TOWNSEND (C.R.), HARPER (J.L.), 2006.- *Ecology*: from individuals to ecosystems. 4th ed. Blackwell Publishing Oxford, United Kingdom.

BENGIS (R.G.), LEIGHTON (F.A.), FISCHER (J.R.), ARTOIS (M.), MÖRNER (T.), & TATE (C. M.), 2004.- The role of wildlife in emerging and re-emerging zoonoses. *Revue Scientifique et Technique - Office International des Epizooties* 23 (2): 497-511.

BENGOLEA (J.), BACIGALUPO (J.) & VELAZCO SUAREZ (G.), 1927.- Distomatosis por *Fasciola hepatica*. *Archivos Argentinos de Enfermedades del Aparato Digestivo y de la Nutrición*, 3 (1): 151.

BHALERAO (G.D.), 1933.- The trematode parasites of the Indian elephant, *Elephas indicus*. *Indian Journal of Veterinary Science and Animal Husbandry*, 3: 103-155.

BONINO (N.), COSSIOS (D.), & MENEGHETTI (J.), 2010.- Dispersal of the European hare, *Lepus europaeus* in South America. *Folia Zoologica,* 59 (1): 9-15.

BORAY (J.C.), 1982.-. Fascioliasis. In: Hillyer, GV, Hopla CE. CRC Handbook Series in Zoonoses; Section C, Volume III. CRC Press. Boca Raton, Florida. pp. 71-88.

BOTO (D.), 1939.- Distomatosis humana por *Fasciola hepatica*. Tesis de Doctorado, Universidad de Buenos Aires, No. 5193: 46 pp.

BOYAZOGLU (J.), HATZIMINAOGLOU (I.), MORAND-FEHR (P.), 2005.- The role of the goat in society: past, present and perspectives for the future. *Small Ruminant Research*, 60 (1-2): 13-23.

BRADY (M.T.), O`NEILL (S.M.), DALTON (J.P.) & MILLS (K.H.), 1999.- *Fasciola hepatica* supresses a protective Th1 response against *Bordetella pertussis*. *Infection and Immunity*, 67: 5372-5378.

BUNDY (D.A.P.), ARAMBULO (P.V.), & GREY (C.L.), 1984.- La fascioliasis en Jamaica: aspectos epidemiológicos y económicos de una zoonosis parasitaria transmitida por caracoles. *Boletín de la Oficina Sanitaria Panamericana*, 96(1): 1-19.

CABADA (M.M.), LOPEZ (M.), CRUZ (M.), DELGADO JR, HILL & WHITE (A.C.), 2016.- Treatment Failure after Multiple Courses of Triclabendazole amongst Patients with Fascioliasis in Cusco, Peru: A Case Series. *Plos Neglected Tropical Diseases*, 10 (1): e0004361.

CACERES (M.A.), 1955.- Distomatosis hepatica - Síndrome coledociana por *Fasciola hepatica*. *Sociedad Argentina de Cirujanos, Boletines y Trabajos*, 19: 528-534.

CAFRUNE (M.M.), REBUFFI (G.E.), CABRERA (R.H.) & AGUIRRE (D.H.), 1996.- *Fasciola hepatica* en llamas (*Lama glama*) de la Puna Argentina. *Veterinaria Argentina*, 13 (128): 570-574.

CAFRUNE (M.M.), REBUFFI (G.E.), GAIDO (A.B.), AGUIRRE (D.H.), 1996.- *Fasciola hepatica* in semi-captive vicunas (*Vicugna vicugna*) in north west Argentina. *Veterinary Record*, 139 (4): 97.

CAFRUNE (M.M.), AGUIRRE (D.H.), FREYTES (I.), 2004.- Fasciolosis en vicuña (*Vicugna vicugna*) en semi-cautiverio de Molinos, Salta, Argentina, con notas de otros helmintos en este hospedador. *Veterinaria Argentina*; 21 (207): 513-520.

CAMES (O.J.), 1940.- Distomatosis por *Fasciola hepatica*. *Anales de Cirugía de Rosario*, 6 (1): 143-147.

CAMES (O.J.), CID (J.M.) & ALVARES (A.), 1947.- *Anales de Cirugía*, 13: 1 (cited in TROSSERO & NOCETTI, 1969).

CAÑAS (R.F.), ESCOLA (M.A.) & ROSSINI (A.P.), 1964.- Distomatosis hepatobiliar por *Fasciola hepatica*. *Prensa Médica Argentina*, 51 (10): 590-595.

CARENA (E.J.), TRAKAL (E.), ORTIZ (G.A.), BUTTI (A.L.), CARENA (F.L.) & ROBIN DE AUGIER (M.R.), 1972.- Infestación humana por *Fasciola hepatica* (nuestra experiencia en 13 casos). *Revista Española de las Enfermedades del Aparato Digestivo,* 36 (5): 531-542.

CARNEVALE (S.), RODRIGUEZ (M.I.), GUARNERA (E.A.), CARMONA (C.), TANOS (T.) & ANGEL (S.O.), 2001a.- Immunodiagnosis of fasciolosis using recombinant procathepsin L cysteine proteinase. *Diagnostic Microbiology and Infectious Disease*, 41 (1-2): 43-49.

CARNEVALE (S.), RODRIGUEZ (M.I.), SANTILLAN (G.), LABBE (J.H.), CABRERA (M.G.), BELEGARDE (E.J.), VELASQUEZ (J.N.), TRGOVCIC (J.E.) & GUARNERA (E.A.), 2001b.- Immunodiagnosis of human fascioliasis by an Enzyme-Linked Immunosorbent Assay (ELISA) and a Micro-ELISA. *Clinical and Diagnostic Laboratory Immunology*, 8 (1): 174-177.

CARNEVALE (S.), CABRERA (M.G.), CUCHER (M.A.), DI RISIO (C.A.), MALANDRINI (J.B.), KAMENETZKY (L.) ALAZRAQUI (M.), ETCHART (C.B.) PANTANO (M.L.), VELASQUEZ (J.N.), 2013.- Direct, immunological and molecular techniques for a fasciolosis survey in a rural area of San Luis, Argentina. Journal of parasitic Diseases 37 (2): 251-259.

CASTELLANOS (Z.A.) & LANDONI (N.A.), 1981.- Mollusca Gastropoda Lymnaeidae. In: Fauna de Agua Dulce de la Republica Argentina. Fundación para la Educación, la Ciencia y la Cultura, Buenos Aires, Argentina, 15 (5): 55-82.

CASTEX (M.) & GREENWAY (D.), 1937.- Distomatosis hepatica con ejemplar errático eliminado por uretra. *Boletín de la Academia de Medicina de Buenos Aires*, Abril: 461-464.

CATS (A.), SCHOLTEN (P.), MEUWISSEN (G.M.) & KUIPERS (E.J.), 2000.- Acute *Fasciola hepatica* infection attributed to chewing khat. *Gut* 47, 584-585.

CEDEÑO (D.A.), MARTÍNEZ (G.), & CILIMA (R.), 2012.- Principales causas de decomisos de vísceras rojas en bovinos en el frigorífico del municipio de Pasto. *Revista Investigación Pecuaria*, 1(1): 8-12.

CERVI (L.), RUBINSTEIN (H.), MASIH (D.T.), 1996.- Involvement of excretion-secretion products from *Fasciola hepatica* inducing suppression of the cellular immune responses. *Veterinary Parasitology*, 61 (1-2): 97-111.

CHEN (M.G.) & MOTT (K.E.), 1990.- Progress in assessment of morbidity due to *Fasciola hepatica* infection: a review of recent literature. *Tropical Diseases Bulletin*, 87: R1-R38.

CHOWANIEC (W.) & DARSKI (J.), 1970.- Investigations on excretion time of liver fluke eggs after killing the parasite. *Bullettin of the Veterinary Institute Pulway*, 14:108-110.

CID (J.M.), 1947.- Granuloma tuberculoide y gomoide peritoneal por Fasciola errática. *Archivos de la Sociedad Argentina de Anatomía Normal y Patológica*, 9: 389-401.

CIOCCO (N.F.) & SCHEIBLER (E.), 2008.- Malacofauna of the littoral benthos of a saline lake in southern Mendoza, Argentina. *Fundamental and Applied Limnology.* (*Archiv fur. Hydrobiologie*) 172 (2): 87-98.

CORDOVA (E.), NAQUIRA (F.) & NAQUIRA (C.), 1961.- *Lymnaea diaphana* King como huéspedintermediariode *Fasciola hepatica* en Arequipa (Perú). *Archivos Peruanos de Patología y Clínica*, 15: 165-172.

CORNEJO (J.A.) & CASTILLO (E.), 1964.- Mioglobinemia secundaria a distomatosis por *Fasciola*. *Revista de la Facultad de Medicina de Tucumán*, 7: 241-257.

CORREA (A.J.), RUGGIERI (F.) & MARTINEZ (E.), 1969.- *Fasciola hepatica* de localización intracraneal. *Revista Médica de Santa Fé*, 3: 38-41.

CORTI (M.E.), PALMIERI (O.J.), MENDEZ (N.), GANCEDO (E.), SAWICKI (M.) & MILLET (S.), 2006.- Diagnóstico por imágenes en la fasciolosis hepática. *Revista Argentina de Radiología*, 70 (3): 197-200.

CUCHER (M.A.), CARNEVALE (S.), PREPELITCHI (L.), LABBE (J.H.), & WISNIVESKY-COLLI (C.), 2006.- PCR diagnosis of *Fasciola hepatica* in field-collected *Lymnaea columella* and *Lymnaea viator* snails. *Veterinary Parasitology*, 137 (1-2): 74-82.

CUENYA (C.), 1944.- Parasitosis raras en el Norte Argentino: *Fasciola hepatica*. *Archivos de Farmacia y Bioquímica de Tucumán*, 1 (2): 235-239.

CUERVO (P.), MERA Y SIERRA (R.L.), DEIS (E.) & ZARCO (A.), 2008.-. La introducción de *Fasciola hepatica* en América: ¿un posible riesgo para la conservación de especies en peligro de extinción de Argentina? In: III Congreso Nacional de Conservación de la Biodiversidad Buenos Aires, 11-14 Agosto 2008, Resúmenes de Trabajos: 123.

CUERVO (P.), SIDOTI (L.), FANTOZZI (M.C.), NEIRA (G.), GERBENO (L.), MERA Y SIERRA (R.), 2013.- *Fasciola hepatica* infection and association with gastrointestinal parasites in Creole goats from western Argentina. *Revista Brasileira de Parasitologia Veterinaria*, 22 (1): 53-57.

CUERVO (P.), CATALDO (S.D.), FANTOZZI (M.C.), DEIS (E.), ISENRATH (G.D.), VIBERTI (G.), ARTIGAS (P.), PEIXOTO (R.), VALERO (M.A.),MERA Y SIERRA (R.), MAS-COMA (S.), 2015.- Liver fluke (*Fasciola hepatica*) naturally infecting introduced European brown hare (*Lepus europaeus*) in northern Patagonia: phenotype, prevalence and potential risk. Acta Parasitologica, 60 (3): 536-543.

DAS CHAGAS BERNARDO (C.), BATISTA CARNEIRO (M.), RAUTA DE AVELAR (B.), MOLINARI DONATELE (D.), FREIRE MARTINS (I.V.), & SALIM PEREIRA (M.J.), 2011. Prevalence of liver condemnation due to bovine fasciolosis in Southern Espírito Santo: temporal distribution and economic losses. *Revista Brasileira de Parasitologia Veterinaria*, 20 (1): 49-53.

DASZAK (P.), CUNNINGHAM (A.A.) & HYATT (A.D.), 2000.- Emerging infectious diseases of wildlife - Threats to biodiversity and human health. *Science,* 287: 443-449.

DAYENOFF (P.), 2001.- La veranada caprina. Comunicación Técnica EEA INTA Rama Caída, San Rafael, Mendoza; 2001.

DE (N.V.), HOA (L.T.), WAIKAGUL (J.), 2006.- Plant-borne trematode (fascioliasis) in Vietnam. In: V Seminar on Food- and Water-borne Parasitic Zoonoses (5th FBPZ), Mahidol University, Bangkok, Thailand.

DE BREE (C.J.), BODELIER (G.L.) & VERBURG (G.P.), 2013.- *Fasciola hepatica* as a cause of jaundice after chewing khat: a case report. *The Netherlands Journal of Medicine* 71: 478-479.

DE GEA G, PETRYNA AM, MELLANO A, BONVILLANI A, TURIELLO P.), 2005.- *El ganado caprino en la Argentina:* Antecedentes para su estudio. Córdoba: Universidad Nacional de Río Cuarto; Argentina.

DE RIJK (P.), NEEFS (J.M.), VAN DE PEER (Y.) & DE WACHTER (R.), 1992.- Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Research*, 20 (suppl.): 2075-2089.

DE RIJK (P.) & DE WACHTER (R.), 1993.- DCSE, an interactive tool for sequence alignment and secondary structure research. *Computer Applications in Bioscience*, 9 (6): 735-740.

DEAN (M.D.) & BALLARD (J.W.), 2004.- Linking phylogenetics with population genetics to reconstruct the geographic origin of a species. *Molecular Phylogenetics and Evol*ution, 32 (3): 998-1009.

DEL VALLE (D.) & DONOVAN (R.), 1928.- Síndrome de cólico hepático provocado por *Fasciola hepatica*. *Archivos Argentinos de Enfermedades del Aparato Digestivo y de la Nutrición*, 4: 697-710.

DI CATALDO (S.D.), SOHAEFER (N.), CUERVO (P.), GONZALEZ (M.), NEIRA (G.), SIDOTI (L.), MERA Y SIERRA (R.), 2015.- Factores climáticos y su relación con la presencia de *Fasciola* hepatica en caprinos de las distintas regiones productivas de la provincia de Mendoza, Argentina. In: III Congreso Panamericano de Zoonosis (La 10.13. Plata, Argentina, 4-6 June 2014), published in *Revista Argentina de Zoonosis y Enfermedades Infecciosas Emergentes,* 2015*,* 10 (1): 45-46.

DINNIK (J.A.) & DINNIK (N.N.), 1961.- On the morphology and life history of *Fasciola nyanzae* Leiper from the hippopotamus. *Journal of Helminthology*, (Leiper Suppl.): 53-62.

DITTMAR (K.), 2002.- Arthropod and helminth parasites of the wild guinea pig*, Cavia aperea*, from the Andes and the Cordillera in Perú, South America. *Journal of Parasitology*; 88 (2): 409-411.

DIXON, (K.E.), 1965.- The structure and histochemistry of the cyst wall of the metacercaria of *Fasciola hepatica* L. *Parasitology,* 55: 215-226.

D'ORBIGNY (A.), 1835.- Synopsis terrestrium et fluviatilium molluscorum, in suo per Americam Meridionalem itinere collectorum. *Magazin de Zoologie*, 5, Classe 5 (61-62): 1-44.

DREYFUSS (G.). & RONDELAUD (D.), 1994.- *Fasciola hepatica*: a study of the shedding of cercariae from *Lymnaea truncatula* raised under constant conditions of temperature and photoperiod. *Parasite,* 1 (4): 401-404.

DUBEUF (J.P.), MORAND-FEHR (P.), RUBINO (R.), 2004.- Situation, changes, and future of goat industry around the world. *Small Ruminant Research*. 51 (2): 165‑173.

DUFFY (T.), KLEIMAN (F.), PIETROKOVSKY (S.), ISSIA (L.), SCHIJMAN (A.G.) & WISNIVESKY-COLLI (C.), 2009.- Real-time PCR strategy for rapid discrimination amongst main lymnaeid species from Argentina. *Acta Tropica*, 109 (1): 1-4.

DURAND (P.), POINTIER (J.P.), ESCOUBEYROU (K.), ARENAS (J.A.), YONG (M.), AMARISTA (M.), BARGUES (M.D.), MAS-COMA (S.) & RENAUD (F.), 2002.- Occurrence of a sibling species complex within Neotropical lymnaeids, snail intermediate hosts of fascioliasis. *Acta Tropica*, 83 (3): 233-240.

DWINGER (R.H.), LE RICHE (P.D.) & KUHNE (G.I.), 1982.- Fascioliasis in beef cattle in north-west Argentina. *Tropical Animal Health and Production*, 14 (3): 167-171.

EGERTON (J.R.), OSTLIND (D.A.), BLAIR (L.S.), EARY (C.H.), SUHAYDA (D.), CIFELLI (S.), RIEK (R.F.) & CAMPBELL (W.C.), 1979.- Avermectins, new family of potent anthelmintic agents: efficacy of the B1a component. *Antimicrobial Agents and Chemotherapy,* 15 (3): 372-378.

EL-KOUBA (M.M.), MARQUES (S.M.), PILATI (C.), & HAMANN (W.), 2005.-. Presence of *Fasciola hepatica* in feral nutria (*Myocastor coypus*) living in a public park in Brazil. *Zoo and Wildlife Medicine*, 40(1): 103-106.

ESCLAIRE (F.), AUDOUSSET (J.C.), RONDELAUD (D.), & DREYFUSS (G.), 1989.- Les metacercaires 'flottantes' de *Fasciola hepatica* L. à propos de quelques observations sur leur structure et leurs variations numériques au cours d'une infestation expérimentale chez *Lymnaea truncatula* Müller. *Bulletin de la Société française de parasitologie***,** 7: 225-228.

ESPINOZA (J.R.), MACO (V.), MARCOS (L.), SAEZ (S.), GONZALEZ (C.), NEYRA (V.), TERASHIMA (A.), SAMALVIDES (F.), GOTUZZO (E.), CHAVARRY (E.), HUAMAN (C.), BARGUES (M.D.), VALERO (M.A.) & MAS-COMA (S.), 2007.- Evaluation of Fas2-ELISA for the serological detection of *Fasciola hepatica* infection in humans. *American Journal of Tropical Medicine and Hygiene*, 76 (5): 977-982.

ESPINOZA (J.R.), TERASHIMA (A.), HERRERA-VELIT (P.), & MARCOS (L.A.), 2010.- Fasciolosis humana y animal en el Perú: impacto en la economía de las zonas endémicas. *Revista Peruana de Medicina Experimental y Salud Pública*, 27(4): 604-12.

ESTEBAN (J.G.) FLORES (A.), AGUIRRE (C.), STRAUSS (W.), ANGLES (R.) & MAS-COMA (S.), 1997a.- Presence of very high prevalence and intensity of infection with *Fasciola hepatica* amongst Aymara children from the Northern Bolivian Altiplano. *Acta Tropica*, 66 (1): 1-14.

ESTEBAN (J.G.), FLORES (A.), ANGLES (R.), STRAUSS (W.), AGUIRRE (C.) & MAS-COMA (S.), 1997b.- A population-based coprological study of human fascioliasis in a hyperendemic area of the Bolivian Altiplano. *Tropical Medicine and International Health*, 2 (7): 695-699.

ESTEBAN (J.G.), BARGUES (M.D) & MAS-COMA (S.), 1998.- Geographical distribution, diagnosis and treatment of human fascioliasis: a review. *Research and Reviews in Parasitology*, 58 (1): 13-48.

ESTEBAN (J.G.), FLORES (A.), ANGLES (R.) & MAS-COMA (S.), 1999.- High endemicity of human fascioliasis between Lake Titicaca and La Paz valley, Bolivia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 93 (2): 151-156.

ESTEBAN (J.G.), GONZALEZ (C.), BARGUES (M.D.), ANGLES (R.), SANCHEZ (C.), NAQUIRA (C.) & MAS-COMA (S.), 2002.- High fascioliasis infection in children linked to a man-made irrigation zone in Peru. *Tropical Medicine and International Health*, 7 (4): 339-348.

ESTEBAN (J.G.), GONZALEZ (C.), CURTALE (F.), MUÑOZ-ANTOLI (C.), VALERO (M.A.), BARGUES (M.D.), EL SAYED (M.), EL WAKEEL (A.), ABDEL-WAHAB (Y.), MONTRESOR (A.), ENGELS (D.), SAVIOLI (L.) & MAS-COMA (S.), 2003.- Hyperendemic fascioliasis associated with schistosomiasis in villages in the Nile Delta of Egypt. *American Journal of Tropical Medicine and Hygiene*, 69 (4): 429-437.

EUZEBY (J.), 1971. - Ecologie et biologie de *Lymnea truncatula* en Europe. *Les Cahiers de Medecine Veterinaire,* 40: 283-289.

FAIRWEATHER (I.), 1999.- Development of *F. hepatica* in the Mammalian Host. In: *Fasciolosis* (J.P. Dalton edit.), CAB International Publishing, Wallingford, Oxon, United Kingdom, pp. 47-111.

FAIRWEATHER (I.), 2011a.- Reducing the future threat from (liver) fluke: realistic prospect or quixotic fantasy? *Veterinary Parasitology*, 180 (1-2):, 133-143.

FAIRWEATHER (I.), 2011b.- Raising the bar on reporting 'triclabendazole resistance'. *Veterinary Record*, 168 (19): 514-515.

FETENE (A.) & MEKONNEN (A.), 2014.- An abattoir survey on the prevalence and monetary loss of fasciolosis amongst calttle slaughtered at Dangila Municipal Abattoir, Ethiopia. *Journal of Veterinary Medicine and Animal Health*, 6 (12): 309-316.

FIEL (C.A.), STEFFAN (P.E.), & FERREYRA (D.), 2011.- Diagnóstico de las Parasitosis más Frecuentes de los Rumiantes: Técnicas de Laboratorio e Interpretación de Resultados. (1ra ed.) Editorial Abad Benjamín. Tandil, Argentina.

FOLMER (O.), BLACK (M.), HOCH (W.), LUTZ (R.) & VRIJENHOEK (R.), 1994.- DNA primers for amplification of mitochondrial cytochrome c oxidase subunit 1 from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3 (5): 294-299.

FOREYT, (WJ.) &, TODD (A.C.), 1972. The occurrence of *Fascioloides magna* and *Fasciola hepatica* together in the livers of naturally infected cattle in south Texas and the incidence of the flukes in cattle, white-tailed deer, and feral hogs. *Journal of Parasitology*, 58 (5): 1010-1011.

FORMICA-CORSI (A.), 1900-1901.- Moluscos de la República Oriental del Uruguay. Anales del Museo Nacional de Montevideo, 2 (15/17): 293-525.

FUENTES (M.V.), VALERO (M.A.), BARGUES (M.D.), ESTEBAN (J.G.), ANGLES (R.) & MAS-COMA (S.), 1999.- Analysis of climatic data and forecast indices for human fascioliasis at very high altitude. *Annals of Tropical Medicine and Parasitology*, 93 (8): 835-850.

FUENTES (M.V.), MALONE (J.B.) & MAS-COMA (S.), 2001.- Validation of a mapping and predicting model for human fasciolosis transmission in Andean very high altitude endemic areas using remote sensing data. *Acta Tropica*, 79 (1): 87-95.

GASSER (R.B.), CHILTON (N.B.), HOSTE (H.) & BEVERIDGE (I.), 1993.- Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. *Nucleic Acids Research*, 21 (10): 2525-2526.

GAYO (V.), CUERVO (P.), ROSADILLA (D.), BIRRIEL (S.), DELL'OCA (L.), TRELLES (A.), CUORE (U.), MERA Y SIERRA (R.). 2011.- Natural *Fasciola hepatica* infection in "Nutria" (*Myocastor coypus*), in Uruguay. *Journal of Zoo and Wildlife Medicine,* 42 (2): 354-356.

GIFFONIELLO (A.H.), MIRAVET (S.V.), D'ANGELO (J.C.) & NOGARO (E.), 1983.- Distomatosis por *Fasciola hepatica*. *Prensa Médica Argentina*, 70: 70-73.

GIL (L.C.), DÍAZ (A.), RUEDA (C.), MARTÍNEZ (C.), CASTILLO (D.) & APT (W.), 2014.- Resistant human fasciolasis: report of four patients. *Revista Médica de Chile*, 142 (10): 1330-1333.

GIRONES (N.), VALERO (M.A.), GARCIA-BODELON (M.A.), CHICO-CALERO (M.I.), PUNZÓN (C.), FRESNO (M.) & MAS-COMA (S.), 2007.- Immune supression in advanced chronic fascioliasis: an experimental study in a rat model. *Journal of infectious Diseases*, 195 (10): 1504-1512.

GONZALEZ (M.S.), DI NUCCI (D.), SIDOTI (L.), & MERA Y SIERRA (R.L.), 2006.- Decomiso de hígados de bovinos provenientes de Mendoza en frigorífico debidoa *Fasciola hepatica*. In: XII Jornadas Argentinas de Microbiología, Mendoza, Argentina, June 2006. 102.

GONZÁLEZ (R.), PÉREZ RUANO (M.), & BRITO (R.), 2007. Fasciolosis bovina. Evaluación de las principales pérdidas provocadas en una empresa ganadera. *Revista Salud Animal*, 29 (3): 167-175.

GONZALEZ (L.C.), ESTEBAN (J.G.), BARGUES (M.D.), VALERO (M.A.), ORTIZ (P.), NAQUIRA (C.) & MAS-COMA (S.), 2011.- Hyperendemic human fascioliasis in Andean valleys: An altitudinal transect analysis in children of Cajamarca province, Peru. *Acta Tropica*, 120 (1-2): 119-129.

GREENWAY (D.), 1924.- Distomatosis humana por *Fasciola hepatica* en la Argentina. Primer diagnóstico coproscópico. *La Prensa Médica*, 11: 369.

GÜLHAN (B.), KANIK YÜKSEK (S.), TEZER (H.), ÖZKAYA PARLAKAY (A.), DALGIÇ (B.), DALGIÇ (A.) & YILMAZ (G.), 2015.- Partial hepatectomy for the resistant *Fasciola hepatica* infection in a child. *APSP Journal of Case Reports*, 6 (3): 27.

GUTELL (R.R.), SCHNARE (M.N.) & GRAY (M.W.), 1992.- A compilation of large subunit (23S- and 23S-like) ribosomal RNA structures. *Nucleic Acids Research*, 20 (suppl): 2095-2109.

GUTIERREZ (A.), POINTIER (J.P.), FRAGA (J.), JOBET (E.), MODAT (S.), PEREZ (R.T.), YONG (M.), SANCHEZ (J.), LOKER (E.S.) & THERON (A.), 2003.- *Fasciola hepatica*: identification of molecular markers for resistant and susceptible *Pseudosuccinea columella* snail hosts. *Experimental Parasitology*, 105 (3-4): 211-218.

HIJMANS (R.J.), CAMERON (S.E.), PARRA (J.L.), JONES (P.G.) & JARVIS (A.), 2005.- Very high resolution interpolated climate surfaces for global land areas. *International Journal of Climatology*, 25: 1965-1978.

HILLYER (G.V.), 1999.- Immunodiagnosis of human and animal fasciolosis. In: *Fasciolosis* (J.P. Dalton edit.), CAB International Publishing, Wallingford, Oxon, United Kingdom, pp. 435-447.

HOSTE (H.), TORRES-ACOSTA (J.F.), PAOLINI (V.), AGUILAR-CABALLERO (A.), ETTER (E.), LEFRILEUX (Y.), 2005.- Interactions between nutrition and gastrointestinal infections with parasitic nematodes in goats. *Small Ruminant Research,* 60 (1–2): 141–151.

HOSTE (H.), TORRES-ACOSTA (J.F.), AGUILAR-CABALLERO (A.J.), 2008.- Nutrition-parasite interactions in goats: is immunoregulation involved in the control of gastrointestinal nematodes? *Parasite Immunology*, 30 (2): 79-88.

HOSTE (H.), SOTIRAKI (S.), YAN LANDAU (S.), JACKSON (F.), BEVERIDGE (I.), 2010.- Goat-nematode interactions: think differently. *Trends Parasitology*, 26 (8): 376-381.

HOWELL (A.), MUGISHA (L.), DAVIES (J.), LACOURSE (E.), CLARIDGE (J.), WILLIAMS (D.J.L.), KELLY-HOPE (L.), BETSON (M.), KABATEREINE (N.B.), & STOTHARD (J.R.), 2012.- Bovine fasciolosis at increasing altitudes: Parasitological and malacological sampling on the slopes of Mount Elgon, Uganda. *Parasites & Vectors*, 5:196.

HUBENDICK (B.), 1951.- Recent Lymnaeidae. Their variation, morphology, taxonomy, nomenclature, and distribution. *Kungliga Svenska Vetenskapsakademiens Handlingar*, Fjärde Serien, 3 (1): 1-223 + 5 pl.

INABA (A.), 1969.- Cytotaxonomic studies of lymnaeid snails. *Malacologia*, 7: 143-168.

ISSIA (L.), PIETROKOVSKY (S.), SOUSA-FIGUEIREDO (J.), RUSSEL STOTHARD (J.) & WISNIVESKY-COLLI (C.), 2009.- *Fasciola hepatica* infections in livestock flock, guanacos and coypus in two wildlife reserves in Argentina. *Veterinary Parasitology* 165 (3-4): 341-344.

JABBOUR-ZAHAB (R.), POINTIER (J.P.), JOURDANE (J.), JARNE (P.), OVIEDO (J.A.), BARGUES (M.D.), MAS-COMA (S.), ANGLES (R.), PERERA (G.), BALZAN (C.), KHALLAAYOUNE (K.) & RENAUD (F.), 1997.- Phylogeography and genetic divergence of some lymnaeid snails, intermediate hosts of human and animal fascioliasis, with special reference to lymnaeids from the Bolivian Altiplano. *Acta Tropica*, 64 (3): 191-203.

JARNE (P.) & LAGODA (P.J.L), 1996.- Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution*, 11 (10): 424-429.

KABIL (S.M.), EL ASHRY (E.), & ASHRAF (N.K.), 2000.- An open-label clinical study of nitazoxanide in the treatment of human fascioliasis. *Current Therapeutic Research*, 61 (6): 339-345.

KACZORKIEWICZ (A.J.), 1983.- Distomatosis en la provincia de Neuquén. *Revista de Medicina Veterinaria*, 64: 354-356.

KAPLAN (R.M.), 2001.- *Fasciola hepatica* : a review of the economic impact in cattle and considerations for control. *Veterinary Theriogenology*, 2 (1): 40-50.

KEISER (J.) & UTZINGER (J.), 2005.- Emerging foodborne trematodiasis. *Emerging Infectious Diseases*, 11 (10): 1507-1514.

KELLEY (J.M.), ELLIOTT (T.P.), BEDDOE (T.), ANDERSON (G.) SKUCE (P.), & SPITHILL (T.W.), 2016.- Current Threat of triclabendazole resistance in *Fasciola hepatica. Trends in Parasitology,* 32 (6): 458-469.

KELLY (D.W.), PATERSON (R.A.), TOWNSEND (C.R.) & POULIN (R.), TOMPKINS (D.M.), 2009.- Parasite spillback: a neglected concept in invasion ecology? *Ecology,* 90 (8): 2047-2056.

KITHUKA (J.M.), MAINGI (N.), NJERUH (F.M.), & OMBUI (J.N.), 2002.- The prevalence and economic importance of bovine fasciolosis in Kenya-an analysis of abattoir data. *Onderstepoort Journal of Veterinary Research*, 69 (4): 255-262.

KLEIMAN (F.), GONZALEZ (N.), RUBEL (D.), WISNIVEKSY (C.), 2004.- *Fasciola hepatica* (Linnaeus, 1758) (Trematoda, Digenea) en liebres europeas (*Lepus europaeus*, Pallas 1778) (Lagomorpha, Leporidae) en la región Cordillerana Patagónica, Chubut, Argentina. *Parasitologia Latinoamericana*, 59: 68-71.

KNUBBEN-SCHWEIZER (G.) & TORGERSON (P.), 2015.- Bovine fasciolosis: Control strategies based on the location of *Galba truncatula* habitats on farms. *Veterinary Parasitology*, 208 (1-2): 77-83.

KRUSE (H.), KIRKEMO (A.), & HANDELAND (K.), 2004. Wildlife as source of zoonotic infections. *Emerging Infectious Diseases,* 10 (12): 2067-2072.

KUTZER (E.) & FREY (H.), 1976.- Die Parasiten der Feldhasen (*Lepus europaeus*) in Österreich. *Berliner und Münchener Tierärztliche Wochenschrift,* 89: 480-483.

LA POOK (J.D.), MAGUN (A.M.), NICKERSON (K.G.) & MELTZER (J.I.), 2000.- Sheep, watercress, and the Internet. *Lancet*, 356 (9225): 218.

LARREA (H.), OVIEDO (M.L.) & HUAMAN (P.), 1994.- Estudio anatómico de *Lymnaea diaphana* King, 1830, agente transmisor de la fascioliasis en la zona Sur del Perú. *Boletín de Lima*, 16 (91-96): 95-100.

LE (T.H.), DE (N.V.), AGATSUMA (T.), NGUYEN (T.G.T.), NGUYEN (Q.D.), & MCMANUS (D.P.), *et al.,* 2008. Human fascioliasis and the presence of hybrid/introgressed forms of *Fasciola hepatica* and *Fasciola gigantica* in Vietnam. *International Journal of Parasitology*, 38 (6): 725-730.

LEBBIE (S.H.), 2004.- Goats under household conditions. *Small Ruminant Research* 51 (2): 131-136.

LEGUÍA (P.G.), 1997.- Acute and subacute Fasciolosis of alpacas (*Lama pacos*) and treatment with triclabendazole. *Tropical Animal Health and Production*, 29 (1): 31-2.

LLORET (L.), DE JONG (L.), SALOMON (S.), & CARENA (J.), 2005.-Hipereosinofilia en un brote urbano de distomatosis hepática. XIV Congreso Nacional de Medicina: 21-24 November 2005; Buenos Aires, Argentina. Conference Abstracts: No. 2.

LOGALDO (E.), 1955.- Distomatosis de las vias biliares, litiasis vesicular. *Revista de la Asociación Médica Argentina*, Agosto: 260-261.

LOMBARDERO (O.J.), MORIENA (R.A.), RACIOPPI (O.), COPPO (J.A.), & SCHIFFO (H.P.), 1979a.- Distomatosis hepática de los bovinos en la Provincia de Corrientes. *Revista Veterinaria*, 2 (2): 21-29.

LOMBARDERO (O.J.), MORIENA (R.A.), RACIOPPI (O.) & QUIROZ (L.), 1979b.- Bionomía de *Limnaea* sp. en la Provincia de Corrientes. *Veterinaria*, 2 (2): 5-19.

LONGO (O.F.) & DARAIO (M.), 1953.- Distoma del colédoco. Importancia de la colangiografía operatoria. *Prensa Médica Argentina*, 43 (40): 2910-2911.

LOPEZ LEMES (M.H.), HERNANDEZ (S.), ACUÑA (A.M.), & NARI (A.), 1996.- Fascioliasis en la República Oriental del Uruguay. *Revista Médica del Uruguay*, 12 (1): 37-43.

LUMBRERAS (H.), CANTELLA (R.) & BURGA (R.), 1962.- Acerca de un procedimiento de sedimentación rápida para investigar huevos de *Fasciola hepatica* en las heces, su evaluación y uso en el campo. *Revista de Medicina Peruana*, 31: 167-174.

MAILLES (A.), CAPEK (I.), AJANA (F.), SCHEPENS (C.), ILEF (D.) & VAILLANT (V.), 2006.- Commercial watercress as an emerging source of fascioliasis in Northern France in 2002: results from an outbreak investigation. *Epidemiology and Infection* 134: 942-945.

MAJUL (E.J.), SALICH (J.C.) & BENDER (A.L.), 1981.- Distomatosis hepatobiliar por *Fasciola hepatica. Prensa Médica Argentina*, 68: 81-83.

MALANDRINI (J.B.), CARNEVALE (S.), VELAZQUEZ (J.) & SORIA (C.C.), 2009a.- Diagnóstico de *Fasciola hepatica* con la técnica de ELISA en el Departamento de Tinogasta. *Ciencia,* 4 (10): 143-151.

MALANDRINI (J.B.), SORIA (C.C.), CARNEVALE (S.), VELAZQUEZ (J.) & PIZARRO (M.C.), 2009b.- Estudio de la fascioliasis desde una perspectiva multidisciplinaria. In: XXVIII Jornadas de Actualización en Ciencias Veterinarias (Villa Giardino, Córdoba 25-27 September 2009).

MALEK (E.A.), 1985.- Snail hosts of schistosomiasis and other snail-transmitted diseases in tropical America: a manual. Pan American Health Organization, Washington, Scientific Publication No. 478: 325 pp.

MALONE (J.B.), GOMMES (R.), HANSEN (J.), YILMA (J.M.), SLINGENBERG (J.), SNIJDERS (F.), NACHTERGAELE (F.) & ATAMAN (E.), 1998.- A geographic information system on the potential distribution and abundance of *Fasciola hepatica* and *F. gigantica* in east Africa based on Food and Agriculture Organization databases. *Veterinary Parasitology*, 78 (2): 87-101.

MANGOLD (A.J.), BARGUES (M.D.) & MAS-COMA (S.), 1998.- 18S rRNA gene sequences and phylogenetic relationships of European hard-tick species (Acari: Ixodidae). *Parasitology Research*, 84 (1): 31-37.

MARCOS (L.A.) & TERASHIMA (A.), 2007.- Update on human fascioliasis in Peru: diagnosis, treatment and clinical classification proposal. *Neotropical Helminthology*, 1 (2): 85-103.

MARTÍNEZ-DÍAZ (R.A), MARTELLA (M.B.), NAVARRO (J.L.) & PONCE-GORDO (F.), 2013.- Gastrointestinal parasites in greater rheas (*Rhea americana*) and lesser rheas (*Rhea pennata*) from Argentina. *Veterinary Parasitology*, 194 (1): 75-78.

MASCHERONI (H.A.), 1933.- Colecistitis parasitaria: sobre un caso de distomatosis por *Fasciola hepatica*. *Revista Médica Quirúrgica de Patologías Femeninas*, 2: 710.

MAS-COMA (S.), 2004.- Human fascioliasis. In: World Health Organization (WHO), Waterborne Zoonoses: Identification, Causes and Control. Cotruvo (J.A.) *et al.* (eds), IWA Publishing, London, United Kingdom: 305-322.

MAS-COMA (S.), 2005.- Epidemiology of fascioliasis in human endemic areas. *Journal of Helminthology*, 79 (3): 207-216.

MAS-COMA (S.) & BARGUES (M.D.), 1997.- Human liver flukes: a review. *Research and Reviews in Parasitology*, 57 (3-4): 145-218.

MAS-COMA (S.) & BARGUES (M.D.), 2009.- Populations, hybrids and the systematic concepts of species and subspecies in Chagas disease triatomine vectors inferred from nuclear ribosomal and mitochondrial DNA. *Acta Tropica*, 110 (2-3): 112-136.

MAS-COMA (S.), FONS (R.), FELIU (C.), BARGUES (M.D.), VALERO (M.A.), & GALAN- PUCHADES (M.T.), 1988.- Small mammals as natural definitive hosts of the liver fluke, *Fasciola hepatica* Linnaeus, 1758 (Trematoda: Fasciolidae): a review and two new records of epidemiologic interest on the island of Corsica. *Rivista di Parasitologia,* 5: 73-78.

MAS-COMA (S.), ANGLES (R.), STRAUSS (W.), ESTEBAN (J.G.), OVIEDO (J.A.) & BUCHON (P.), 1995.- Human fasciolasis in Bolivia: a general analysis and a critical review of existing data. *Research and Reviews in Parasitology*, 55 (2): 73-93.

MAS-COMA (S.), RODRIGUEZ (A.), BARGUES (M.D.), VALERO (M.A.), COELLO (J.R.) & ANGLES (R.), 1997.- Secondary reservoir role of domestic animals other than sheep and cattle in fascioliasis transmission in the Northern Bolivian Altiplano. *Research and Reviews in Parasitology*, 57 (1): 39-46.

MAS-COMA (S.), ESTEBAN (J.G.) & BARGUES (M.D.), 1999a.- Epidemiology of human fascioliasis: a review and proposed new classification. *Bulletin of the World Health Organization*, 77 (4): 340-346.

MAS-COMA (S.), BARGUES (M.D.) & ESTEBAN (J.G.), 1999b.- Human Fasciolosis. In: Fasciolosis, Dalton (J.P.) (ed.), CAB International Publishing, Wallingford, Oxon, United Kingdom, pp. 411-434.

MAS-COMA (S.), ANGLES (R.), ESTEBAN (J.G.), BARGUES (M.D.), BUCHON (P.), FRANKEN (M.) & STRAUSS (W.), 1999c.- The Northern Bolivian Altiplano: a region highly endemic for human fascioliasis. *Tropical Medicine and International Health*, 4 (6): 454-467.

MAS-COMA (S.), FUNASTSU (I.R.) & BARGUES (M.D.), 2001.- *Fasciola hepatica* and lymnaeid snails occurring at very high altitude in South America. *Parasitology*, 123: (Suppl.): 115-127.

MAS-COMA (S.), BARGUES (M.D.), VALERO (M.A.), FUENTES (M.V.), 2003.- Adaptation capacities of *Fasciola hepatica* and their relationships with human fascioliasis: from below sea level up to the very high altitude. In: Taxonomy, Ecology and Evolution of Metazoan Parasites, Combes (C.) & Jourdane (J.) (eds.), Presses Universitaires de Perpignan, Perpignan, France, pp. 81-123.

MAS-COMA (S.), BARGUES (M.D.) & VALERO (M.A.), 2005.- Fascioliasis and other plant-borne trematode zoonoses. *International Journal for Parasitology*, 35 (11-12): 1255-1278.

MAS-COMA (S.), BARGUES (M.D.) & VALERO (M.A.), 2007.- Plantborne trematode zoonoses: fascioliasis and fasciolopsiasis. In: World Class Parasites, Vol. 11. Food-Borne Parasites, , Fish and Plant-Borne Parasites (D. Murrell & B. Fried eds.), Springer Verlag, New York,United States of America, Vol. 11: pp. 293-334.

MAS-COMA (S.), VALERO (M.A.) & BARGUES (M.D.), 2008.- Effects of climate change on animal and zoonotic helminthiases. In: Climate Change: Impact on the Epidemiology and Control of Animal Diseases (S. de La Rocque, G. Hendrickx & S. Morand coord.). Scientific and Technical Review, World Organisation for Animal Health (OIE), Paris. *Revue Scientifique et Technique de l'Office Internationale des Epizooties*, 27 (2): 443-457.

MAS-COMA (S.), VALERO (M.A.) & BARGUES (M.D.), 2009a.- *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Advances in Parasitology*, 69: 41-146.

MAS-COMA (S.), VALERO (M.A.) & BARGUES (M.D.), 2009b.- Climate change effects on trematodiases, with emphasis on zoonotic fascioliasis and schistosomiasis. *Veterinary Parasitology*, 163 (4): 264-280.

MAS-COMA (S.), VALERO (M.A.) & BARGUES (M.D.), 2010.- Climate change effects on trematode and nematode diseases affecting children in rural areas of developing countries. *International Public Health Journal,* 2 (4): 405-430.

MAS-COMA (S.), BARGUES (M.D.), & VALERO (M.A.), 2014.- Diagnosis of human fascioliasis by stool and blood techniques: Update for the Climate change effects on trematode and nematode diseases affecting children in rural areas of developing countries present global scenario. *Parasitology*, 141 (Special Issue): 1918-1946.

MAS-COMA (S.), BARGUES (M.D.), & VALERO (M.A.), 2018.- Human fascioliasis infection sources, their diversity, incidence factors, analytical methods and prevention measures. *Parasitology*, 1-35.

MATHEWS (D. H.), SABINA (J.), ZUKER (M.) & TURNER (D. H.), 1999.- Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *Journal of Molecular Biology*, 288 (5):911-40.

MCKENNA, (P.B.), 1997.-. Checklist of helminth parasites of terrestrial mammals in New Zealand. *New Zealand Journal of Zoology*, 24: 277-290.

MELERO (M.), RIGOU (R.C.), LLOVERAS (J.) & GENNARO (O.), 1991.- Fascioliasis hepática Causa infrecuente de síndrome febril prolongado con hipereosinofilia e imágenes hipodensas en la tomografía computada de hígado. *Medicina, Buenos Aires*, 51 (3): 244-248.

MENARD (A.), AGOULON (A.), L'HOSTIS (M.), RONDELAUD (D.),COLLARD (S.), & CHAUVIN (A.), 2001.- *Myocastor coypus* as a reservoir host of *Fasciola hepatica* in France. *Veterinary Research*, 32 (5): 499-508.

MERA Y SIERRA, (R.L.), 2001.- Distribución de Lymnaeidos en el sistema hidrográfico andino de la provincia de Mendoza, Argentina. Director: Bargués, M. D. Tesis de Maestría, Master Internacional en Enfermedades Parasitarias Tropicales. Facultad de Farmacia, Universitat de. València.

MERA Y SIERRA (R.), 2012.- Rumiantes: Fasciolosis. In: Parasitología y Enfermedades Parasitarias en Veterinaria (2º Parte: Enfermedades Parasitarias). Rosa (A.B.) & Ribicich (M.M.) (eds). Editorial Hemisferio Sur S.A., Buenos Aires, Argentina. pp. 145-149.

MERA Y SIERA (R.L.), SCIBILIA (C.), PABST (A.), IRRAZABAL (G.), & SENAR (M.), 2005a.- Abattoir condemnation of bovine livers due to *Fasciola hepatica* in Tupungato, Mendoza. In: XXIII Reunión Científica Anual, Sociedad Biológica de Cuyo, BIOCELL 29: 371.

MERA Y SIERRA (R.), MORALES (J.J.), GUIÑAZU (M.), SIDOTI (L.) & ARTIGAS (P.), 2005b.- Altitudinal distribution of Lymnaeid molluscs in Mendoza province, Argentina. Presented in XXIII Reunión Científica Anual, Sociedad Biológica de Cuyo. *Biocell, 29* (3): 371.

MERA Y SIERRA (R.L.), ARTIGAS (P.) & MAS-COMA (S.), 2006.- Lymnaeid vectors of fascioliasis in endemic areas of Mendoza, Argentina. In: XI Inter-national Congress of Parasitology-ICOPA XI (Glasgow, UK, 6-11 August 2006): CD-ROM.

MERA Y SIERRA (R.), ARTIGAS (P.), CUERVO (P.), DEIS (E.), SIDOTI (L.), MAS-COMA (S.) & BARGUES (M.D.), 2009.- Fascioliasis transmission by *Lymnaea neotropica* confirmed by nuclear rDNA and mtDNA sequencing in Argentina. *Veterinary Parasitology*, 166: 73-79.

MERA Y SIERRA (R.), AGRAMUNT (V.H.), CUERVO (P.), MAS-COMA (S.), 2011.- Human fascioliasis in Argentina: retrospective overview, critical analysis and baseline for future research. *Parasite & Vectors* 4: 104.

MERA Y SIERRA (R.), CANTERO (F.). GONZALEZ (M.), 2015.- *Fasciola hepatica* en guanacos y llamas en un establecimiento de Malargue, provincia de Mendoza. In: III Congreso Panamericano de Zoonosis (La Plata, Argentina, 4-6 June 2014), published in *Revista Argentina de Zoonosis y Enfermedades Infecciosas Emergentes,* 2015*,* 10 (1): 46-47.

MERA Y SIERRA (R.), GONZALEZ (M.), NEIRA (G.), 2016.- Distribución Altitudinal de *Fasciola hepatica* en Bovinos de la Provincia de Mendoza, Argentina. Investigación, Ciencia y Universidad, 1 (1): 14-18.

MEUNIER (C.), TIRARD (C.), HURTREZ-BOUSSES (S.), DURAND (P.), BARGUES (M.D.), MAS-COMA (S.), POINTIER (J.P.), JOURDANE (J.) & RENAUD (F.), 2001.- Lack of molluscan host diversity and the transmission of an emerging parasitic disease in Bolivia. *Molecular Ecology*, 10 (5): 1333-1340.

MEZO (M.), GONZÁLEZ-WARLETA (M.), CASTRO-HERMIDA (J.A.), MANGA-GONZÁLEZ (M.Y.), PEIXOTO (R.), MAS-COMA (S.), VALERO (M.A.) 2013.- The wild boar (*Sus scrofa* Linnaeus, 1758) as secondary reservoir of *Fasciola hepatica* in Galicia (NW Spain). *Veterinary Parasitology,* 198 (3-4): 274-283.

MIGUEL (C.M.), MALLEA GIL (M.S.), BASILE (M.A.) & MAURO (E.L.), 1985.- Distomatosis por *Fasciola hepatica*. *Prensa Médica Argentina*, 72 (6): 192-195.

MINOPRIO (J.E.), DONNA (R.A.), BORREMANS (C.G.), PASSARDI (A.H.), GLATSTEIN DE WOLF (T.), OLINDO (A.L.) & JORG (M.E.), 1995.- Epidemia familiar de distomatosis humana por *Fasciola hepatica*. Localización errática (cutánea). *Prensa Médica Argentina*, 82: 869-877.

MOGHADDAM (A.S.), MASSOUD (J.), MAHMOODI (M.), MAHVI (A.H.), PERIAGO (M.V.), ARTIGAS (P.), FUENTES (M.V.), BARGUES (M.D.) & MAS-COMA (S.), 2004.-Human and animal fascioliasis in Mazandaran province, northern Iran. *Parasitology Research*, 94 (1): 61-69.

MONTES (M.C.), CARMANCHAHI (P.D.), REY (A.), FUNES (M.C.), 2006.- Live shearing free-ranging guanacos (*Lama guanicoe*) in Patagonia for sustainable use. *Journal of Arid Environments*, 64: 616-625.

MORAND-FEHR (P.), BOUTONNET (J.P.), DEVENDRA (C.), DUBEUF (J.P), HAENLEIN (G.F.), HOLST (P.), 2004, Strategy for goat farming in the 21st century. *Small Ruminant Research,* 51 (2): 175-183.

MORGAN (J.A.) & BLAIR (D.), 1998 - Mitochondrial ND1 gene sequences used to identify echinostome isolates from Australia and New Zealand. *International Journal* for *Parasitology*, 28 (3): 493-502

MORIENA (R.A.), RACIOPPI (O.), ALVAREZ (J.D.) & LOMBARDERO (O.J.), 2001.- *Fasciola hepatica* y otros trematodos de bovinos. Prevalencia según decomisos en plantas faenadoras en la provincia de Corrientes (Argentina). *Veterinaria Argentina*, 17 (177): 493-498.

MORIENA (R.A.), RACIOPPI (O.), ALVAREZ (J.D.) & LOMBARDERO (O.J.), 2002.- Distomatosis hepática (*Fasciola hepatica*) y otros trematodos de los rumiantes. Distribución en la provincia de Corrientes. Comunicaciones Científicas y Tecnológicas de la Universidad Nacional del Nordeste, Ciencias Veterinarias, Corrientes, Argentina.

MORIENA (R.A.), RACIOPPI (O.), & ALVAREZ (J.D.), 2004.- Fasciolosis en bovinos del nordeste argentino. Prevalencia según edad. *Revista Veterinaria*, 15 (1): 3-4.

MORIENA (R.A.), ALVAREZ (J.D.), PIETROKOVSKY (S.), RUBEL (D.), PREPELITCHI (L.), RACIOPPI (O.) & WISNIVESKY (C.). 2008.- Presencia de *Pseudosuccinea columella* naturalmente infestada con *Fasciola hepatica* en Santo Tomé (Corrientes, Argentina). *Revista Veterinaria*, 19 (2): 147-149.

MORRONE (J.J.), 2006.- Biogeographic areas and transition zones of Latin America and the Caribbean islands based on pangeographic and cladistic analyses of the entomofauna. *Annual Review of Entomology,* 51: 467-494.

MUSTERS (W.), BOON (K.), Van Der SANDE (C.), Van HEERIKHUIZEN (H.) & PLANTA (R.J.), 1990 - Functional analysis of transcribed spacers of yeast ribosomal DNA. *The EMBO Journal*, 9 (12): 389-396.

NGUYEN (T.G.), (L.E.) (T.H.), DAO (T.H.), TRAN (T.L.), PRAET (N.), SPEYBROECK (N.), VERCRUYSSE (J.) & DORNY (P.), 2011.- Bovine fasciolosis in the human fasciolosis hyperendemic Binh Dinh province in Central Vietnam. *Acta Tropica* 117 (1): 19-22.

NICKEL (S.), & GOTTWALD (A.), 1979.- Parasites of the GDR. 3. Endoparasites of the hare (*Lepus europeus*). *Angewandte Parasitologie*, 20: 57-62.

NIEC (R.), 1968.- Cultivo e identificación de larvas infectantes de nemátodes gastrointestinales del bovino y ovino.: INTA, Manual técnico n. 3. Buenos Aires, Argentina.

NIETO SOSA (L.), BARNES (A.), JIMENEZ (G.) & CICAERLLI (G.), 2008.-Fasciolosis: evaluación clínica y terapéutica. In: *III Congreso Latinoamericano de Zoonosis y VI Congreso Argentino de Zoonosis* (Buenos Aires, Argentina, Junio 2008). Conference abstracts: No 35.

NIÑO (F.), 1965.- Parasitología. Zooparásitos y Patología de las Zooparasitosis Humanas. Editorial Beta, Buenos Aires, Argentina, 656 pp.

NOVARO (A.J.), CAPURRO (A.F.), TRAVAINI (A.), FUNES (M.C.), RABINOVICH (J.E.), 1992.- Pellet-count sampling based on spatial distribution: a case study of the European hare in Patagonia. *Ecología Austral,* 2: 11-18.

NOVOBILSKÝ (A.), KAŠNÝ (M.), BERAN (L.), RONDELAUD (D.), HÖGLUND (J.), 2013.- *Lymnaea palustris* and *Lymnaea fuscus* are potential but uncommon intermediate hosts of *Fasciola hepatica* in Sweden**.** *Parasite & Vectors, 6 (1): 251.*

OLAECHEA (F.V.), 1994.- Epidemiología y control de *Fasciola hepatica* en la Argentina. In: Enfermedades Parasitarias de Importancia Económica en Bovinos (Nari A & Fiel C. edit.). Editorial Agropecuaria, Hemisferio Sur SRL, Montevideo, Uruguay: 213-232.

OLAECHEA (F.V.), 2007. Trematodes y Céstodes: *Fasciola hepatica*. In: (Eds. V. H. Suárez, F. V. Olaechea, C. E. Rossanigo, and J. R. Romero), Enfermedades parasitarias de los ovinos y otros rumiantes menores en el cono sur de América, Buenos Aires, Argentina, Instituto Nacional de Tecnología Agropecuaria - INTA, 159-168.

OLAECHEA (F.V.), LOVERA (V.), LARROZA (M.), RAFFO (F.) & CABRERA (R.), 2011.- Triclabendazole-resistant liver fluke in Patagonian (Argentina) cattle. In: XII International Congress of Parasitology ICOPA XII (Melbourne, Australia, 16-20 August 2010), Conference Abstracts: No. 1783.

OLAZARRI (J.), 1988.- Los transmisores del “saguaypé” en el Uruguay. *Almanaque BSE Montevideo.* 1988; 212-219

OLLERENSHAW (C.B.), 1971.- The influence of climate on the life cycle of *Fasciola hepatica* in Britain with some observations on the relationship between climate and the incidence of fascioliasis in the Netherlands. In: Facts and Reflections, Symposium Proceedings (3 June 1971), Parasitology Department of the Centraal Diergeneeskundig Institut, Lelystad: 41-63.

O'NEILL (S.M.), PARKINSON (S.M.), STRAUSS (W.), ANGLES (R.) & DALTON (J.P.), 1998.- Immunodiagnosis of *Fasciola hepatica* (Fascioliasis) in a human population in the Bolivian Altiplano using purified cathepsin L cysteine proteinase. *American Journal of Tropical Medicine and Hygiene*, 58 (4): 417-423.

ORTIZ (P.), SCARCELLA (S.), CERNA (C.), ROSALES (C.), CABRERA (M.), GUZMÁN (M.), LAMENZA (P.), SOLANA (H.), 2013.- Resistance of *Fasciola hepatica* against Triclabendazole in cattle in Cajamarca (Peru): A clinical trial and an *in vivo* efficacy test in sheep. *Veterinary Parasitology*, 95 (1-2):118-121.

OSSOLA (A.), SICILIANO (C.) & GARCIA FAURE (J.), 1972.- Distomatosis hepática, tres epidemias familiares en Córdoba, Argentina. *Revista Asociación Bioquímica Argentina*, 37 (201-201):100-106.

OVEREND (D.J.) & BOWEN (F.L.), 1995.- Resistance of *Fasciola hepatica* to triclabendazole. *Australian Veterinary Journal*, 72: 275-276.

OVIEDO (J.A.), BARGUES (M.D.) & MAS-COMA (S.), 1995a.- Lymnaeid snails in the human fascioliasis high endemic zone of the Northern Bolivian Altiplano. *Research and Reviews in Parasitology*, 55 (1): 35-43.

OVIEDO (J. A), BARGUES (M. D.) & MAS-COMA (S.), 1995b.- Ecological characteristics of lymnaeid snails in the human fascioliasis high endemic zone of the Bolivian Northern Altiplano. In Unitas Malacologica, Guerra, A., Rolán, E. & Rocha, F. eds. Instituto de Investigaciones Marinas, Vigo, Spain, pp. 463-465.

OVIEDO (J. A), BARGUES (M. D.) & MAS-COMA (S.), 1996.- The intermediate snail host of *Fasciola hepatica* on the mediterranean island of Corsica. *Research and Reviews in Parasitology*, 56 (4): 217-220.

PADILLA ANTONI (F.), SALEME (A.) & JORRATT (M.), 1970.- *Fasciola hepatica*. A propósito de una observación. *Prensa Médica Argentina*, 57: 521-525.

PALADINO (J.E.) & GALARCE (J.), 1939.- Parasitosis apendicular. *La Semana Médica*, 2: 689-690.

PARAENSE (W.L.), 1976.- *Lymnaea viator*: a study of topotypic specimens (Mollusca: Lymnaeidae). *Revista Brasileira de Biologia*, 36: 419-428.

PARAENSE (W.L.), 1982a.- *Lymnaea viator* and *Lymnaea columella* in the Neotropical region: A distributional outline. *Memórias do Instituto Oswaldo Cruz*, 77 (2): 181-188.

PARAENSE (W.L.), 1982b.- *Lymnaea rupestris* sp. n. from southern Brazil (Pulmonata: Lymnaeidae). *Memórias do Instituto Oswaldo Cruz*, 77 (4): 437-443.

PARAENSE (W.L.), 1983.- *Lymnaea columella* in northern Brazil. *Memorias do Instituto Oswaldo Cruz*, 78 (4): 477-482.

PARAENSE (W.L.), 1984.- *Lymnaea diaphana:* a study of topotypic specimens (Pulmonata: Lymnaeidae). *Memorias do Instituto Oswaldo Cruz*, 79 (1): 75-81.

PARAENSE (W.L.), 2005.-). Planorbidae, Lymnaeidae and Physidae of Argentina (Mollusca: Basommatophora). *Memórias do Instituto Oswaldo Cruz*, 100 (5): 491-493.

PEIRETTI (J.A.), 1969.- Distomatosis hepática, contribución al diagnóstico precoz. *Día Médico*, 10: 248.

PEIRETTI (J.A.) & MORALES (J.R.), 1973.- Epidemia familiar de distomatosis por *Fasciola hepatica*. *Prensa Médica Argentina*, 60: 262-264.

PERIAGO (M.V.), VALERO (M.A.), PANOVA (M.) & MAS-COMA (S.), 2006.- Phenotypic comparison of allopatric populations of *Fasciola hepatica* and *Fasciola gigantica* from European and African bovines using a computer image analysis system (CIAS). *Parasitology Research,* 99 (4): 368-378.

PETRAGLIA (A.A.), 1954.- Parasitosis humana por *Fasciola hepatica*: primer caso que se describe en el Noreste Argentino. *Actas y Trabajos de la Asociación Argentina para el Estudio de Enfermedades Transmisibles*, 3: 47-48.

PFEIFFER (L.), 1839.- Bericht *über* die Ergebnisse meiner Reise nach Cuba im winter 1838-1839. *Archiv für Naturgeschichte*, 5: 346-359.

PILSBRY (H.A.), 1898.- Notes on Uruguay and Argentine fresh-water shells supplemental to the list in Nautilus, X, 76-81, *Nautilus*; 12 (4): 46.

PILSBRY (H.A.), 1911.- Non-marine mollusca of Patagonia. *Reports of Princeton University Expeditions to Patagonia*, 1896-1899, 3 (5): 513-633.

PILSBRY (H.A.) & RUSH (W.H.), 1896.- List, with notes, of land and fresh water shells collected by Dr. Wm. H. Rush in Uruguay and Argentina. *Nautilus*, 10 (7): 76-81.

PIZZI (H.), ZUNILDA NAVARRO (D.), PIZZI (D.), & BENVISSUTO (G.), 1984.- Estudio epidemiológico e implicancias económicas de la distomatosis hepática en la provincia de Córdoba. *Gaceta Veterinaria*, 374: 944-947.

POINTIER (J.P.), CAZZANIGA (N.J.), GONZALEZ-SALAS (C.), GUTIERREZ (A.), ARENAS (J.A.), BARGUES (M.D.) & MAS-COMA (S.), 2006.- Anatomical studies of sibling species within Neotropical lymnaeids snail intermediate hosts of fascioliasis. *Memorias do Instituto Oswaldo Cruz*, 101 (4): 431-435.

POINTIER, (J.P.), COUSTAU (C.), RONDELAUD (D.) & THERON. (A.), 2007.- *Pseudosuccinea columella* (Say, 1817) (Gastropoda, Lymnaeidae), snail host of *Fasciola hepatica*: first record for France in the wild. *Parasitology Research,* 101(5): 1389-1392.

POLLEY (L.), 2005.- Navigating parasite webs and parasite flow: Emerging and re-emerging parasitic zoonoses of wildlife origin. *International Journal for Parasitology,* 35 (11-12): 1279-1294.

POSADA (D.) & CRANDALL (K. A.), 1998.- Modeltest: testing the model of DNA substitution. *Bioinformatics*, 14: 817-818.

POSADA (D.) & BUCKLEY (T.R.), 2004.- Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. *Systematic Biology*, 53 (5): 793-808.

POULIN (R.), 1997.- Egg production in adult trematodes: adaptation or constraint. *Parasitology,* 11:195-204.

POULIN (R.), PATERSON (R.A.), TOWNSEND (C.R.), TOMPKINS (D.M.), KELLY (D.W.) 2011. Biological invasions and the dynamics of endemic diseases in freshwater ecosystems. *Freshwater Biology,* 56: 676-688.

PREPELITCHI (L.), KLEIMAN (F.), PIETROKOVSKY (S.M.), MORIENA (R.A.), RACIOPPI (O.), ALVAREZ (J.) & WISNIVESKY-COLLI (C.), 2003.- First report of *Lymnaea columella* Say, 1817 (Pulmonata: Lymnaeidae) naturally infected with *Fasciola hepatica* (Linnaeus, 1758) (Trematoda: Digenea) in Argentina. *Memorias do Instituto Oswaldo Cruz*, 98 (7): 889-891.

QUIJADA (T.), ARAQUE (C.), JIMÉNEZ (M.), PACHECO (A.), QUIJADA (J), DURAN (M.), & BOHÓRQUEZ (R.), 2005.- Prevalencia de *Fasciola hepatica* en bovinos en un matadero industrial del Estado Lara. Venezuela. *Gaceta de Ciencias Veterinarias*, 10 (2): 114-118.

QURESHI (A.W.), TANVEER (A.), MAS-COMA (S.), 2016.-. Epidemiological analysis of human fascioliasis in northeastern Punjab, Pakistan. *Acta Tropica*, 156: 157-164.

RACHOWICZ (L.J.), HERO (J.M.), ALFORD (R.A.), TAYLOR (J.W.), MORGAN (J.A.T.), VREDENBURG (V.T.), COLLINS (J.P.) & BRIGGS (C.J.), 2005.- The novel and endemic pathogen hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. *Conservation Biology,* 19: 1441-1448.

RANGUEL RUIZ (L.J.) & MARTÍNEZ DURAN (E.), 1994.- Pérdidas económicas por decomisos de hígados y distribución geográfica de la fasciolosis bovina en el estado de Tabasco, México. *Veterinaria México,* 25 (4): 327-331.

REMIGIO (E.A.) & BLAIR (D.), 1997a.- Relationships amongst problematic North American stagnicoline snails (Pulmonata: Lymnaeidae) reinvestigated using nuclear ribosomal DNA internal transcribed spacer sequences. *Canadian Journal of Zoology*, 75 (9): 1540-1545.

REMIGIO (E.A.) & HEBERT (P.D.), 2003.- Testing the utility of partial COI sequences for phylogenetic estimates of gastropod relationships. *Molecular Phylogenetics and Evolution*, 29 (3): 641-647.

RIOS (N.), PAVIA (M.), MEANA (M.), SERRANO (G.) & NAVARRO (R.), 2008.- Un caso de fascioliasis hepática. In: III Congreso Latinoamericano de Zoonosis y VI Congreso Argentino de Zoonosis (Buenos Aires, Argentina, Junio 2008). Resúmenes de Comunicaciones, 35.

RITCHIE (L.S.), 1948.- An ether sedimentation technique for routine stool examination. *Bulletin of the US Army Medical Department,* 8 (4): 326.

RODRIGUEZ (C.), 1952.- Parasitosis por *Fasciola hepatica*, Consideraciones. Experiencia Personal. *Revista Médica de Córdoba*, 40: 9-12.

RODRIGUEZ (C.), 1954.- *Fasciola hepatica*. Biología del Parásito. *Revista Médica de Córdoba*, 42: 213-216.

RODRIGUEZ (C.), 1961.- Parasitosis intestinales en Córdoba. Estadística sobre diez mil casos. *Revista Medica de Córdoba*, 49: 37-40.

ROFFO (A.H.), 1913.- Distomatosis humana, primer caso observado en el país. Imprenta y Editora Coni Hermanos, Buenos Aires, Argentina, 38 pp.

RONDELAUD (D.), VIGNOLES (P.), DREYFUSS (G.), 2014.- Adaptation of preadult *Lymnaea fuscus* and *Radix balthica* to *Fasciola hepatica* through the infection of several successive generations of snails. *Parasites & Vectors*, 7: 296.

ROOS (M.H.), HOEKSTRA (R.), PLAS (M.E.), OTSEN (M.) & LENSTRA (J.A.), 1998.- Polymorphic DNA markers in the genome of parasitic nematodes. *Journal of Helminthology,* 72 (4): 291-294.

ROSS (I.C.) & MCKAY (A.C.), 1929.- The bionomics of *Fasciola hepatica* in New South Wales and of the intermediate host Limnaea brazieri. *Bulletin of the Council of Scientific and Industrial Research Australia*. No. 43, 62 pp.

ROSSANIGO (C.E.), AVILA (J.D.), VASQUEZ (R.), SAGER (R.L.), 1983.- Incidencia, distribución e identificación del huésped intermediario de la distomatosis bovina en la provincia de San Luis. *Gaceta Veterinaria* 1983; 45(382): 739-746.

ROSSANIGO (C.E.), 2007.- Parasitosis de las cabras. In: Suárez VH, Olaechea FV, Rossanigo CE, Romero JR. Enfermedades parasitarias de los ovinos y otros rumiantes menores en el cono sur de América. EEA Anguil, INTA Buenos Aires, Argentina pp. 247-270.

ROWAN (W.), 1956.- The mode of hatching of the egg of *Fasciola hepatica*. *Experimental Parasitology*, 5 (2): 118-136.

ROWCLIFFE (S.A.) & OLLERENSHAW (C.B.), 1960.- Observations on the bionomics of the egg of *Fasciola hepatica*. *Annals of Tropical Medicine and Parasitology* 54:172-181.

RUBEL (D.), PREPELITCHI (L.), KLEIMAN (F.), CARNEVALE (S.) & WISNIVESKY-COLLI (C.), 2005.- Estudio del foco en un caso de fasciolosis humana en Neuquén. *Medicina, Buenos Aires*, 65 (3): 207-212.

RUGGIERI (F.), CORREA (A.J.E.) & MARTINEZ (E.), 1967.- Cerebral distomiasis. Case report. *Journal of Neurosurgery*, 27 (3): 268-271.

RUMI, (A.), GREGORIC, (D.E.), NUÑEZ, (V.), & DARRIGRAN, (G.A.), 2008.- Malacologia latinoamericana. Moluscos dulceaquícolas de Argentina. *Revista de Biología Tropical*, 56: 77-111.

SAH (R.), KHADKA (S.), KHADKA (M.), GURUBACHARYA (D.), SHERCHAND (J.B.), PARAJULI (K.), SHAH (N.P.), KATTEL (H.P.), POKHAREL (B.M.) & RIJAL (B.), 2017.- Human fascioliasis by *Fasciola hepatica*: the first case report in Nepal. *BMC Research Notes*, 10 (1): 439.

SAHLU (T.), GOETSCH (A.L.), 2005.- A foresight in goat research. *Small Ruminant Research* 2005; 60(1-2): 7-12.

SALIH (T.), AL-HABBIBW (O.), AL-HABBIB (W.), AL-ZAKOT (S.) & ALI (T.), 1981.- The effects of constant and changing tempertures on the development of eggs of the freshwater snail *Lymnaea auricularia* (L.). *Journal of Thermal Biology,* 6 (4): 379-388.

SALOMON (M.C.), DE JONG (L.), TONELLI (R.L.), BORREMANS (C.G.), BERTELLO (D.), JOFRE (C.A.), PAGELLA (H.), LLORET (L.), CARNEVALE (S.) & CARENA (J.), 2006.- Confirmación etiológica de un brote de distomatosis hepática en Mendoza. In: *XII Jornadas Argentinas de Microbiología* (Mendoza, Argentina, 14-17 June 2006), Resúmenes de las Comunicaciones: 108.

SAMADI (S.), ROUMEGOUX (A.), BARGES (M.D.), MAS-COMA (S.), YONG (M.), & POINTIER (J.P.) 2000.- Morphological studies of lymnaeid snails from the human fascioliasis endemic zone of Bolivia. *Journal of Molluscan Studies, 66*: 31-44.

SAMBROOK (J.), FRITSCH (E.F.) & MANIATIS (T.), 1989.- Molecular Cloning. A Laboratory Manual. 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA, Vols. I, II & III, 1647 pp.

SANGER (F.), NICKLEN (S.) & COULSON (A.R.), 1977.- DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*. U.S.A. 74: 5463-5467.

SANTAREM, (V. A.), TOSTES (R.A.), ALBERTI (H.), & CARVALHO SANCHES (O.), 2006.- *Fasciola hepatica* in capybara. *Acta Tropica*, 98: 311-313.

SARIOZKAN, (S.) & YALCIN (C.), 2011.- Estimating the total cost of bovine fasciolosis in Turkey. *Annals of Tropical Medicine & Parasitology*, 105 (6): 439-445.

SAVOYAT (M.D.), 1867.- El saguaipé. Epizootía causada por el Saguaipé vulg. Duva o *Fasciola* de Linnéo. *Anales de la Sociedad Rural Argentina*, Año 1867: 173-176.

SAWICKI (M.), GANCEDO (E.), PETRAROIA (C.), TITTARELLI (C.) & MENDEZ (N.), 2017.- Fasciolosis: Formas de presentación ecográfica en nuestra experiencia. In XVIII Simposio Internacional sobre enfermedades desatendidas (Buenos Aires, Argentina, 5-6 October 2017). Conference abstrats: 142.

SCARABINO (F.), 2004.-. Lista sistemática de los Gastropoda dulceacuícolas vivientes de Uruguay. Comunicaciones de la Sociedad Malacológica del Uruguay, 8(84-85 / 86-87): 347-356.

SCHWEIZER, (G.), BRAUN (U.), DEPLAZES (P.) & TORGERSON (P.R.), 2005.- Estimating the financial losses due to bovine fasciolosis in Switzerland. *Veterinary Record*, 157 (7):188-93.

SCOTT (M.I.S.), 1953.- Sobre Limnaeidae argentinos. *Physis*, 20: 401-408.

SENASA, 2006.- Anuario 2006. Servicio Nacional de Sanidad y Calidad Agroalimentaria, Secretaria de Agricultura, Ganadería y Pesca.

SHEATHER (A.L.), 1923.- The detection of intestinal protozoa and mange parasites by a flotation technique. *Journal of Comparative Pathology,* 36: 266-275.

SHIMALOV (V.V.), 2001.- Helminth fauna of the hare (*Lepus europaeus* Pallas, 1778) in the southern part of Belarus. *Parasitology Research,* 87 (1): 85.

SHIMALOV (V.V.) & SHIMALOV (V.T.), 2003.-. Helminth fauna of cervids in Belorussian Polesie. *Parasitology Research*, 89 (1): 75-76.

SICILIANO (C.), 1982.- Distomatosis hepática, perfil clínico. Thesis Doctoral. Universidad Nacional de Córdoba, Argentina, 118 pp.

SICILIANO (C.), CHALUB (E.), SOSA (L.), BARNES (A.) & SHICTON (G.), 1989.- Distomatosis hepática, nuevos casos en Córdoba, Argentina. *Prensa Médica Argentina*, 76: 106-109.

SIELFELD (W.), 2001.- Phylum Mollusca. Guías de identificación y biodiversidad. Fauna Chilena*,* Universidad Arturo Prat, Iquique, Chile, 15 pp.

SIMON (M.P.), BUSTOS ARAUJO (A.) & SOSA LARA (E.), 1964.- Colecistitis crónica alitiásica (cólico hepático) por distomatosis hepática. In: VII Jornada Quirúrgica de Cuyo (Hospital Central, Mendoza, Argentina, 11 Julio 1964), Resúmenes de las Comunicaciones: 1-15.

SOARES (M.P.), SILVA DA SILVA (S.), QUINTANA NIZOLI (L.), RODRIGUES FELIX (S.) & SCHILD (A.L.), 2007.- Chronic fascioliasis in farmed and wild greater rheas (*Rhea americana*). *Veterinary Parasitology*,145 (1-2): 168-171.

SOLANAS (M.V.), MERA Y SIERRA (R.), SCARCELLA (S.), NEIRA (G.) & SOLANAS (H.), 2016.- *In vivo* assessment of closantel ovicidal activity in *Fasciola hepatica* eggs. *Experimental Parasitlology*, 160: 49-53.

SOLARI (A.V.) & CANEPA (L.), 1943.- Consideraciones sobre distomatosis hepática por *Fasciola hepatica*, un nuevo caso de parasitismo humano. *Revista de Medicina y Ciencias Afines*, 55: 795-799.

SONZINI ASTUDILLO (C.), CUENCA PEREZ (M.), TIGLIO (M.) & CANDIZANO (H.), 1973.- Distomatosis hepática *Fasciola hepatica*. *La Semana Médica,* 143 (17): 507-510.

SOSA (H.) 1993.- Situación de la Avifauna Acuática de Laguna Blanca "Coihue-co" Malargüe, Mendoza. *Multequina*, 2:243-249.

SOSA (H.), 1995.- Actualización de la Lista de Avifauna de la Reserva Provincial Laguna Llancanelo Malargüe Mendoza. Presencia estacional, Preferencia de Hábitats y Nidificación. *Multequina*, 4 :65-75.

SOSA (H.), l999.- Inventario de Humedales de Mendoza. *Revista Territorio Científico (Colegio de Agrimensores de Mendoza)*, 4 (6): 15-20.

SOSA (M.A.) & ROMERO (A.E.), 1967.- Distomatosis hepática. *Acta Médica de Córdoba*, 12 (2): 78-83.

SOULSBY (E.J.L.), 1987.- Parasitología y enfermedades parasitarias en los animales domésticos. 7th ed. Nueva Editorial Interamericana, D.F. México, 823 pp.

STADEN (R.), JUDGE (D.P.) & BONFIELD (J.K.), 2001.- Sequence assembly and finishing methods. *Methods of Biochemical Analysis*, 43: 302-322.

STOTHARD (J.R.), BREMOND (Ph.), ANDRIAMARO (L.), LOXTON (N.J.), SELLIN (B.) & ROLLINSON (D.), 2000.- Molecular characterization of the freshwater snail *Lymnaea natalensis* (Gastropoda: Lymnaeidae) on Madagascar with an observation of an unusual polymorphism in ribosomal small subunit genes. *Journal of Zoology*, 252: 303-315.

SUKHDEO (M.V.) & METTRICK (D.F.), 1986.- The behaviour of the juvenile *Fasciola hepatica*. *Journal of Parasitology* 72 (4): 492-497.

STRADA (L.), 1961.- Fascioliasis hepática humana*. Prensa Medica Argentina*, 48:2985-2992.

SWOFFORD (D.L.), 2002.- PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0 Beta. Sinauer Associates, Sunderland, United States of America.

TAMURA (K.), PETERSON (D.), PETERSON (N.), STECHER (G.), NEI (M.) & KUMAR (S.), 2011.- MEGA5: Molecular Evolutionary Genetic Analyses using likelihood, distance and parsimony methods. *Molecular Biology and Evolution*, 10: 2731-2739.

TANTALEAN (M.), HUIZA (A.) & CAPUÑAY (R.), 1974.- Los hospederos intermediarios de *Fasciola hepatica*en el Perú. I. Estudio de la infección natural y experimental de *Lymnaea viator*, *L. diaphana*y *Physa venustula*. *Biota*, 10 (81): 243-250.

THIELTGES (D.W.), REISE (K.), PRINZ (K.), JENSEN (K.T.), 2009.- Invaders interfere with native parasite-host interactions. *Biological Invasions,* 11: 1421-1429.

THOMPSON (J.D.), HIGGINS (D.G.) & GIBSON (T.J.), 1994.- CLUSTAL W: improving the sensitivity and progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22: 4673-4680.

TOMPKINS (D.M.), POULIN (R.), 2006.-. Parasites and biological invasions.In: Biological Invasions in New Zealand, R. B. Allen, & W. G. Leen eds.), Springer, Berlin, Germany, pp. 67-86.

TORGERSON (P.) & CLAXTON (J.), 1999.- Epidemiology and control. In Fasciolosis, Dalton (J.P.) ed. CAB International, Wallingford, United Kingdom, pp. 113-149.

TROPILO (J.), 1964.- Intensity and extensity of invasion by *Cysticercus pisiformis* and *Fasciola hepatica* in hares (in Polish). *Medycyna Weterynaryjna Warszawa,* 20: 259-269.

TROSSERO (A.I.) & NOCETTI (V.M.), 1969.- Distomatosis por *Fasciola hepatica*. *Semana Médica*, Septiembre: 879-882.

UBEIRA (F.M.), MUIÑO (L.), VALERO (M.A.), PERIAGO (M.V.), PEREZ-CRESPO (I.), MEZO (M.), GONZALEZ-WARLETA (M.), ROMARIS (F.), PANIAGUA (E.), CORTIZO (S.), LLOVO (J.) & MAS-COMA (S.), 2009.- MM3-ELISA detection of *Fasciola hepatica* coproantigens in preserved human stool samples. *American Journal of Tropical Medicine and Hygiene*, 81 (1): 156-162.

UENO (H.), ARANDIA (R.) MORALES (G.) & MEDINA (G.), 1975.- Fascioliasis of livestock and snail host for *Fasciola* in the Altiplano region of Bolivia. *National Institute of Animal Health Quarterly*, 15 (2): 61-67.

URRUTIA (J.M.) & FERRARIS (L.V.), 1962.- Distomatosis del hepatocoledoco. Importancia de la colangiografia operatoria. In: Primera Conferencia Nacional de Anatomia Normal y Patologica, Histologia, Embriologia y Tecnica Quirurgica (Córdoba, October 1962).

VALDOVINOS (C.), 2006.- Estado de conocimiento de los gastropodos dulceaquícolas de Chile. *Gayana,* 70: 88-95.

VALERO (M.A.) & MAS-COMA (S.), 2000.- Comparative infectivity of *Fasciola hepatica* metacercariae from isolates of the main and secondary reservoir animal host species in the Bolivian Altiplano high human endemic region. *Folia Parasitologica*, 47 (1): 17-22.

VALERO (M.A.), MARCOS (M.D.), FONS (R.) & MAS-COMA (S.), 1998.- *Fasciola hepatica* development in the experimentally infected black rat *Rattus rattus*. *Parasitology Research,* 84 (3): 188-194.

VALERO (M.A.), DARCE (N.A.), PANOVA (M.) & MAS-COMA (S.), 2001a.- Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the Northern Bolivian Altiplano hyperendemic region. *Veterinary Parasitology*, 102 (1-2): 85-100.

VALERO (M.A.), PANOVA (M.) & MAS-COMA (S.). 2001b.- Developmental differences in the uterus of *Fasciola hepatica* between livestock liver fluke populations from Bolivian Highlands and European lowlands. *Parasitology Research,* 87 (4): 337-342.

VALERO (M.A.), PANOVA (M.), COMES (A.M.), FONS (R.) & MAS-COMA (S.), 2002.- Patterns in size and shedding of *Fasciola hepatica* eggs by naturally and experimentally infected murid rodents. *Journal of Parasitology,* 88 (2): 308-313.

VALERO (M.A.), SANTANA (M.), MORALES (M.), HERNANDEZ (J.L.) & MAS-COMA (S.), 2003.- Risk of gallstone disease in advanced chronic phase of fascioliasis: an experimental study in a rat model. *Journal of Infectious Diseases*, 188 (5): 787-793.

VALERO (M.A.), PANOVA (M.), & MAS-COMA (S.), 2005.- Phenotypic analysis of adults and eggs of *Fasciola hepatica* by computer image analysis system. *Journal of Helminthology,* 79 (3): 217-225.

VALERO (M.A.), NAVARRO (M.), GARCIA-BODELON (M.A.), MARCILLA (A.), MORALES (M.), GARCIA (J.E.), HERNANDEZ (J.L.) & MAS-COMA (S.), 2006a.- High risk of bacterobilia in advanced experimental chronic fasciolosis. *Acta Tropica*, 100 (1-2) 17-23.

VALERO (M.A.), DE RENZI (M.), PANOVA (M.), GARCÍA-BODELON (M.A.), PERIAGO (M. V.), ORDOÑEZ (D.) & MAS-COMA (S.), 2006b.- Crowding effect on adult growth, pre-patent period and egg sheding of *Fasciola hepatica*. *Parasitology,* 133 (4): 453-463.

VALERO (M.A.), GIRONES (N.), GARCIA-BODELON (M.A.), PERIAGO (M.V.), CHICO-CALERO (I.), KHOUBBANE (M.), FRESNO (M.) & MAS-COMA (S.), 2008.- Anemia in advanced chronic fasciolosis. *Acta Tropica*, 108 (1): 35-43.

VALERO (M.A.), PEREZ-CRESPO (I.), PERIAGO (M.V.), KHOUBBANE (M.) & MAS-COMA (S.), 2009a.- Fluke egg characteristics for the diagnosis of human and animal fascioliasis by *Fasciola hepatica* and *F. gigantica*. *Acta Tropica*, 111 (2): 150-159.

VALERO (M.A.), UBEIRA (F.M.), KHOUBBANE (M.), ARTIGAS (P.), MUIÑO (L.), MEZO (M.), PEREZ-CRESPO (I.), PERIAGO (M.V.) & MAS-COMA (S.), 2009b.- MM3-ELISA evaluation of coproantigen release and serum antibody production in sheep experimentally infected with *Fasciola hepatica* and *F. gigantica*. *Veterinary Parasitology*, 159 (1): 77-81.

VALERO (M.A.), PANOVA (M.), PÉREZ-CRESPO (I.), KHOUBBANE (M.) & MAS-COMA (S.), 2011.- Correlation between egg-shedding and uterus development in *Fasciola hepatica* human and animal isolates: applied implications. *Veterinary Parasitology,* 183 (1-2): 79-86.

VALERO (M.A.), PEREZ-CRESPO (I.), KHOUBBANE (M.), ARTIGAS (P.), PANOVA (M.), ORTIZ (P.), MACO (V.), ESPINOZA (J.R.) & MAS-COMA (S.), 2012.- *Fasciola hepatica* phenotypic characterization in Andean human endemic areas: valley versus altiplanic patterns analysed in liver flukes from sheep from Cajamarca and Mantaro, Peru. *Infection*, *Genetics and Evolution,* 12 (2): 403-410.

VALERO (M.A.), BARGUES (M.D.), KHOUBBANE (M.), ARTIGAS (P.), QUESADA (C.), BERINDE (L.), UBEIRA (F.M.), MEZO (M.), HERNANDEZ (J.L.), AGRAMUNT (V.H.) & MAS-COMA (S.), 2016.- Higher physiopathogenicity by *Fasciola gigantica* than by the genetically close *F. hepatica*: experimental long-term follow-up of biochemical markers.*Transactions of the Royal Society of Tropical Medicine and Hygiene*, 110 (1): 55-66.

VAN DER SANDE (C.A.), KWA (M.), VAN NUES (R.W.), VAN HEERIKHUIZEN (H.), RAUE (H.A.) & PLANTA (R.J.), 1992.- Functional analysis of internal transcribed spacer 2 of *Saccharomyces cerevisiae* ribosomal DNA. *Journal of Molecular Biology*, 223 (4): 899-910.

VAUGHAN (J.L.), CHARLES (J.A.), BORAY (J.C.), 1997.- *Fasciola hepatica* infection in farmed emus (Dromaius novaehollandiae). *Australian Veteterinary Journal*, 75: 811-813.

VENTURINI (L.), 1978.- Revisión del ciclo biológico de *Fasciola hepatica. Analecta Veterinaria*, 10: 13-20.

VIGNOLES (P.), RONDELAUD (D.), DREYFUSS (G.), 2016.- Aptitude of *Lymnaea palustris* and *L. stagnalis* to *Fasciola hepatica* larval development through the infection of several successive generations of 4-mm-high snails. *Parasitology Research*, 115 (6): 2263-22638.

WALKER (S.M.), JOHNSTON (C.), HOEY (E.M.), FAIRWEATHER (I.), BORGSTEEDE (F.H.M.), GAASENBEEK (C.P.H.), PRODOHL (P.A.), & TRUDGETT (A.), 2011.- Potential role of hares in the spread of liver fluke in the Netherlands. *Veterinary Parasitology* 177 (1-2): 179-181.

WERNICKE (R.), 1888.- Informe sobre los trabajos llevados a cabo en el laboratorio para el estudio de las enfermedades contagiosas. Facultad de Ciencias Médicas, Universidad de Buenos Aires: Informe Nº 16599.

WETHINGTON (A.R.) & LYDEARD (C.), 2007.- Molecular phylogeny of Physidae (Gastropoda: Basommatophora) based on mitochondrial DNA sequences. *Journal of Molluscan Studies*, 73 (3): 241-257.

WILSON (G.I.) & SAMSON (K.S.), 1971.- The incidence of fascioliasis of sheep and cattle in the southwest with observations on the snail vectors. *Proceedings of the Helminthology Society of Washingto*n, 38 (1): 52-56.

WILSON (R.A.), SMITH (G.) & THOMAS (M.R.), 1982.- Fascioliasis. In: The Population Dynamics of Infectious Diseases: Theory and Applications, R. M. Anderson (ed.) Chapman and Hall. London, United Kingdom, pp. 262-361.

WINKELHAGEN (A.J), MANK (T.), DE VRIES (P.J.) & SOETEKOUW (R.), 2012.- Apparent triclabendazole-resistant human *Fasciola hepatica* infection, the Netherlands. *Emerging Infectious Diseases,* 18(6):1028-1029.

WINNEPENNICKX (B.), BACKELJAU (T.), VAN DE PEER (Y.) & DE WACHTER (R.), 1992.- Structure of the small ribosomal subunit RNA of the pulmonate snail, *Limicolaria kambeul*, and phylogenetic analysis of the Metazoa. *FEBS Letters*, 309 (2): 123-126.

WORLD HEALTH ORGANIZATION, 2006.-: Report of the WHO Informal Meeting on use of triclabendazole in fascioliasis control. WHO Press, Geneva, Switzerland, 39 pp.

WORLD HEALTH ORGANIZATION, 2010.- Working to overcome the global impact of neglected tropical diseases. First WHO report on neglected tropical diseases, WHO Press, Geneva, Switzerland, 184 pp.

WORLD HEALTH ORGANIZATION, 2013.- Sustaining the drive to overcome the global impact of neglected tropical diseases. Second WHO report on neglected diseases. World Health Organization, WHO Press, Geneva, Switzerland, 138 pp.

WORLD HEALTH ORGANIZATION, 2015.- Investing to overcome the global impact of neglected tropical diseases: Third WHO report on neglected tropical diseases. WHO Press, Geneva, Switzerland, 191 pp.

YAMAGUTI (S.), 1971.- Synopsis of Digenetic Trematodes of Vertebrates, Vol. 1 and 2. Tokyo, Japan: Keigaku Publishing Co.

YILMA (J.M.) & MALONE (J.B.), 1998.- A geographic information system forecast model for strategic control of fascioliasis in Ethiopia. *Veterinary Parasitology*, 78 (2): 103-127.

ZIEGE (S.), BRAUNEIS (M.), VON KEYSERLINGK (M.), & WOHLSEIN (P.), 2009.- Fasziolose beim Europäischen Feldhasen (*Lepus europaeus*) in Nordwestdeutschland. *Deutsche Tierarztliche Wochenschrifte,* 116 (2):60-63.

ZILCH (A.), 1959-1960.- Gastropoda (Euthyneura). In O Schindewolf, *Handbuch der paleozoologie*, Berlin, Germany: Borntraeger, p. 91-102.

ZUMAQUERO-RÍOS (J.L.), SARRACENT-PÉREZ (J.), ROJAS-GARCÍA (R.), ROJAS-RIVERO (L.), MARTÍNEZ-TOVILLA (Y.), VALERO (M.A.), & MAS-COMA (S.), 2013.- Fascioliasis and intestinal parasitoses affecting schoolchildren in Atlixco, Puebla State, Mexico: epidemiology and treatment with nitazoxanide. *Plos Neglected Tropical Diseases*, 7 (11): e2553.

ABBREVIATIONS

ABBREVIATIONS

AL	Aperture Length
AW	Aperture Width
*cox*1	Mitochondrially encoded cytochrome c oxidase 1 gene
CRT	coproantigen reduction test
EDTA	Ethylenediaminetetraacetic acid
EHA	egg hatch assay test
epg	Egg per gram of faeces
FECRT	fecal egg count reduction test
ITS1	rDNA Internal Transcribed spacer sequence
LSL	Last Spire Length
mtDNA	Mitochondrial deoxyribonucleic acid
*ND*1	NADH dehydrogenase subunit 1 gene
NIS	Non Indiginous Species
NWA	North West Argentina
PCR	polymerase chain reaction
rRNA	Ribosomal deoxyribonucleic acid
SENASA	Servicio Nacional de Sanidad y Calidad Agroalimentaria
SL	Shell Length
SNP	Single Nucleotide Polymorphism
SW	Shell width
TE	Tris EDTA buffer
TRIS	tris(hydroxymethyl)aminomethane
WHO	World Health Organization

ANNEX

Ribosomal DNA ITS–1 sequencing of *Galba truncatula* (Gastropoda, Lymnaeidae) and its potential impact on fascioliasis transmission in Mendoza, Argentina

M. D. Bargues, R. L. Mera y Sierra, H. G. Gómez, P. Artigas & S. Mas–Coma

Bargues, M. D., Mera y Sierra, R. L., Gómez, H. G., Artigas P. & Mas–Coma, S., 2006. Ribosomal DNA ITS–1 sequencing of *Galba truncatula* (Gastropoda, Lymnaeidae) and its potential impact on fascioliasis transmission in Mendoza, Argentina. *Animal Biodiversity and Conservation*, 29.2: 191–194.

Abstract

Ribosomal DNA ITS–1 sequencing of Galba truncatula *(Gastropoda, Lymnaeidae) and its potential impact on fascioliasis transmission in Mendoza, Argentina*.— Sequencing of the rDNA ITS–1 proved that the lymnaeid snail species *Galba truncatula* is present in Argentina and that it belongs to the haplotype HC, the same as that responsible for the fascioliasis transmission in the human hyperendemic area with the highest human prevalences and intensities known, the Northern Bolivian Altiplano.

Key words: *Galba truncatula*, Lymnaeid vectors, Human and animal fascioliasis, Transmission, Mendoza, Argentina.

Resumen

Secuenciación del ITS–1 del ADN ribosomal de Galba truncatula *(Gastropoda, Lymnaeidae) y su impacto potencial en la transmisión de la fascioliasis en Mendoza, Argentin*a.— La secuenciación del ITS–1 del ADNr demostró que la especie de gasterópodo lymnaeido *Galba truncatula* se encuentra en Argentina y que pertenece al haplotipo HC, el mismo responsable de la transmisión de la fascioliasis en el área de hiperendemia humana con las mayores prevalencias e intensidades de fascioliasis conocidas, el Altiplano Norte Boliviano.

Palabras clave: *Galba truncatula*, Vectores Lymnaeidae, Fascioliasis humana y animal, Transmisión, Mendoza, Argentina.

(Received: 15 XI 06; Final acceptance: 15 I 07)

M. D. Bargues, R. L. Mera y Sierra, H. G. Gómez, P. Artigas & S. Mas–Coma, Depto. de Parasitología, Fac. de Farmacia, Univ. de Valencia, Av. Vicente Andrés Estellés s/n, 46100 Burjassot (Valencia), España (Spain).– R. L. Mera y Sierra, Fac. de Ciencias Médicas, Univ. Nacional de Cuyo, Av. Libertador 80 Centro Universitario, P. O. Box 33, 5500 Mendoza, Argentina (Argentina).

Corresponding author: M. D. Bargues. E–mail: M.D.Bargues@uv.es

ISSN: 1578–665X

Introduction

Fascioliasis is a well–known parasitic disease transmitted by freshwater snail species of the family Lymnaeidae (Gastropoda) (Mas–Coma et al., 2005). *Fasciola hepatica* is believed to be of European origin, with the lymnaeid *Galba truncatula* as the original vector species. *G. truncatula* is a species which reproduces by selfing and spread to other continents, most probably together with the commercial export of livestock (Mas–Coma et al., 2001).

When carrying out animal fascioliasis studies in the province of Mendoza, Argentina, Mera y Sierra (2001) collected lymnaeids which he classified as belonging to the species *G. truncatula*, by comparing with morphological descriptions (Oviedo et al., 1995; Samadi et al., 2000).

This was the first time that *G. truncatula* was cited in Argentina. Studies performed during sub- sequent years showed that morphology is not sufficient to differentiate lymnaeid species be- longing to the so–called Galba–Fossaria group (Bargues et al., 2001; Durand et al., 2002). Problems in morphological differentiation of lymnaeid species have already been detected in Mendoza (Mera y Sierra et al., 2005, 2006; Artigas et al., 2005).

The present paper aimed to verify the species classification of the lymnaeids collected by Mera y Sierra (2001) in Mendoza, by analysis of the complete sequence of the first internal transcribed spacer (ITS–1) of the nuclear ribosomal DNA (rDNA). This molecular marker has recently proved its usefulness in Lymnaeidae (Bargues & Mas–Coma, 2005; Bargues et al., 2006).

Materials and methods

The rDNA ITS–1 sequence was obtained from each of 5 lymnaeid specimens collected in the locality of El Salto, province of Mendoza, Argentina. DNA extraction procedure steps were performed according to methods outlined previously (Bargues et al., 2001). Total DNA was isolated according to the phenol–chloroform extraction and ethanol precipitation method, the ITS–1 fragment was amplified by the Polymerase Chain Reaction (PCR), sequencing performed by the dideoxy chain–termination method, and sequences were aligned using CLUSTAL–W version 1.8 (Bargues et al., 2001; Mas–Coma et al., 2001). Homologies were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST). The following ITS–1 sequences present in GenBank were used for comparisons: *Galba truncatula* haplotype A (AJ243018), haplotype B (AJ296270), and haplotype C (AJ272052) (Mas–Coma et al., 2001).

Results

The five ITS–1 sequences from the 5 lymnaeid specimens analyzed presented the same length of 504 base pairs (bp) and a scarcely biased GC content of 57.5%. The nucleotide sequence was in all cases the same and it is shown in table 1. The Argentinian lymnaeids presented an ITS–1 sequence showing a 99.6% similitude with the *G. truncatula* haplotype A (HA) from Europe. The nucleotide differences detected were only two mutations: the transition G / A in position 74 and the transversion T / G in position 75. Concerning *G. truncatula* haplotype B

Table 1. Complete nucleotide sequence of the first internal transcribed spacer ITS–1 of the nuclear ribosomal DNA of lymnaeids from the population of El Salto, Mendoza, Argentina.

Tabla 1. Secuencia nucleotídica completa del primer espaciador transcrito interno ITS–1 del ADN ribosomal nuclear de los lymnaeidos de la población de El Salto, Mendoza, Argentina.

```
  1 ATCATTAACG AGCAGCCAAC CGAGCGTTGA CTACTTTGTT GTCTCAGTCA GTCAGTCAGT
 61 CAGGCCCCGC GGCGTGCACG CATGAAGCGCTGTCGCGGGG CTGTGTCCGC TTCGTCTTTC
121 GGGGTACCTA TTACTGTCCT CGATGCGACC CACGGTGACG GCTTAGAGCC CGTGTGCTCG
181 CCGGGTCGCG ACGGTTCAAA GAGTGGCCGG CTTGGCTCAG CTCGAGAGTC AGCCGGCGAC
241 CGCCCCGCCG TCGCAAAAAA ACAGGAGGTT AGTCCGGGGT ACCTATGCCC TGCCCGCGCT
301 CGCTCTCGCG CCGGCAAGGC GGTAGCTCCA GCTCGCTATT TGGCCGCGAG GTTCAAAGAG
361 ACGACCGTGC CTTAACTTGC TCTCTCCGTG GGCAACGGTC GCCGCCCCGG GCCTCCTAAA
421 ATTTCCTTTA ATAAAACGAA ATTATTTTTT AAAAATGTGT GTCGGCTCGA TCGTGGCACA
481 CGAAAAACAA ACAAAAGTC TTAT
```

Table 2. Sequence length, nucleotide contents and differences found in the comparison of the sequences of ITS–1 of the nuclear ribosomal DNA of lymnaeid populations from Argentina and known haplotypes of *Galba truncatula*.

Tabla 2. Longitud, composición y diferencias nucleotídicas encontradas en la comparación de las secuencias del primer espaciador transcrito interno ITS–1 del ADN ribosomal nuclear de los lymnaeidos argentinos y los haplotipos conocidos de Galba truncatula.

G. truncatula haplotypes	Origin	Lenght	% GC	Positions 74	75	132	GenBank Acc. No.
HA	Spain, Portugal, Switzerland, Corsica	504 pb	57.5	A	G	T	AJ243018
HB	Morocco	504 pb	57.5	A	G	C	AJ296270
HC	Northern Bolivian Altiplano	504 pb	57.5	G	T	T	AJ272052
HC	El Salto, Mendoza, Argentina	504 pb	57.5	G	T	T	present work

(HB) from Morocco, the similitude was of 99.4% and a total of three mutations were detected: two transitions G / A and T / C in positions 74 and 132, respectively, and one transversion T / G in position 75 (table 2). When comparing the ITS–1 sequence of the Mendoza lymnaeid specimens with that of *G. truncatula* haplotype C (HC) from the Northern Bolivian Altiplano, no nucleotide differences appeared (table 2).

Discussion

The present work demonstrates that the lymnaeids of Mendoza belong to *G. truncatula* haplotype HC. The presence of *G. truncatula* HC in the Andean area of Argentina represents a great potential risk of fascioliasis. The haplotype HC of this lymnaeid is the same as that responsible for transmission of the disease in the endemic area with the highest prevalences and intensities known in humans, the Northern Bolivian Altiplano (Mas–Coma et al., 1999). Although of a somewhat lower level, prevalence and intensity situations found in other Andean areas of Peru are similar (Mas–Coma et al., 2005).

The province of Mendoza is also located in the Andean area and although the altitudes are not as high as those of hyperendemic zones in Bolivia and Peru, temperatures are similar because of the southern latitude (Fuentes et al., 1999). This suggests a high disease transmission capacity in Mendoza.

The wide ecological features of *G. truncatula* do allow it to come close to human settings (Mas–Coma et al., 1999). This explains why this lymnaeid is in the background of many human infections. Moreover, *G. truncatula* is markedly linked to areas where livestock is present, enabling its passive transportation by domestic animals from one place to another. The disease–spreading risk in Mendoza is evident.

Acknowledgements

Studies funded by Projects No. BOS2002–01978 and No. SAF2006–09278 of the Ministry of Education and Science, Madrid, and Red de Investigación de Centros de Enfermedades Tropicales–RICET (Projects No. C03/04, No. ISCIII2005–PI050574 and RD06/0021/0017, Programa de Redes Temáticas de Investigación Cooperativa), FIS, Ministry of Health, Madrid. Studies in Argentina funded by Universidad Nacional de Cuyo. Technical support provided by the Servicio Central de Secuenciación para la Investigación Experimental (SCSIE) of the Universidad de Valencia (Dr. A. Martínez).

References

Artigas, P., Mera y Sierra, R. Correa, A. P., Morales, J. R., Zuriaga, M. A., Jiménez, M., Khoubbane, M., Bargues, M. D. & Mas–Coma, S., 2005. Comparison of the conchiologic morphology of lymnaeid molluscs from eastern and western Andean slopes of the endemic zones of fasciolosis in Argentina and Chile. *Acta Parasitologica Portuguesa*, 12: 243–244.

Bargues, M. D. & Mas–Coma, S., 2005. Reviewing lymnaeid vectors of fascioliasis by ribosomal DNA sequence analyses. *Journal of Helminthology*, 79: 257–267.

Bargues, M. D., Vigo, M., Horak, P., Dvorak, J., Patzner, R. A., Pointier, J. P., Jackiewicz, M., Meier–Brook, C., & Mas–Coma, S., 2001. European Lymnaeidae (Mollusca: Gastropoda), intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS–2 sequences. *Infection, Genetics and Evolution*, 1: 85–107.

Bargues, M. D., Artigas, P., Jackiewicz, M., Pointier, J. P. & Mas–Coma, S., 2006. Ribosomal DNA ITS–1 sequence analysis of European

stagnicoline Lymnaeidae (Gastropoda). *Heldia, München*, 6: 29–40.

Durand, P., Pointier, J. P., Escoubeyrou, K., Arenas, J. A., Yong, M., Amarista, M., Bargues, M. D., Mas–Coma, S. & Renaud, F., 2002. Occurrence of a sibling species complex within Neotropical lymnaeids, snail intermediate hosts of fascioliasis. *Acta Tropica*, 83: 233–240.

Fuentes, M. V., Valero, M. A., Bargues, M. D., Esteban, J. G., Angles, R. & Mas–Coma, S., 1999. Analysis of climatic data and forecast indices for human fascioliasis at very high altitude. *Annals of Tropical Medicine and Parasitology*, 93: 835–850.

Mas–Coma, S., Angles, R., Esteban, J. G., Bargues, M. D., Buchon, P., Franken, M. & Strauss, W., 1999. The Northern Bolivian Altiplano: a region highly endemic for human fascioliasis. *Tropical Medicine and International Health*, 4: 454–467.

Mas–Coma, S., Funatsu, I. R. & Bargues, M. D., 2001. *Fasciola hepatica* and lymnaeid snails occurring at very high altitude in South America. *Parasitology*, 123: S115–S127.

Mas–Coma, S., Bargues, M. D. & Valero, M. A., 2005. Fascioliasis and other plant–borne trematode zoonoses. *International Journal for Parasitology*, 35: 1255–1278.

Mera y Sierra, R. L., 2001. Distribución de Lymnaeidos en el sistema hidrográfico andino de la provincia de Mendoza, Argentina. Tesis de Maestría, Master Internacional en Enfermedades Parasitarias Tropicales (XIV Edición). Tutor: M. D. Bargues. Depto. de Parasitología, Fac. de Farmacia, Univ. de Valencia, 22 Junio 2001.

Mera y Sierra, R. L., Artigas, P., Abraham, C., Hynes, V., González, M., Jiménez, M., Khoubbane, M., Bargues, M. D. & Mas–Coma, S., 2005. Conchiologic morphology of lymnaeid molluscs in the endemic zones of fasciolosisis in Mendoza province, Argentina. *Acta Parasitologica Portu–guesa*, 12: 313–314.

Mera y Sierra, R. L., Artigas, P. & Mas–Coma, S., 2006. Lymnaeid vectors of fascioliasis in endemic areas of Mendoza, Argentina. In: *XI International Congress of Parasitology–ICOPA XI (Glasgow, UK, 6–11 August 2006)*: CD–ROM.

Oviedo, J. A., Bargues, M. D. & Mas–Coma, S., 1995. Lymnaeid snails in the human fascioliasis high endemic zone of the Northern Bolivian Altiplano. *Research and Reviews in Parasitology*, 55: 35–43.

Samadi, S., Roumegoux, A., Bargues, M. D., Mas–Coma, S., Yong, M. & Pointier, J. P., 2000. Morphological studies of lymnaeid snails from the human fascioliasis endemic zone of Bolivia. *Journal of Molluscan Studies*, 66: 31–44.

Annals of Tropical Medicine & Parasitology, Vol. 101, No. 7, 621–641 (2007)

Characterisation of *Lymnaea cubensis*, *L. viatrix* and *L. neotropica* n. sp., the main vectors of *Fasciola hepatica* in Latin America, by analysis of their ribosomal and mitochondrial DNA

M. D. BARGUES*, P. ARTIGAS*, R. L. MERA Y SIERRA*,{, J. P. POINTIER{ and S. MAS-COMA*

*Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Avenida Vicente Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain

{Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Avenida Libertador 80, Centro Universitario, P.O. Box 33, 5500 Mendoza, Argentina

{UMR 5244 CNRS-EPHE-UPVD, Biologie et Ecologie Tropicale et Méditerranéenne, Université de Perpignan, Via Domitia, 52 avenue Paul Alduy, 66860 Perpignan Cedex, France

Received 15 December 2006, Revised 26 February 2006, Accepted 1 March 2007

Although, in the endemic areas throughout the world, human fascioliasis presents varying patterns in its epidemiology, the species of lymnaeid snail that act as intermediate hosts and vectors are always crucial in the transmission of the causative parasites. Species in the *Galba/Fossaria* group of snails, such as *Lymnaea cubensis*, *L. viatrix* var. A ventricosa, *L. viatrix* var. B elongata and *Galba truncatula*, appear to be frequently involved in the transmission of *Fasciola hepatica* in Central and South America, although specific classification within this morphologically and anatomically confusing group is often very difficult. To explore the potential use of molecular analyses in the identification of vector snails, regions of the ribosomal DNA — the small subunit (18S) gene and internal transcribed spacers (ITS-2 and ITS-1) — and of the mitochondrial DNA — the cytochrome c oxidase subunit I (COI) — of wild-caught lymnaeid snails of *L. cubensis*, *L. viatrix* var. A ventricosa, *L. viatrix* var. B elongata and *G. truncatula* have been sequenced. The samples of the Latin American species included specimens from the respective type localities.

The genetic distances observed and the results of phylogenetic analyses demonstrate that two different species exist within *L. viatrix*. *Lymnaea neotropica* n. sp. (5*L. viatrix* var. B elongata) is here proposed for specimens from Lima, Peru, and is differentiated from *L. viatrix* (5*L. viatrix* var. A ventricosa), *L. cubensis* and *G. truncatula*. The data collected on the 18S ribosomal-RNA gene indicate that the snails investigated may cover more than one supraspecific taxon. The ITS-2, ITS-1 and COI nucleotide sequences are clearly useful markers for the differentiation of these morpho–anatomically similar lymnaeid species. The numerous microsatellite repeats found within ITS-2 are potential tools for differentiation at population level.

Fascioliasis is a parasitic disease that is emerging in many areas of the world (Mas-Coma, 2004). Caused by two fluke species, *Fasciola hepatica* and *Fa. gigantica*, the disease has long been recognised as a very important veterinary problem, in livestock (Torgerson and Claxton, 1999). The parasites show a marked specificity in the snail species they use as intermediate hosts or vectors. The original vector of *Fasciola hepatica*, which is believed to be of European origin, was probably the lymnaeid snail *Galba truncatula* (Mas-Coma *et al.*, 2001). Beyond Europe, other lymnaeid species of the so-called *Galba/Fossaria* group usually transmit *Fa. hepatica*, whereas *Fa. gigantica*, found in Africa and Asia, is linked to

Reprint requests to: S. Mas-Coma.
E-mail: s.mas.coma@uv.es; fax: 34 96 354 4769.

DOI: 10.1179/136485907X229077

lymnaeids of the *Radix* group (Bargues *et al.*, 2001). In certain circumstances, particularly when the main vector species are absent, members of other vicariant lymnaeid groups, such as stagnicolines for *Fa. hepatica* (Bargues *et al.*, 2003) and *Pseudosuccinea columella* for both fasciolid species (Bargues *et al.*, 2001), can be involved in transmission.

Fascioliasis was not considered a disease of great public-health importance until the 1990s, when the reported incidence of human infection with *Fasciola* began to rise (Chen and Mott, 1990). Areas of endemic human fascioliasis have since been described, first in Latin America and subsequently in Africa and Asia (Mas-Coma *et al.*, 1999*b*, *c*, 2005). The prevalences and intensities of human fascioliasis appear to be increasing in several countries, with the number of people infected with *Fasciola* estimated to be 17 million even before large areas of the world had been surveyed (Hopkins, 1992). In terms of its latitudinal, longitudinal and altitudinal distribution, human fascioliasis is now more wide-spread than any other vector-borne parasitic disease (Mas-Coma, 2004). The epidemiology of the disease varies with geographical region and, particularly, the species of freshwater lymnaeid snail that are involved in transmission (Mas-Coma, 2005). The correct classification of the lymnaeid species acting as vectors in each endemic area is therefore very important to those planning interventions to reduce transmission.

The highest known prevalences and intensities of human fascioliasis occur in areas of Latin America (Mas-Coma, 2004, 2005; Mas-Coma *et al.*, 2005), where three New-World species of lymnaeid, *Lymnaea cubensis*, *Ly. viatrix* and *P. columella*, appear to be the main vectors of the causative parasite, *Fa. hepatica* (Malek, 1985). A fourth species, *G. truncatula*, has recently been added to the list of important Latin American vectors. First detected and morphologically classified in a hyper-endemic area for human fascioliasis in the northern Bolivian Altiplano (Oviedo *et al.*, 1995*a*), Bolivian *G. truncatula* have subsequently been the subject of studies based on the characterisation of the snail's small-subunit ribosomal-RNA (18S rRNA) gene (Bargues and Mas-Coma, 1997; Bargues *et al.*, 1997), the sequencing of the internal-transcribed-spacer regions 1 and 2 (ITS-1 and ITS-2) of the snail's ribosomal DNA (rDNA; Mas-Coma *et al.*, 2001), iso-enzymatic phenotyping (Jabbour-Zahab *et al.*, 1997), eco–epidemiological analysis (Oviedo *et al.*, 1995*b*; Mas-Coma *et al.*, 1999*a*; Fuentes *et al.*, 1999), anatomy (Samadi *et al.*, 2000), and analysis of the genetic variability in the snail's DNA microsatellite markers (Meunier *et al.*, 2001). The results of the rDNA studies indicate that this snail species was introduced from Europe relatively recently, most probably by the first Spanish colonizers (Mas-Coma *et al.*, 2001). It is not confined to Bolivia but also occurs, in areas with endemic human fascioliasis, in Peru (Esteban *et al.*, 2002).

Unfortunately for those investigating the epidemiology and control of human fascioliasis in Latin America, although *P. columella* is a very peculiar species that can easily be identified by shell morphology and anatomy (Paraense, 1982; Malek, 1985), *Ly. cubensis* and *Ly. viatrix* belong to the morphologically and anatomically confusing *Galba*/*Fossaria* group. Their marked intraspecific morphological variability makes several 'sibling' species within this group difficult or even impossible to separate without genetic analysis (Bargues *et al.*, 2001; Durand *et al.*, 2002), and the taxonomy of the whole group remains unclear (Bargues *et al.*, 1997). The division of *Ly. viatrix* into two varieties (A and B) dates right back to the description of the species (D'Orbigny, 1835). The aims of the present study, based on analyses of the sequences of the snails' 18S, ITS-1 and ITS-2 rDNA, and their cytochrome-c- oxidase-subunit-I (COI) mitochondrial DNA (mtDNA), were to identify the mem- bers of the *Galba*/*Fossaria* group involved in transmission in Central and South America,

and develop molecular tools for the future identification, to species level, of lymnaeid populations belonging in this group. Of the different genetic markers already applied to lymnaeids, DNA sequencing appears to give the most accurate results. Although mtDNA has also been analysed and may offer complementary information (Remigio and Hebert, 2003), nuclear rDNA markers, particularly 18S, ITS-2 and ITS-1, have consistently been found useful in the identification of gastropod snails to genus, species or even population level (Bargues and Mas-Coma, 2005).

In the present study, in order to be systematically conclusive, specimens from the '*terra typica*' of each Latin American species of lymnaeid snail were used whenever possible. The exact type locality of *Ly. cubensis*, which was originally described from Cuba (Pfeiffer, 1839), is unknown but specimens from Vaqueria 21, Cuba, were selected for the present study. The original description of *Ly. viatrix* (D'Orbigny, 1835) includes two varieties: var. A ventricosa from the Rio Negro area, Patagonia, Argentina, and var. B elongata from Lima, Peru. In the present study, European and South American specimens of *G. truncatula* were used for comparison. The results of the molecular analyses indicate that a new species, for which the name *Lymnaea neotropica* n. sp. is proposed and a diagnostic description provided, may be involved in the transmission of *Fa. hepatica* to humans and other mammals in Latin America.

MATERIALS AND METHODS

Lymnaeid Samples

The snail specimens investigated were of *Ly. cubensis* (from Vaqueria 21, La Habana, Cuba; 23u019N, 82u329W), *Ly. viatrix* var. A ventricosa (from Frias, Rio Negro, Argentina; 40u149S, 64u109W), *Ly. viatrix* var. B elongata (from Rio Rimac, Lima, Peru; 12u029S, 76u569–77u089W), *Ly. viatrix* var. B elongata (from Rio Lurin, Lima, Peru; 12u039S, 77u049W), and *G. truncatula* (from Benicasim, Castellon, Spain; 40u069N, 00u079E), the latter just being used in the sequencing of COI mtDNA.

DNA was extracted from more than one specimen of a given population when this was deemed necessary for sequence verification. Only snails that appeared free of helminth infection were used in the molecular analyses. To reduce further the risk of contamination of DNA from helminths (which are more likely to be localized in other tissues), DNA was only isolated from the foot of each snail. Use of just the feet, rather than all the soft tissues, also prevented the development in the DNA pellets of the white flocculate substance (probably of polysaccharides) and melanic pigments that can inhibit PCR and cause amplification of non-specific products (Gasser *et al.*, 1993; Bargues *et al.*, 1997).

Molecular Techniques

DNA EXTRACTION

The phenol–chloroform method (Sambrook *et al.*, 1989) was used to extract total DNA from snail feet that had been fixed in 70% ethanol and stored at 4uC for several weeks (Bargues and Mas-Coma, 1997). After dissection under a microscope, half of each foot was suspended in 400 ml lysis buffer [10 mM Tris-HCl (pH 8.0), 100 mM EDTA, 100 mM NaCl, and 1% sodium dodecyl sulphate] containing 500 mg proteinase K (Promega, Madison, WI)/ml, and digested for 2 h at 55uC, with shaking every 15 min. The lysate was then gently mixed and incubated for another 4 h at 55uC, again with shaking every 15 min. In the three-step DNA extraction, an equal volume of phenol, then 200 ml phenol and 200 ml chloroform:isoamyl alcohol (24:1, by vol.) and finally 400 ml of the same chloroform: isoamyl-alcohol mixture were sequentially employed. After each extraction step, the phases were separated by centrifugation (12,000*g* for 3 min). The final aqueous phase was precipitated with 0.1 vol. of 3 M

sodium acetate and 2.5 vol. 100% ethanol, at 220uC. The resultant DNA pellet was washed in 70% ethanol, centrifuged down (at 12,000–13,000*g* and 4uC for 5–10 min), briefly air-dried, re-dissolved in a small volume (20–50 ml) of sterile TE buffer [10 mM Tris-HCl (pH 7.6), 1 mM EDTA], and stored at 220uC until use.

RDNA SEQUENCE AMPLIFICATION

The complete rDNA small subunit (18S rRNA gene), the rDNA internal transcribed spacers (ITS-1 and ITS-2) and the COI mtDNA of each lymnaeid were amplified by PCR, using 4–6 ml of lymnaeid genomic DNA in each 50-ml reaction mixture (Bargues and Mas-Coma, 1997; Bargues *et al.*, 2001; Mas-Coma *et al.*, 2001). Eight conserved oligonucleotide primers were used for the amplification of five superimposed fragments of the 18S rRNA gene (Bargues *et al.*, 1997), a 9600 thermocycler (Perkin Elmer, Waltham, MA) and a standard protocol (Bargues *et al.*, 1995) being employed to amplify the specific 18S rDNA regions. The fragments corresponding to ITS-1 and ITS-2 were amplified using primers designed to match the conserved positions of the 18S and 5.8S rRNA genes and 5.8S and 28S rRNA genes of several species of eukaryote, respectively (Bargues *et al.*, 2001, 2006). A COI gene fragment was amplified using universal primers (Folmer *et al.*, 1994). For ITS-2 and ITS-1, the Peltier thermal cycler (MJ Research, Watertown, MA) used was set to give 30 s at 94uC, followed by 30 cycles, each of 30 s at 94uC, 30 s at 50uC and 1 min at 72uC, and then a final 7 min at 72uC. For COI, however, the thermocycler was set to give 2.5 min at 94uC, followed by 40 cycles, each of 30 s at 90uC, 1 min at 48uC and 1 min at 72uC, and then a final 10 min at 72uC.

The products of each PCR (10 ml) were separated by electrophoresis in a gel of 1% NusieveH GTG agarose (FMC Bioproducts, Rockland, ME) and visualized by staining with ethidium bromide and trans-illuminating with ultra-violet light. A set of molecular-weight markers (Marker VI; Boehringer Mannheim, Mannheim, Germany) containing 0.1 mg DNA/ml was run in each gel.

PURIFICATION AND QUANTIFICATION OF PCR PRODUCTS

Primers and nucleotides were removed from the PCR products using a commercial DNA purification system (Wizard™ PCR Preps; Promega) before the products were resuspended in 50 ml 10 mM TE buffer (pH 7.6). The final DNA concentration was determined by measuring absorbances at 260 and 280 nm.

DNA SEQUENCING

The sequencing, of the entire 18S rRNA gene, the complete ITS-1 and ITS-2 of the rDNA, and a fragment of the COI gene of the mtDNA, was performed on both strands by the dideoxy chain-termination method (Sanger *et al.*, 1977). The Taq dye-terminator chemistry kit for the ABI 373A and ABI 3700 capillary system (Perkin Elmer) and the PCR primers were employed.

DNA HAPLOTYPE NOMENCLATURE

The nomenclature proposed by Bargues and Mas-Coma (2005) and Bargues *et al.* (2006) for the ITS-1 and ITS-2 haplotypes of lymnaeid snails is used throughout. An 'H' followed by a lower-case letter (e.g. Ha) is used to identify the mtDNA COI haplotypes.

Software

SEQUENCE ALIGNMENT

Sequences were aligned using version 1.8 of the CLUSTAL-W programme (Thompson *et al.*, 1994), with the sequences introduced in random order, to reduce bias (Lake, 1991). Homologies were investigated using the BLAST programme, via the website of the United States National Center for

Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST). Genetic distances were estimated using parameters provided by version 4.0b10 of the PAUP programme (Swofford, 2002).

Alignments included not only the sequences determined in the present study but also relevant reference sequences stored in the GenBank databank. These reference sequences were those of the 18S rRNA genes of *G. truncatula* (accession Z73985; Bargues and Mas-Coma, 1997) and *Ly. cubensis* (Z83831; Bargues *et al.*, 1997), ITS-2, of haplotype H1, from *G. truncatula* from Spain, Portugal and Corsica, France (AJ296271), ITS-2, of haplotype H2, from *G. truncatula* from Spain, Portugal and Switzerland (AJ243017), ITS-2, of haplotype H3, from *G. truncatula* from Bolivia [5*Ly. viatrix sensu* Ueno *et al.* (1975) and *Ly. cubensis sensu* Ueno *et al.* (1975); accession AJ272051; Bargues *et al.*, 2001; Mas-Coma *et al.*, 2001], ITS-1, of haplotype HA, from *G. truncatula* from Spain, Portugal, France and Switzerland (AJ243018), ITS-1, of haplotype HB, from *G. truncatula* from Morocco (AJ296270), and ITS-1, of haplotype HC, from *G. truncatula* from Bolivia (AJ272052; Mas-Coma *et al.*, 2001).

REPRESENTATION OF THE 18S RRNA SECONDARY STRUCTURE

The previously published secondary-structure prediction for *Limicolaria kambeul* 18S rRNA (Winnepennickx *et al.*, 1992), which was based on the general eukaryote-18S-rRNA secondary structure (De Rijk *et al.* 1992), was used and extended to encompass the lymnaeid sequences. Version 2.54 of the DCSE programme (De Rijk and de Wachter, 1993) was used to examine potential secondary structures, with the RNAstructure programme (Mathews *et al.*, 1999) employed to predict parts of the structure and the RnaViz programme (De Rijk and de Wachter, 1997) employed for the graphical representation.

Phylogenetic Inference

Phylogenies were inferred, from ITS-1 and ITS-2 rDNA sequences, using maximum-likelihood (ML) estimates with PAUP. Maximum-likelihood parameters such as model, base frequencies, transition/transversion ratio (ts/tv), the shape parameter for the gamma distribution, and the proportion of invariant sites, were optimised using the hierarchical likelihood ratio test (hLRT) and the Akaike information criterion (AIC; Akaike, 1974; Posada and Buckley, 2004), implemented in Modeltest 3.7 (Posada and Crandall, 1998). Starting branch lengths were obtained using the least-squares method with ML distances. To provide an assessment of the reliability of the nodes of the trees, a quartet-puzzling analysis was employed, with 1000 puzzling steps.

Phylogenetic analyses were performed after adding reference sequences of ITS-2 and ITS-1 of lymnaeid rDNA stored in the GenBank databank. These sequences were of *Ly.* (*Stagnicola*) *palustris palustris* ITS-2 (AJ319620) and ITS-1 (AJ626849), *Ly.* (*S.*) *palustris turricola* ITS-2 (AJ319618) and ITS-1 (AJ626853), *Ly.* (*S.*) *fuscus* ITS-2 (AJ319621) and ITS-1 (AJ626856), *Catascopia occulta* ITS-2 (AJ319642) and ITS-1 (AJ626858), ITS-2 (AJ296271, AJ243017 and AJ272951, respectively) and ITS-1 (AJ243018, AJ272052 and AJ296270, respectively) of the H1, H2 and H3 haplotypes of *G. truncatula*, and the sequence from *P. columella* that includes both spacers (AY186751). The sequence (AY030361) including both spacers of a planorbid species, *Biomphalaria pfeifferi*, was used as an outgroup.

Phenotypic Study

Shells of Peruvian *Ly. viatrix* var. B elongata were measured, according to traditional malacological methods (Oviedo *et al.*, 1995*a*; Samadi *et al.*, 2000), using a computerized image-analysis system (Valero *et al.*, 2005). This system was based on a

DXC-930P colour video camera (Sony, Tokyo) fitted to a stereomicroscope, and connected to a computer running image-analysis software (ImagePro® Plus 4.5; Media Cybernetics Inc., Silver Spring, MD).

For anatomical studies, adult Peruvian *Ly. viatrix* var. B elongata were collected in the field and allowed to relax overnight in water containing menthol. They were then immersed for 40 s in hot water (70°C) before transfer to water at room temperature. The soft parts were drawn from the shells with forceps applied to the cephalopedal mass, and fixed in slightly modified Railliet–Henry's fluid (930 ml distilled water, 6 g NaCl, 50 ml 40% formalin, and 20 ml glacial acetic acid). The fixed snails were then dissected under a stereomicroscope, so that drawings of the reproductive system could be made using a camera lucida (Pointier *et al.*, 2006).

RESULTS

18S rRNA Gene

The 18S rDNA sequence of *Ly. cubensis* from Cuba is 1860-bp long, with a GC content of 51.82%, and has base frequencies of 0.236 (A), 0.235 (G), 0.283 (C) and 0.245 (T). This sequence is identical to that deposited in GenBank by Bargues *et al.* (1997).

The sequence of the 18S rRNA gene of *Ly. viatrix* var. A ventricosa from Argentina is 1860-bp long, shows a GC content of 51.82%, and has base frequences of 0.236 (A), 0.236 (G), 0.282 (C) and 0.245 (T).

The sequences of the 18S of *Ly. viatrix* var. B elongata from the two localities in Lima, Peru, were identical, base to base, and did not differ, even by a single nucleotide, from the corresponding sequence of *Ly. viatrix* var. A ventricosa. This 18S sequence has been deposited in GenBank as accession AM412222.

The 18S sequence of *Ly. cubensis* differs from that of *Ly. viatrix* var. A ventricosa and *Ly. viatrix* var. B elongata by a nucleotide divergence of only 0.32%, represented by six nucleotide differences [one transition (ts), three transversions (tv), and two insertions/deletions (indels)] in a 1861-bp-long alignment. According to the secondary structure, the four mutations appear to be located in helix E10-1 of the V2 variable area whereas the two indels appear to be scattered throughout the rest of the 18S sequence (Table 1).

The 18S sequence of *Ly. cubensis* differs much more from that of *G. truncatula*, with a nucleotide divergence of 1.50%, represented by 28 nucleotide differences (five ts, four tv and 19 indels) in a 1861-bp-long alignment. Again, the secondary structure indicated that the differences were concentrated in E10-1 of V2 (Table 1).

Curiously, the 18S sequences indicate the same level of difference between *Ly. cubensis* and *G. truncatula* as between *Ly. viatrix* and *G. truncatula*, the latter comparison also showing a nucleotide divergence of 1.50%, represented by 28 nucleotide differences (six ts, three tv and 19 indels) in a 1861-bp-long alignment. The majority of these differences also appear in helix E10-1 of V2 (Table 1). The triple-comparison alignment, including the 18S sequences of *Ly. cubensis*, *Ly. viatrix* and *G. truncatula*, was 1862-bp long and included 31 variable nucleotide positions, giving a nucleotide divergence of 1.66% (Table 1).The majority (24) of the modified positions appeared to be grouped in the short sequence between positions 231 and 263, whereas the other seven modified positions appeared isolated and scattered throughout the rest of the 18S sequence (Table 1).

ITS-2 rDNA

The three ITS-2 sequences of the four lymnaeid populations studied were deposited in GenBank as accessions AM412223 (*Ly. cubensis* haplotype H1 from Cuba), AM412224 (*Ly. viatrix* var. A ventricosa haplotype H1 from Argentina), and

AM412225 (*Ly. viatrix* var. B elongata haplotype H1 from Rio Rimac and Rio Lurin, Lima, Peru). The lengths and slightly GC-biased mean nucleotide compositions of these ITS-2 sequence were, respectively, 534 bp and 56.22% in *Ly. cubensis*, 452 bp and 54.20% in *Ly. viatrix* var. A ventricosa, and 417 bp and 56.83% in *Ly. viatrix* var. B elongata.

The numbers and percentages of nucleotide differences that appeared in the two-sequence alignments of the ITS-2 sequences, and details of the ts, tv and indels, are shown in Table 2. The numbers of repeats of each microsatellite found in these comparisons are noted in Table 3. The ITS-2 sequence of *Ly. cubensis* was unusually long because of a tetranucleotide microsatellite (CTTG) that appears to be repeated 25 times, consecutively, in positions 51–150.

The ITS-2 sequences of the specimens of *Ly. viatrix* var. B elongata from the populations of Rio Lurin and Rio Rimac were identical. They differed from those of *Ly. viatrix* var. A ventricosa at 53 positions, giving a relatively large nucleotide divergence of 11.67%. Eleven of the 39 indels found were not related to microsatellite repeats. The CTTG microsatellite seen in *Ly. cubensis* also appears in both *Ly. viatrix* var. A ventricosa and *Ly. viatrix* var. B elongata, together with other two tetranucleotide repeats (TTCA and TGAA).

The number of variable positions (124) and level of nucleotide divergence (22.67%) were higher in the comparison between *Ly. cubensis* and *Ly. viatrix* var. A ventricosa. Among the numerous indels, most (76) were related to the CTTG repeat but other microsatellites, including the trinucleotide GGT and the tetranucleotides GCAA, TGAA and TCGA, were also present (Table 3).

In the comparison of *Ly. cubensis* with *Ly. viatrix* var. B elongata, the number of

TABLE 1. *Nucleotide differences found in comparisons of the complete 18S ribosomal DNA sequences of the lymnaeids, and their location in the secondary structure*

Variable	Positions, code numbers or sequences
Variable area*	V V V V V V V V V V V V V V V V V V V V V V V V V V V V 2
	2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 4 4 5 9
Helix*	E EE E EE EE E EEEEEEEEE E EEEEE 1 E E 2 3 3 4
	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 5 2 2 7 4 6 7
	0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 1 1
	– – –
	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 7
Position[{]	1 1 1 1
	2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 4 7 8 1 2 2 7
	3 3 3 3 3 3 3 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 6 5 2 5 3 3 9 4
	1 2 3 4 5 6 7 2 3 5 6 7 8 9 0 1 2 3 4 5 6 7 8 3 9 1 2 5 0 9 7
Lymnaea cubensis Cuba	G T C G T G C G T C A A G C C G T G G T C G C C C G G – – T C
Ly. viatrix var. A ventricosa Argentina	G T G C C T C G T C A A G C C G T G G T C G C C C – G – C T C
Ly. viatrix var. B elongata Peru	G T C C C T C G T C A A G C C G T G G T C G C C C – G – C T C
Galba truncatula Europe	– – C C T T T A G – – – – – – – – – – – – – – – T – – A C – G T
Difference number[{]	1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 3 3
	1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1

*Code numbers (to be read vertically) refer to the secondary structure proposed for the gastropod *Limicolaria kambeul* (Winnepennickx *et al.*, 1992), which was based on the model and nomenclature for eukaryotes (De Rijk *et al.*, 1992)

[{]Numbers (to be read vertically) refer to positions obtained in the alignment made with CLUSTAL W.

[{]To be read vertically.

TABLE 2. *ITS-2 sequence differences detected in pair-wise comparisons between the lymnaeid species studied*

Comparison	Alignment length (bp)	No. and (%) of nucleotide differences	No. and (%) of substitutions		No. and (%) of insertions/ deletions
			Transitions	Transversions	
Lymnaea cubensis v. Ly. viatrix var. A ventricosa	547	124 (22.67)	8 (1.46)	8 (1.46)	108 (19.74)
Ly. cubensis v. Ly. viatrix var. B elongata	534	143 (26.78)	11 (2.06)	14 (2.62)	118 (22.09)
Ly. viatrix var. A ventricosa *v. Ly. viatrix* var. B elongata	454	53 (11.67)	9 (1.98)	5 (1.10)	39 (8.59)
Ly. cubensis v. Galba truncatula (ITS-2 haplotypes 1, 2 and 3)	548	197–199 (35.94–36.31)	18–20 (3.28–3.65)	17–18 (3.10–3.28)	161 (29.38)
Ly. viatrix var. A ventricosa *v. G. truncatula* (ITS-2 haplotypes 1, 2 and 3)	465	113–114 (24.30–24.52)	11–12 (2.36–2.58)	24–25 (5.16–5.38)	77 (16.56)
Ly. viatrix var. B elongata *v. G. truncatula* (ITS-2 haplotypes 1, 2 and 3)	438	93–94 (21.23–21.46)	13–14 (2.97–3.20)	21–22 (4.79–5.02)	58 (13.24)

TABLE 3. *The microsatellites in ribosomal-DNA ITS-2 sequences of the American lymnaeids studied, and number of times each one was repeated in pair-wise comparisons*

Comparison	Alignment length (bp)	Repeat	Start point (position no.)	No. of times each microsatellite appears: *Lymnaea cubensis*	*Ly. viatrix* var. A ventricosa	*Ly. viatrix* var. B elongata
Ly. cubensis v. *Ly. viatrix* var. A ventricosa	547	CTTG	51	25	6	–
		GGT	328	3	1	–
		GCAA	431	2	1	–
		TGAA	487	2	4	–
		TCGA	529	2	1	–
Ly. cubensis v. *Ly. viatrix* var. B elongata	540	CTTG	51	25	–	2
		GGT	328	3	–	0
		GCAA	431	2	–	1
		TTCA	445	2	–	1
		TGAA	487	2	–	1
		TCGA	529	2	–	1
Ly. viatrix var. A ventricosa v. *Ly. viatrix* var. B elongata	454	CTTG	51	–	6	2
		TTCA	352	–	2	1
		TGAA	398	–	3	1

variable positions (143) and level of nucleotide divergence (26.78%) were even higher. Again, most of the indels detected (92) were related to the CTTG repeat. The microsatellites GGT, GCAA, TTCA, TGAA and TCGA, however, did not appear to be repeated in *Ly. viatrix* var. B elongata (Table 3).

When the ITS-2 sequences of the *G. truncatula* haplotypes H1, H2 and H3 (which differed by only one or two mutations) were compared with those of the three taxa of American lymnaeids, a high number of nucleotide differences appeared in the alignments and a notable equidistance in the true mutations was observed (see Table 2). The results of the PAUP-based analysis of the pair-wise comparisons of ITS-2 sequences are shown in Table 4.

ITS-1 rDNA

The three ITS-1 sequences of the four lymnaeid populations studied were

TABLE 4. *Total character differences (above diagonal) and mean character differences adjusted for missing data (below diagonal), as determined using the PAUP programme, in pair-wise comparisons between the ITS-2 sequences of the lymnaeid species*

	Galba truncatula of ITS-2 haplotype: 1	2	3	*Lymnaea viatrix Ly. viatrix* var. var. B elongata	A ventricosa	*Ly. cubensis*
Galba truncatula						
ITS-2 haplotype 1	–	0.00249	0.00249	0.08995	0.09278	0.11340
ITS-2 haplotype 2	1	–	0.00499	0.08730	0.09021	0.11082
ITS-2 haplotype 3	1	2	–	0.08730	0.09021	0.11082
Ly. viatrix var. B elongata	34	33	33	–	0.03373	0.07090
Ly. viatrix var. A ventricosa	36	35	35	14	–	0.07289
Ly. cubensis	44	43	43	29	32	–

deposited in GenBank as accessions AM412226 (*Ly. cubensis* haplotype HA from Cuba), AM412227 (*Ly. viatrix* var. A ventricosa haplotype HA from Argentina) and AM412228 (*Ly. viatrix* var. B elongata haplotype HA from Rio Rimac and Rio Lurin, Lima, Peru). The lengths and slightly GC-biased mean nucleotide compositions of the ITS-1 sequences were, respectively, 524 bp and 56.29% in *Ly. cubensis*, 568 bp and 54.58% in *Ly. viatrix* var. A ventricosa, and 533 bp and 56.66% in *Ly. viatrix* var. B elongata.

The numbers and percentages of nucleotide differences that appeared in the two-sequence alignments of the ITS-1 sequences, and details of the ts, tv and indels, are shown in Table 5.

The ITS-1 sequence of *Ly. cubensis* shows no microsatellite repeat. Those of the specimens of *Ly. viatrix* var. B elongata from the populations of Rio Rimac and Rio Lurin in Lima, Peru, were identical but differed from the ITS-1 sequence of *Ly. viatrix* var. A ventricosa at 98 positions, giving a considerable nucleotide divergence of 17.19%. This divergence is markedly higher than that seen in ITS-2, with no microsatellite repeats found in the ITS-1 sequences.

Although the ITS-1 comparisons between *Ly. cubensis* and either *Ly. viatrix* var. A ventricosa or *Ly. viatrix* var. B elongata showed numerous nucleotide differences, the levels of nucleotide divergence were less than those seen with ITS-2. A large number of nucleotide differences was also apparent in the alignments of the ITS-1 sequences of *G. truncatula* haplotypes HA, HB or HC (the sequences of which differed by only one, two or three mutations) with those of the three taxa of American lymnaeid (see Table 5).

The results of the PAUP-based analysis of the pair-wise comparisons of ITS-2 sequences are shown in Table 6.

COI mtDNA

The three COI sequences of the four lymnaeid populations studied were deposited in GenBank as accessions AM494009 (*Ly. cubensis* haplotype Ha from Cuba), AM494010 (*Ly. viatrix* var. A ventricosa haplotype Ha from Argentina), AM494008 (*Ly. viatrix* var. B elongata haplotype Ha from Rio Rimac and Rio Lurin, Lima, Peru), and AM494011 (*G. truncatula* Ha — corresponding to snails belonging to the rDNA ITS combined haplotype CH1A — from Benicasim, Castellon, Spain). The lengths and highly AT-biased mean nucleotide compositions of the COI sequence were, respectively, 672 bp and 68.45% in *Ly. cubensis*, 672 bp and 69.39% in *Ly. viatrix* var. A ventricosa, 672 bp and 69.93% in *Ly. viatrix* var. B elongata, and 672 bp and 68.45% in *G. truncatula* Ha.

The numbers and percentages of nucleotide differences that appeared in the two-sequence alignments of the COI sequences, and details of the ts, tv and indels, are shown in Table 7.

The COI sequences of the specimens of *Ly. viatrix* var. B elongata from the populations of Rio Rimac and Rio Lurin in Lima, Peru, were identical but differed from those of *Ly. viatrix* var. A ventricosa at 29 positions, giving a nucleotide divergence of 4.31%. In the COI comparisons of *Ly. cubensis* with either *Ly. viatrix* var. A ventricosa or *Ly. viatrix* var. B elongata, numerous nucleotide differences were apparent although the levels of divergences were less than those seen in ITS-2 and ITS-1, mainly because of the absence of indels. Large numbers of nucleotide differences were also apparent in the alignments of the COI sequence of *G. truncatula* Ha with those of the three taxa of American lymnaeid (Table 7).

The results of the PAUP-based analysis of the pair-wise comparisons of COI sequences are shown in Table 8.

For each of the four lymnaeid taxa studied, the amino-acid sequence of the COI gene fragment that was obtained was 224 acids long. Pair-wise comparison at the level of these COI amino-acid sequences

TABLE 5. *ITS-1 sequence differences detected in pair-wise comparisons between the lymnaeid species studied*

Comparison	Alignment length (bp)	No. and (%) of nucleotide differences	No. and (%) of substitutions		No. and (%) of insertions/deletions
			Transitions	Transversions	
Lymnaea cubensis v. Ly. viatrix var. A ventricosa	569	86 (15.11)	18 (3.16)	22 (3.87)	46 (8.08)
Ly. cubensis v. Ly. viatrix var. B elongata	544	72 (13.23)	20 (3.67)	21 (3.87)	31 (5.69)
Ly. viatrix var. A ventricosa *v. Ly. viatrix* var. B elongata	570	98 (17.19)	30 (5.26)	29 (5.08)	39 (6.84)
Ly. cubensis v. Galba truncatula (ITS-1 haplotypes A, B and C)	549	126–128 (22.95–23.31)	27–28 (4.92–5.10)	29–30 (5.28–5.46)	70 (12.75)
Ly. viatrix var. A ventricosa *v. G. truncatula* (ITS-1 haplotypes A, B and C)	569	139–141 (24.43–24.78)	34–35 (5.97–6.15)	39–40 (6.85–7.03)	66 (11.59)
Ly. viatrix var. B elongata *v. G. truncatula* (ITS-1 haplotypes A, B and C)	544	131–133 (24.08–24.45)	40–41 (7.35–7.54)	40–41 (7.35–7.54)	51 (9.37)

indicated 100% identity between *Ly. cubensis* and *Ly. viatrix* var. B elongata, and only one amino-acid difference (isoleucine/valine, at position 11) between them and *Ly. viatrix* var. A ventricosa. In terms of these amino-acid sequences, *G. truncatula* was identical to *Ly. cubensis* and *Ly. viatrix* var. B elongata.

Phylogenetic Analysis

The combination of the two internal transcribed spacers in a single data-set generated a robust tree, indicating phylogenetic accordance between the two spacers. The ML model best fitting this data-set was HKY85ΓG, using a ts/tv ratio of 1.12 (kappa52.2016), base frequencies for A,

TABLE 6. *Total character differences (above diagonal) and mean character differences adjusted for missing data (below diagonal), as determined using the PAUP programme, in pair-wise comparisons between the ITS-1 sequences of the lymnaeid species*

	Galba truncatula of ITS-1 haplotype:					
	A	B	C	*Ly. cubensis*	*Ly. viatrix* var. A ventricosa	*Lymnaea viatrix* var. B elongata
Galba truncatula						
ITS-1 haplotype A	–	0.00198	0.00397	0.12343	0.15339	0.16057
ITS-1 haplotype B	1	–	0.00595	0.12552	0.15538	0.16260
ITS-1 haplotype C	2	3	–	0.12762	0.15737	0.16463
Ly. cubensis	59	60	61	–	0.07648	0.06126
Ly. viatrix var. A ventricosa	77	78	79	40	–	0.09943
Ly. viatrix var. B elongata	79	80	81	31	52	–

TABLE 7. *COI sequence differences detected in pair-wise comparisons between the lymnaeid species studied*

Comparison	Alignment length (bp)	No. and (%) of nucleotide differences	No. and (%) of substitutions: Transitions	Transversions	No. and (%) of insertions/deletions
Lymnaea cubensis v. Ly.viatrix var. A ventricosa	672	38 (5.65)	32 (4.76)	6 (0.89)	0 (0)
Ly. cubensis v. Ly. viatrix var. B elongata	672	14 (2.08)	14 (2.08)	0 (0)	0 (0)
Ly. viatrix var. A ventricosa *v. Ly. viatrix* var. B elongata	672	29 (4.31)	23 (3.42)	6 (0.89)	0 (0)
Ly. cubensis v. Galba truncatula (COI haplotype Ha)	672	75 (11.16)	43 (6.39)	32 (4.76)	0 (0)
Ly. viatrix var. A ventricosa *v. G. truncatula* (COI haplotype Ha)	672	68 (10.11)	38 (5.65)	30 (4.46)	0 (0)
Ly. viatrix var. B elongata *v. G. truncatula* (COI haplotype Ha)	672	67 (9.97)	35 (5.21)	32 (4.76)	0 (0)

TABLE 8. *Total character differences (above diagonal) and mean character differences adjusted for missing data (below diagonal), as determined using the PAUP programme, in pair-wise comparisons between the COI sequences of the lymnaeid species*

	Lymnaea viatrix var. B elongata	*Ly. cubensis*	*Ly. viatrix* var. A ventricosa	*Galba truncatula* (COI haplotype Ha)
Ly. viatrix var. B elongata	–	0.02083	0.04315	0.09968
Ly. cubensis	14	–	0.05655	0.11158
Ly. viatrix var. A ventricosa	29	38	–	0.10117
Galba truncatula (COI haplotype Ha)	67	75	68	–

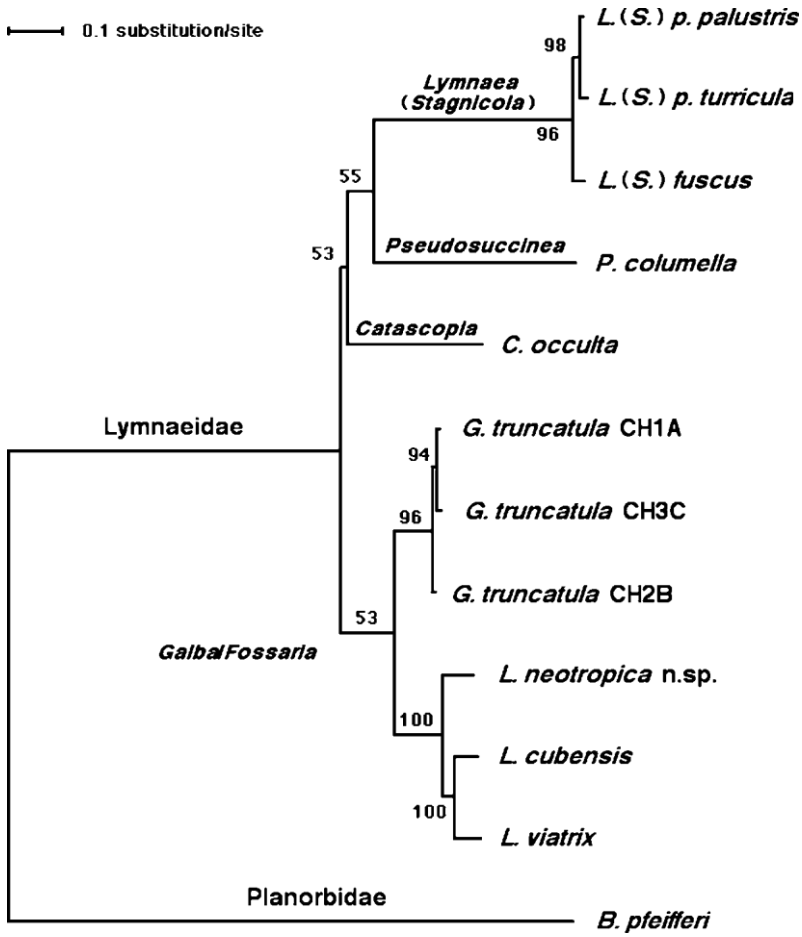


FIG. 1. A phylogenetic tree of lymnaeid snail species, based on ITS-1 and ITS-2 ribosomal-DNA sequences. The tree was derived from a maximum-likelihood model (HKY85≂G), using a transition/transversion ratio of 1.12, with the planorbid species *Biomphalaria pfeifferi* as an outgroup. The numbers on the nodes represent the percentage of 1000 puzzling replicates.

C, G and T of 0.3361, 0.1419, 0.1787 and 0.3433, respectively, a proportion of invariable sites50, and a gamma-distribution shape parameter of 0.59. The resulting phylogeny (2Ln56314.7671) was evaluated using a least-squares method with ML distances, with high puzzle values supporting the reliability of the nodes of the main branches.

In the tree obtained, *Ly. viatrix* var. B elongata appears as sister species, with maximum support (puzzle value5100%), in the same clade as *Ly. cubensis* and *Ly. viatrix* var. A ventricosa, the latter two taxa also clustering with maximum support. The branch holding all three haplotypes of *G. truncatula* is grouped within the same *Galba/Fossaria* clade as *Ly. viatrix* var. B elongata, *Ly. cubensis* and *Ly. viatrix* var. A ventricosa. Stagnicolines are grouped in the other main clade. The European *Lymnaea* (*Stagnicola*) species are grouped together in a well supported branch, with the American *P. columella* and the Palaearctic *C. occulta* appearing as basal species (Fig. 1).

Diagnostic Description of *Lymnaea neotropica* n. sp.

SYNONYM

Lymnaea viatrix var. B elongata D'Orbigny, 1835.

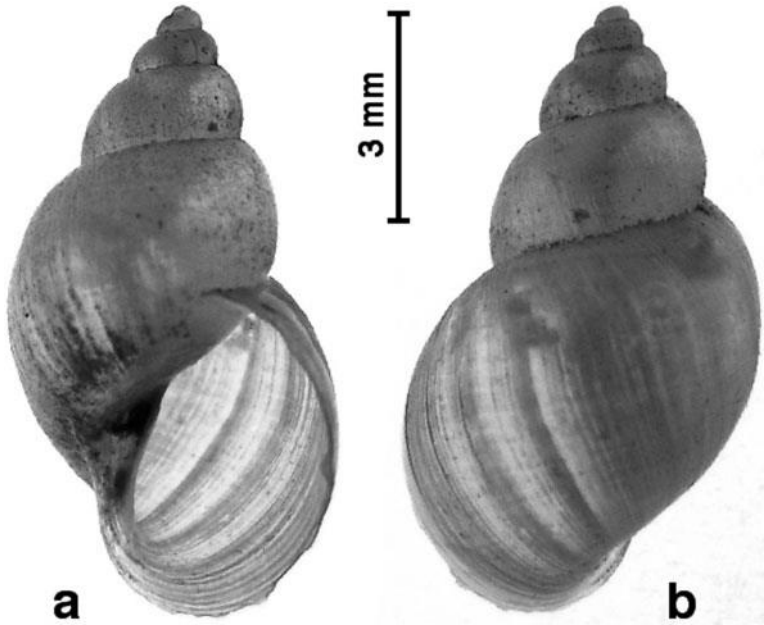


FIG. 2. The shell of *Lymnaea neotropica* n.sp., showing ventral (a) and dorsal (b) views.

TYPE LOCALITY

Irrigation canals running from the Rio Rimac, near Lima and Callao, Peru (12u029S, 76u569–77u089W).

OTHER LOCALITY

Rio Lurin, Lima, Peru (12u039S, 77u049W).

TYPE SPECIMENS

Holotype and a paratype deposited in the *Muse´um National d'Histoire Naturelle* (MNHN) in Paris, France. Other voucher specimens deposited in the parasite collection of the Department of Parasitology, University of Valencia, Valencia, Spain.

SHELL

The form of the shell is illustrated in Figure 2. Shell length in the specimens measured varied between 5.89 and 8.74 mm, with a mean (S.D.) of 7.13 (1.20) mm. Maximum width varied from 3.29–4.56 mm, with a mean (S.D.) of 3.95 (0.54) mm. The length/width ratio varied from 1.69–1.92, with a mean (S.D.) of 1.80 (0.07). The shell is formed of 5.5 convex and deeply sutured whorls that gradually increase in diameter, with a shell spiral angle of 38.64u–46.99u [mean (S.D.)542.73 (2.44)u]. The last whorl is 4.47- to 6.78-mm long [mean (S.D.)55.58 (0.91) mm], with a shell/last-whorl length ratio of 1.21–1.38 [mean (S.D.)51.28 (0.05)]. The spire is more-or-less pointed, or short with a rather obtuse apex. Growth lines and umbilicus are slightly pronounced. The spiral sculpture is very faint and not easily seen. The aperture is oval and almost round, with a thin peristome. The aperture measures 2.90–4.53 mm [mean (S.D.)53.75 (0.64) mm] in length and 1.97–2.99 mm [mean (S.D.)52.46 (0.44) mm] in width. The length of the aperture is about half the shell length, with a shell/aperture length ratio of 1.76–2.03 [mean (S.D.)51.91 (0.09)].

Shells up to 10 mm long and 5.6 mm wide, with length/width ratios varying from 1.61–1.85 (with a mean of 1.75), a whorl increase ratio of 1.71–2.09 (with a mean of 1.88), and shell/aperture length ratios of 1.81–2.02 (with a mean of 1.90) were recorded by Paraense (1976).

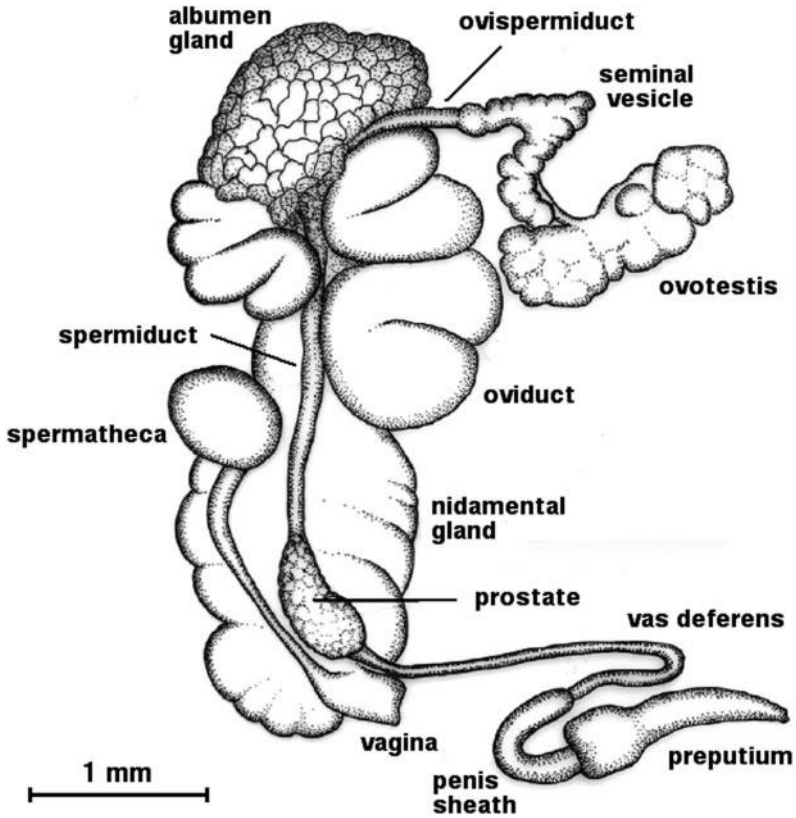


FIG. 3. Anatomy of the reproductive system of *Lymnaea neotropica* n.sp.

ANATOMY

The morphology of the reproductive system is illustrated in Figure 3. The ovotestis, which has a lobulate surface formed by numerous acini, has a collecting canal that continues into the ovispermiduct. The latter, which appears to be a short and very thin tube, empties into the wider seminal vesicle, which has a bosselated surface and leads into an egg-shaped chamber which is located between the albumen gland, oviduct and spermiduct.

The voluminous albumen gland is roughly bean-shaped and has a funnel-shaped duct that opens into the egg-shaped chamber. The oviduct is so markedly contorted that it nearly describes a complete circle around the spermiduct. It is in contact with the albumen gland, and, near to its cephalic end, has a wrinkle-walled, expanded pouch projecting from its right dorsal side. The final, thick-walled part of the oviduct is roughly cylindrical and continues into the nidamental gland. The nidamental gland is oblong, with rounded shoulders, and is initially wider than the cephalic portion of the oviduct before narrowing into a short uterus followed by a short vagina.

The spermatheca is round–ovoid, with a narrow and relatively long duct. Although the first expanded part of the spermiduct shows a granular surface, the duct becomes gradually thinner and smoother, as it runs across the ventral surface of the nidamental gland, before again widening to form a prostate with a granular surface. The prostate, which is generally ovoid but not infrequently elongate or pear- or spindle-shaped, measures 580–1120 μm [mean (S.D.)5782 (1.12) μm] in length and is flattened dorsoventrally at its caudal end.

The vas deferens emerges from the opposite part of the prostate, runs for short distance across the vagina and then forms a long caudal loop before emptying in a curved, cylindrical penis sheath. The penis is very slender, acicular, unarmed, with a terminal outlet, and about as long as the sheath. The penis sheath is frequently more-or-less invaginated into the preputium, from which it is separated, internally, by the sarcobelum, a perforated papilla mainly consisting of a gland-cell aggregate. The preputium is markedly wider and longer than the penis sheath, the latter appearing to be more than half as long as the preputium in around 40% of the specimens examined. The preputium/penis-sheath length ratio varied from 1.3–3.4 [mean ($_{S.D.}$)52.12 (0.28)] in the present study [and from 1.1–3.9 (mean52.7) in the study by Paraense (1976)].

The central tooth of the radula is bicuspid, with a tiny cusp or protuberance in addition to the main cusp. The lateral teeth are usually bicuspid, although the first laterals may occasionally be tricuspid or very rarely quadricuspid. The intermediate teeth have the mesocone split into two parts, and the marginal teeth are multicuspidate. The teeth often appear irregularly arranged, groups of teeth with well divided cusps sometimes alternating with groups of teeth with relatively undivided cusps.

For additional information on the anatomy of the soft parts of this species see Paraense (1976).

DNA SEQUENCE MARKERS

Specific classification can be based on the sequences of ITS-2 rDNA (GenBank accession AM412225; haplotype H1), ITS-1 rDNA (AM412228; haplotype HA) and COI mtDNA (AM494008; haplotype Ha). For supraspecific classification, the nucleotide sequences of the 18S rDNA (AM412222) and the amino-acid sequence corresponding to the mtDNA COI (AM494008; haplotype Ha) can be employed.

DISCUSSION

Lymnaeids seem to have originated in the Nearctic region, which currently holds the largest number of species (Inaba, 1969). Although there appears to be only one member of the *Galba/Fossaria* group acting as a vector of *Fa. hepatica* in Europe (i.e. *G. truncatula*), in the New World studies on the transmission of *Fa. hepatica* are complicated by the presence of several, morphologically similar members of this group, all of which can act as vectors (Bargues *et al.*, 2001).

A good example of the problems posed by this confusing lymnaeid group comes from the studies on a highly endemic area for human fascioliasis in the northern Bolivian Altiplano. Thirty years ago, it was thought that there were two species acting as vectors of *Fa. hepatica* in this area: *Ly. viatrix* and *Ly. cubensis* (Ueno *et al.*, 1975). In subsequent studies, however, the '*Ly. viatrix*' and '*Ly. cubensis*' from this area showed exactly the same susceptibility to infection with *Fa. hepatica* (an abnormal observation for two sympatric vector species) and their morphological features were found to overlap (Oviedo *et al.*, 1995*a*). Finally, the sequencing of various rDNA markers from the lymnaeid snails in the endemic area showed that there was only one lymnaeid species present, albeit one with a large level of intraspecific morphological variability, and that this species was not *Ly. viatrix* or *Ly. cubensis* but *G. truncatula* (Bargues and Mas-Coma, 1997; Bargues *et al.*, 1997; Mas-Coma *et al.*, 2001). In their phenotyp- ing studies based on iso-enzyme analysis and careful morphometry, Jabbour-Zahab *et al.* (1997) and Samadi *et al.* (2000) reached the same conclusion.

For the Lymnaeidae in general, the four DNA markers used in the present study have the advantage of furnishing information at different levels. The 18S rRNA gene, for example, has a conserved, slowly evolving sequence that is useful mainly for supraspecific analyses. The ITS-2 and

ITS-1 sequences evolve relatively fast and are useful for species differentiation, although haplotyping also enables population characterisation within a species (Bargues and Mas-Coma, 2005). COI appears to evolve faster than 18S and ITS-2 but slower than ITS-1, its amino-acid sequence offering information at a similar or higher level than that furnished by the 18S rRNA gene.

The characteristics of the 18S rDNA sequences of *Ly. cubensis*, *Ly. viatrix* (5*Ly. viatrix* var. A ventricosa) and *Ly. neotropica* n.sp. (5*Ly. viatrix* var. B elongata) determined in the present study appear similar to those known for other lymnaeids, the localization of most nucleotide differences in the E10-1 helix of the variable area V2 being a common feature (Bargues and Mas-Coma, 1997; Bargues *et al.*, 1997; Bargues and Mas-Coma, 2005). Interestingly, in spite of the morpho–anatomical similarity of the lymnaeid species studied, there are differences not only between *Ly. cubensis*, *Ly. viatrix* (5*Ly. viatrix* var. A ventricosa) and *Ly. neotropica* n.sp. (5*Ly. viatrix* var. B elongata), but also numerous differences between *G. truncatula* and the American lymnaeids, indicating that the lymnaeids investigated in detail in the present study may cover more than one supraspecific taxon.

The ITS-2 sequences of the lymnaeids investigated in the present study show a high number of microsatellite repeats. Microsatellites have already been detected in the ITS-2 of other lymnaeids (Bargues *et al.*, 2001, 2003) but the details of their origin, mutation, evolution and function, if any, have yet to be elucidated (Jarne *et al.*, 1998). In theory at least, as microsatellite alleles exhibit extreme intraspecific variability, neutrality, Mendelian inheritance, codominance and high mutation rates, they should form very good polymorphic molecular markers for the differentiation of populations within a given species (Jarne and Lagoda, 1996; Roos *et al.*, 1998). Microsatellites of ITS-2 may thus be useful markers for population differentiation in species of the *Galba/Fossaria* group, although their use must be handled with caution. When the ts, tv and indels not related to microsatellites are considered, the nucleotide differences and genetic distances observed, in the present study, between *Ly. viatrix* (5*Ly. viatrix* var. A ventricosa) and *Ly. neotropica* n.sp. (5*Ly. viatrix* var. B elongata) are similar to those known between some closely related but systematically distinct lymnaeid species and markedly greater than observed within some lymnaeid species that show high levels of intraspecific variability (Bargues *et al.*, 2001, 2003).

As no microsatellites were detected in ITS-1, all nucleotide differences seen in the sequence of this spacer may be considered for species differentiation. In all the pairwise comparisons in the present study, the differences seen in ITS-1 (Table 6) were greater than those in ITS-2 (Table 4). This was the expected result, as ITS-1 evolves faster than ITS-2 (Bargues and Mas-Coma, 2005; Bargues *et al.*, 2006). As with ITS-2, the ITS-1 sequences determined in the present study support species status for *Ly. cubensis*, *Ly. viatrix* var. A ventricosa, *Ly. viatrix* var. B elongata and *G. truncatula* (Bargues *et al.*, 2006). The COI sequences also show a number of nucleotide differences that support a greater differentiation between '*Ly. viatrix* var. A ventricosa' and '*Ly. viatrix* var. B elongata'. In addition, the COI amino-acid sequences, like the rDNA ITS phylogenetic tree (Fig. 1), indicate that *Ly. cubensis*, *Ly. viatrix* (5*Ly. viatrix* var. A ventricosa), *Ly. neotropica* n.sp. (5*Ly. viatrix* var. B elongata) and *G. truncatula* had a common, relatively recent ancestor.

The results of the present DNA sequencing generally support those of recent phenotypic and morpho–anatomical studies performed on lymnaeid samples of the same species, varieties and type localities. In the phenotypic study by Durand *et al.* (2002), for example, an analysis of variability based on 12 iso-enzyme loci revealed significant

differences between *Ly. viatrix* var. B elongata (from the type locality in Lima) and *Ly. viatrix* var. A ventricosa (collected in Bahia Blanca, Argentina, about 350 km north of the type locality of this variety, in Rio Negro), as well as between the *Ly. viatrix* var. B elongata and *Ly. cubensis* from Cuba. In their morpho–anatomical study, Pointier *et al.* (2006) found that, in terms of the respective lengths of the penis sheath and preputium, *Ly. viatrix* var. A ventricosa was markedly different from *Ly. cubensis* or *Ly. viatrix* var. B elongata (although, none of the morpho–anatomical parameters that were studied allowed *Ly. cubensis* to be distinguished from *Ly. viatrix* var. B elongata).

As a variety is not a taxonomic entity and a variety name cannot therefore be raised to species level, the new species *Lymnaea neotropica* n. sp. is here proposed for the lymnaeid specimens from Lima previously known as *Lymnaea viatrix* var. B elongata. To avoid further confusion, the type locality for *Ly. viatrix* var. B elongata (D'Orbigny, 1835) — the Rio Rimac at Lima, Peru — is selected as type locality for the new species and *Ly. viatrix* var B. elongata becomes a synonym of *Ly. neotropica* n.sp. *Lymnaea viatrix* (5*Ly. viatrix* var. A ventricosa) is hence characterised by the features of the lymnaeids coming from Frias, Rio Negro, Argentina.

The three species *Ly. cubensis*, *Ly. viatrix* and *Ly. neotropica* n.sp. are kept within the genus *Lymnaea* s.l. for the time being, awaiting a general review of the Lymnaeidae from Latin America which will include an appropriate systematic–taxonomic analysis of the taxa currently recognised within the *Galba/Fossaria* group. The relevant 18S rDNA sequences (Bargues and Mas-Coma, 1997, 2005; present study) already indicate that — even though these four species have similar shells (some old specimens of *Ly. cubensis* are larger than *G. truncatula*) and are difficult to distinguish anatomically (Samadi *et al.*, 2000) — *Ly. cubensis*, *Ly. viatrix*, *Ly. neotropica* n.sp. and *G. truncatula* should not be placed in the same supraspecific taxon.

The present results show that ITS-2, ITS-1 and COI are good markers not only for identifying *Ly. cubensis*, *Ly. viatrix*, *Ly. neotropica* and *G. truncatula*, in fascioliasis-endemic areas in Central and South America, but also for the classification of samples of these species to haplotype level. This usefulness becomes crucial when considering that, anatomically, *Ly. cubensis* appears to be indistinguishable from *Ly. neotropica* (5 *Ly. viatrix* var. B elongata) and is distinguishable only with difficulty from *Ly. viatrix* (5 *Ly. viatrix* var. A ventricosa) (Paraense, 1982; Pointier *et al.*, 2006). Exhaustive ITS-2 and ITS-1 studies on single nucleotide polymorphisms (SNP) have already proved the value of both spacers for the distinction and identification of these lymnaeids (Bargues *et al.*, 2001, 2003, 2005; Bargues and Mas-Coma, 2005). Curiously, a single mutation in the ITS rDNA of *P. columella* has been associated with susceptibility to *Fa. hepatica* infection (Gutierrez *et al.*, 2003).

ACKNOWLEDGEMENTS. This research was largely funded by the Spanish Ministry of Education and Science (projects BOS2002-01978 and SAF2006-09278) and by the *Red de Investigación de Centros de Enfermedades Tropicales* (projects C03/04, ISCIII2005-PI050574 and. ISCIII-RETIC RD06/0021/0017 of the *Programa de Redes Temáticas de Investigación Cooperativa*) of the Spanish Ministry of Health's *Fondo de Investigación Sanitaria*. The studies in Argentina were, however, funded by Universidad Nacional de Cuyo.

The authors thank Dr M. Yong (Laboratorio de Malacolog´ıa, Instituto de Medicina Tropical 'Pedro Kouri', La Habana, Cuba), Dr C. Náquira (Instituto de Medicina Tropical 'Daniel A. Carrión', Facultad de Medicina, Universidad Nacional Mayor de San Marcos, Lima, Peru), and J. A. Arenas (Laboratorio de Investigación,

Facultad de Ciencias Biológicas, Universidad Ricardo Palma, Lima, Peru) for kindly providing samples of *Ly. cubensis* from Vaqueria 21 (La Habana), *Ly. viatrix* var. B elongata from Rio Rimac (Lima) and *Ly. viatrix* var. B elongata from Rio Lurin (Lima), respectively. The collaboration of L. M. Vigo in the early laboratory procedures in Valencia is greatly acknowledged. Technical support was provided by the *Servicio Central de Secuenciación para la Investi- gación Experimental* of the Universidad de Valencia (Dr A. Mart´ınez).

REFERENCES

Akaike, H. (1974). A new look at the statistical model identification. *IEEE Transactions on Automatic Control*, 19, 716–723.

Bargues, M. D. & Mas-Coma, S. (1997). Phylogenetic analysis of lymnaeid snails based on 18S rDNA sequences. *Molecular Biology and Evolution*, 14, 569–577.

Bargues, M. D. & Mas-Coma, S. (2005). Reviewing lymnaeid vectors of fascioliasis by ribosomal DNA sequence analyses. *Journal of Helminthology*, 79, 257–267.

Bargues, M. D., Marquez, F. J. & Mas-Coma, S. (1995). Rapid non-radioactive and non-fluorescent rDNA sequencing: application to parasites. *Research and Reviews in Parasitology*, 55, 199–205.

Bargues, M. D., Mangold, A. J., Muñoz-Antoli, C., Pointier, J. P. & Mas-Coma, S. (1997). SSU rDNA characterization of lymnaeid snails transmitting human fascioliasis in South and Central America. *Journal of Parasitology*, 83, 1086–1092.

Bargues, M. D., Vigo, M., Horak, P., Dvorak, J., Patzner, R. A., Pointier, J. P., Jackiewicz, M., Meier-Brook C. & Mas-Coma, S. (2001). European Lymnaeidae (Mollusca: Gastropoda), intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS-2 sequences. *Infection, Genetics and Evolution*, 1, 85–107.

Bargues, M. D., Horak, P., Patzner, R. A., Pointier, J. P., Jackiewicz, M., Meier-Brook, C. & Mas-Coma, S. (2003). Insights into the relationships of Palaearctic and Nearctic lymnaeids (Mollusca: Gastropoda) by rDNA ITS-2 sequencing and phylogeny of stagnicoline intermediate host species of *Fasciola hepatica*. *Parasite*, 10, 243–255.

Bargues, M. D., Artigas, P., Jackiewicz, M., Pointier, J. P. & Mas-Coma, S. (2006). Ribosomal DNA ITS-1 sequence analysis of European stagnicoline Lymnaeidae (Gastropoda). *Heldia (Münchner Malakologische Mitteilungen), München*, 6, 29–40.

Chen, M. G. & Mott, K. E. (1990). Progress in assessment of morbidity due to *Fasciola hepatica* infection: a review of recent literature. *Tropical Diseases Bulletin*, 87, R1–R38.

D'Orbigny, A. (1835). Synopsis terrestrium et fluviatilum molluscorum, in suo per Americam Meridionalem itinere collectorum. *Magasin de Zoologie*, 5, 1–44.

De Rijk, P. & de Wachter, R. (1993). DCSE v2.54, an interactive tool for sequence alignment and secondary structure research. *Computer Applications in Bioscience*, 9, 735–740.

De Rijk, P. & de Wachter, R. (1997). RnaViz, a program for the visualisation of RNA secondary structure. *Nucleic Acids Research*, 25, 4679–4684.

De Rijk, P., Neefs, J. M., van de Peer, Y. & de Wachter, R. (1992). Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Research*, 20, 2075–2089.

Durand, P., Pointier, J. P., Escoubeyrou, K., Arenas, J. A., Yong, M., Amarista, M., Bargues, M. D., Mas-Coma, S. & Renaud, F. (2002). Occurrence of a sibling species complex within Neotropical lymnaeids, snail intermediate hosts of fascioliasis. *Acta Tropica*, 83, 233–240.

Esteban, J. G., Gonzalez, C., Bargues, M. D., Angles, R., Sanchez, C., Naquira, C. & Mas-Coma, S. (2002). High fascioliasis infection in children linked to a man-made irrigation zone in Peru. *Tropical Medicine and International Health*, 7, 339–348.

Folmer, O., Black, M., Hoch, W., Lutz, R. & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 3, 294–299.

Fuentes, M. V., Valero, M. A., Bargues, M. D., Esteban, J. G., Angles, R. & Mas-Coma, S. (1999). Analysis of climatic data and forecast indices for human fascioliasis at very high altitude. *Annals of Tropical Medicine and Parasitology*, 93, 835–850.

Gasser, R. B., Chilton, N. B., Hoste, H. & Beveridge, I. (1993). Rapid sequencing of rDNA from single worms and eggs of parasitic helminths. *Nucleic Acids Research*, 21, 2525–2526.

Gutierrez, A., Pointier, J. P., Fraga, J., Jobet, E., Modat, S., Perez, R. T., Yong, M., Sanchez, J., Loker, E. S. & Theron, A. (2003). *Fasciola hepatica*: identification of molecular markers for resistant and susceptible *Pseudosuccinea columella* snail hosts. *Experimental Parasitology*, 105, 211–218.

Hopkins, D. R. (1992). Homing in on helminths. *American Journal of Tropical Medicine and Hygiene*, 46, 626–634.

Inaba, A. (1969). Cytotaxonomic studies of lymnaeid snails. *Malacologia*, 7, 143–168.

Jabbour-Zahab, R., Pointier, J. P., Jourdane, J., Jarne, P., Oviedo, J. A., Bargues, M. D., Mas-Coma, S., Angles, R., Perera, G., Balzan, C., Khallaayoune, K. & Renaud, F. (1997). Phylogeography and genetic divergence of some lymnaeid snails, intermediate hosts of human and animal fascioliasis, with special reference to lymnaeids from the Bolivian Altiplano. *Acta Tropica*, 64, 191–203.

Jarne, P. & Lagoda, P. J. L. (1996). Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution*, 11, 424–429.

Jarne, P., David, P. & Viard, F. (1998). Microsatellites, transposable elements and the X chromosome. *Molecular Biology and Evolution*, 15, 28–34.

Lake, J. A. (1991). The order of sequence alignment can bias the selection of tree topology. *Molecular Biology and Evolution*, 8, 378–385.

Malek, E. A. (1985). *Snail Hosts of Schistosomiasis and Other Snail-transmitted Diseases in Tropical America: a Manual*. Scientific Publication No. 478. Washington, DC: Pan American Health Organization.

Mas-Coma, S. (2004). Human fascioliasis. In *World Health Organization (WHO), Waterborne Zoonoses: Identification, Causes and Control*, eds Cotruvo, J. A., Dufour, A., Rees, G., Bartram, J., Carr, R., Cliver, D. O., Craun, G. F., Fayer, R. & Gannon, V. P. J. pp. 305–322. London: IWA Publishing.

Mas-Coma, S. (2005). Epidemiology of fascioliasis in human endemic areas. *Journal of Helminthology*, 79, 207–216.

Mas-Coma, S., Angles, R., Esteban, J. G., Bargues, M. D., Buchon, P., Franken, M. & Strauss, W. (1999*a*). The Northern Bolivian Altiplano: a region highly endemic for human fascioliasis. *Tropical Medicine and International Health*, 4, 454–467.

Mas-Coma, S., Bargues, M. D. & Esteban, J. G. (1999*b*). Human Fasciolosis. In *Fasciolosis*, ed Dalton, J. P. pp. 411–434. Wallingford, U.K.: CAB International.

Mas-Coma, S., Esteban, J. G. & Bargues, M. D. (1999*c*). Epidemiology of human fascioliasis: a review and proposed new classification. *Bulletin of the World Health Organization*, 77, 340–346.

Mas-Coma, S., Funatsu, I. R. & Bargues, M. D. (2001). *Fasciola hepatica* and lymnaeid snails occurring at very high altitude in South America. *Parasitology*, 123 (Suppl.), S115–S127.

Mas-Coma, S., Bargues, M. D. & Valero, M. A. (2005). Fascioliasis and other plant-borne trematode zoonoses. *International Journal for Parasitology*, 35, 1255–1278.

Mathews, D. H., Sabina, J., Zuker, M. & Turner, D. H. (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. *Journal of Molecular Biology*, 21, 911–940.

Meunier, C., Tirard, C., Hurtrez-Bousses, S., Durand, P., Bargues, M. D., Mas-Coma, S., Pointier, J. P., Jourdane, J. & Renaud, F. (2001). Lack of molluscan host diversity and the transmission of an emerging parasitic disease in Bolivia. *Molecular Ecology*, 10, 1333–1340.

Oviedo, J. A., Bargues, M. D. & Mas-Coma, S. (1995*a*). Lymnaeid snails in the human fascioliasis high endemic zone of the Northern Bolivian Altiplano. *Research and Reviews in Parasitology*, 55, 35–43.

Oviedo, J. A., Bargues, M. D. & Mas-Coma, S. (1995*b*). Ecological characteristics of lymnaeid snails in the human fascioliasis high endemic zone of the Bolivian Northern Altiplano. In *Unitas Malacologica*, eds Guerra, A., Rolán, E. & Rocha, F. pp. 463–465. Vigo, Spain: Instituto de Investigaciones Marinas.

Paraense, W. L. (1976). *Lymnaea viatrix*: a study of topotypic specimens (Mollusca: Lymnaeidae). *Revista Brasileira de Biologia*, 36, 419–428.

Paraense, W. L. (1982). *Lymnaea viatrix* and *Lymnaea columella* in the neotropical region: a distributional outline. *Memórias do Instituto Oswaldo Cruz*, 77, 181–188.

Pfeiffer, L. (1839). Bericht ü ber die ergebnisse meiner reise nach Kuba im winter 1838–1839. *Archiv für Naturgeschichte*, 5, 346–359.

Pointier, J. P., Cazzaniga, N. J., Gonzalez-Salas, C., Gutierrez, A., Arenas, J. A., Bargues, M. D. & Mas-Coma, S. (2006). Anatomical studies of sibling species within Neotropical lymnaeids snail intermediate hosts of fascioliasis. *Memórias do Instituto Oswaldo Cruz*, 101, 431–435.

Posada, D. & Buckley, T. R. (2004). Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. *Systematic Biology*, 53, 793–808.

Posada, D. & Crandall, K. A. (1998). Modeltest: testing the model of DNA substitution. *Bioinformatics*, 14, 817–818.

Remigio, E. A. & Hebert, P. D. N. (2003). Testing the utility of partial COI sequences for phylogenetic estimates of gastropod relationships. *Molecular Phylogenetics and Evolution*, 29, 641–647.

Roos, M. H., Hoekstra, R., Plas, M. E., Otsen, M. & Lenstra, J. A. (1998). Polymorphic DNA markers in the genome of parasitic nematodes. *Journal of Helminthology*, 72, 291–294.

Samadi, S., Roumegoux, A., Bargues, M. D., Mas-Coma, S., Yong, M. & Pointier, J. P. (2000). Morphological studies of lymnaeid snails from the human fascioliasis endemic zone of Bolivia. *Journal of Molluscan Studies*, 66, 31–44.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual*, 2nd Edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors.

Proceedings of the National Academy of Sciences of the United States of America, 74, 5463–5467.

Swofford, D. L. (2002). *PAUP*: Phylogenetic Analysis Using Parsimony (* and Other Methods). [4.0beta]*. Sunderland, MA: Sinauer Associates.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity and progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, 22, 4673–4680.

Torgerson, P. & Claxton, J. (1999). Epidemiology and control. In *Fasciolosis*, ed Dalton, J. P. pp. 113–149. Wallingford, U.K.: CAB International.

Ueno, H., Arandia, R., Morales, G. & Medina, G. (1975). Fascioliasis of livestock and snail host for *Fasciola* in the Altiplano region of Bolivia. *National Institute of Animal Health Quarterly*, 15, 61–67.

Valero, M. A., Panova, M. & Mas-Coma, S. (2005). Phenotypic analysis of adults and eggs of *Fasciola hepatica* by computer image analysis system. *Journal of Helminthology*, 79, 217–225.

Winnepennickx, B., Backeljau, T., van de Peer, Y. & de Wachter, R. (1992). Structure of the small ribosomal subunit RNA of the pulmonate snail, *Limicolaria kambeul*, and phylogenetic analysis of the Metazoa. *FEBS Letters*, 309, 123–126.

María Dolores Bargues[1]
Roberto L. Mera y Sierra[1,2]
Humberto Gustavo Gómez[1]
Patricio Artigas[1]
Santiago Mas-Coma[1]

[1]Departamento de Parasitología
Facultad de Farmacia
Universidad de Valencia. España
[2]Facultad de Ciencias Médicas
Universidad Nacional de Cuyo. Argentina

Correspondencia:
María Dolores Bargues
Departamento de Parasitología
Facultad de Farmacia
Universidad de Valencia
Av. Vicent Andres Estelles s/n
46100 Burjassot. Valencia
E-mail: M.D. Bargues@uv.es

Caracterización molecular de *Galba truncatula,* vector principal de la Fascioliasis, en Argentina. Implicaciones en salud pública

ORIGINAL

Resumen

Objetivo: La Fascioliasis humana ha pasado recientemente a engrosar la lista de las grandes enfermedades parasitarias de la humanidad. Las zonas de endemia humana presentando mayores prevalencias e intensidades se encuentran en Países Andinos como Bolivia y Perú, donde la enfermedad está transmitida por el molusco gasterópodo de la familia Lymnaeidae *Galba truncatula.* El hallazgo y determinación de esta misma especie de vector en Argentina, en 2001, fueron inicialmente realizados con base morfológica, siendo así que estudios posteriores han demostrado que la morfología no basta para la distinción entre especies del grupo Galba/Fossaria. El presente trabajo tiene por finalidad la confirmación de la existencia, en Argentina, del mejor vector de Fascioliasis conocido mediante secuenciación de un marcador específico del ADN.
Material y métodos: Los moluscos fueron recolectados en El Salto, en la zona andina de Mendoza, Argentina, y fijados en etanol al 70%. El ADN genómico fue extraído, amplificado y purificado siguiendo métodos estandarizados. La secuenciación del ITS-2 del ADN ribosomal nuclear fue obtenida mediante secuenciador automático utilizando cebadores específicos. El programa BLAST fue usado para comparación rápida con todas las secuencias de Lymnaeidos disponibles en el GenBank. El análisis comparado final se hizo mediante alineamiento utilizando Clustal W versión 1.8.
Resultados: La secuencia completa del ITS-2 del ADNr resultó ser de una longitud de 401 pares de bases y un contenido en GC del 58,6%. La secuencia obtenida demostró ser idéntica en longitud, contenido y composición de nucleótidos que el haplotipo H3 de *Galba truncatula*, anteriormente descrito en Bolivia.
Conclusiones: El hallazgo en Mendoza del mismo haplotipo de molusco vector responsable de la Fascioliasis humana y animal del Altiplano Norte Boliviano, la mayor zona de endemia humana conocida, constituye un hecho de un enorme potencial de impacto en salud pública dadas las connotaciones de gran capacidad de transmisión que confluyen en la zona andina de Mendoza. Todo indica la necesidad de efectuar un amplio estudio futuro con el fin de llegar a conocer la distribución geográfica de *G. truncatula* H3 en Argentina, para lo cual el ITS-2 se muestra como la herramienta molecular más útil, específica, sensible y práctica.
Palabras clave: Fascioliasis humana y animal. Vectores Lymnaeidae. *Galba truncatula.* Mendoza. Argentina. Transmisión. Epidemiología. Salud pública.

Summary

Objective: Human fascioliasis has recently entered in the list of the most important parasitic diseases of man. The human endemic areas presenting the highest prevalences and intensities are found in Andean countries as Bolivia and Peru, where the disease is transmitted by the molluscan gastropod of the Lymnaeidae family *Galba truncatula.* The finding and determination of this vector species in Argentina in 2001 were initially based on morphology. More recent studies have demonstrated that morphology does not enable to distinguish between species of the Galba/Fossaria group. The aim of this paper is to confirm the presence, in Argentina, of the best fascioliasis vector known by means of specific DNA marker sequencing.
Material and methods: Molluscs were collected in El Salto, on the Andean zone of Mendoza, Argentina, and fixed with ethanol 70%. Total DNA was extracted, amplified and purified following standard methods. The sequence of the nuclear ribosomal DNA ITS-2 was obtained by means of an authomatic sequencer using specific primers. The BLAST programme was used for the fast comparison with all sequences of lymnaeids available at GenBank. The final comparison was made by alignment using Clustal W version 1.8.
Results: The complete sequence of the rDNA ITS-2 showed a lenghth of 401 base pairs and a GC content of 58,6%. The sequence obtained was identical in length, contents and composition of nucleotides than the H3 haplotype of *Galba truncatula*, previously described in Bolivia.
Conclusions: The finding in Mendoza of the same haplotype of the molluscan vector responsible of the human and animal fascioliasis on the Northern Bolivian Altiplano, the most important human endemic area, is a fact of great interest because of its large potential impact on public health, owing to the aspects of great transmission capacity which are present on the Andean zone of Mendoza. All suggests the need to perform a large future study to establish the geographical distribution of *G. truncatula* H3 in Argentina. The ITS-2 appears to be the most useful, specific, sensible and practical molecular tool for such future studies.
Key words:. Human and animal fascioliasis. lymnaeid vectors. Galba truncatula. Mendoza. Argentina. Transmission. Epidemiology. Public health.

Enf Emerg 2007;9(2):77-82

Introducción

La Fascioliasis es una enfermedad parasitaria causada por trematodos digénidos pertenecientes al género *Fasciola* (Fasciolidae): *F. hepatica* se encuentra distribuida por todos los continentes y *F. gigantica* se restringe a África y Asia[1,2]. Los estadios adultos de estos fasciólidos son parásitos de los conductos hepáticos y vesícula biliar. Ponen huevos que salen al exterior vía biliar e intestinal con las heces. Aquellos huevos que llegan al agua dulce se embrionan y el miracidio eclosiona, para nadar en busca de un molusco de la familia Lymnaeidae (Gastropoda) en el cual penetra activamente para metamorfosear en estadios larvarios de multiplicación asexual. Dentro del molusco se originan una generación de esporocisto y de 1 a 4 generaciones de redias, las cuales dan lugar al siguiente estadio larvario de cercaria que es el que abandona al gasterópodo para, gracias a una cola natatoria, nadar hasta encontrar un soporte inerte, esencialmente vegetación dulceacuícola, sobre el cual adherirse y enquistarse. Así se origina el último estadio larvario de metacercaria enquistada que constituye la forma metacíclica infestante para el mamífero hospedador definitivo, el cual adquiere la enfermedad al ingerirla conjuntamente con los vegetales que la transportan[3]. Siempre se había creído que estos trematodos eran parásitos propios del hígado de animales herbívoros, esencialmente ganado doméstico como ovinos, bovinos y caprinos, y que únicamente infectaban a humanos esporádicamente. Así fue como la revisión realizada por la Organización Mundial de la Salud (OMS) en 1990 solamente citaba un total de alrededor de 2000 casos humanos diagnosticados en los 20 años previos[4].

Sin embargo, en la segunda mitad de la década de los 90, una serie de investigaciones pasan a mostrar que en realidad esta distomatosis es mucho más frecuente en humanos de lo que se pensaba. Es entonces cuando se empiezan a describir zonas de verdadera endemia humana[5,6]. Las encuestas realizadas vinieron a demostrar, además, que esta parasitosis afectaba a todas las edades pero esencialmente a niños, con un pico alrededor de los 9-11 años, y con mayor afección en hembras[7]. Los estudios dirigidos al análisis de los efectos de la parasitación por fasciólidos en los humanos demostraron que es causante de serios cuadros patológicos con extensa problemática clínica[3,8]. Más recientemente se ha visto incluso como, en las zonas de endemia humana, en las que los afectados se encuentran mayoritariamente en fase de cronicidad (fase obstructiva en la que el parásito ya ha alcanzado su microhábitat hepático definitivo y ha empezado a producir huevos que salen al exterior con las heces) o de cronicidad avanzada (fase subsiguiente en la que colelitiasis y bacteriobilia son características y los parásitos viejos agotan su capacidad de producir huevos), los cuadros patológicos y clínicos antedichos pueden llegar a complicarse aún mucho más[9].

A principios de los 90 las estimaciones oscilaban ya entre 2,4 millones y 17 millones de afectados en el mundo[10,11]. Ante toda esta situación, la Sede Central de la OMS incluyó la Fascioliasis dentro de la lista de las grandes enfermedades parasitarias de la humanidad y, consecuentemente, decidió lanzar una iniciativa mundial contra la Fascioliasis humana a finales de los 90, en la cual se incluyeron dos ejes fundamentales:

- Estudios epidemiológicos y de transmisión.
- Tratamientos de afectados en zonas de endemia mediante triclabendazol, una droga de muy alta efectividad. En la actualidad, y como consecuencia de los numerosos estudios epidemiológicos realizados en diferentes países de Latinoamérica, África y Asia, se empieza a considerar que las cifras disponibles muy probablemente subestimen la realidad a nivel mundial[1].

Las áreas de endemia humana de Fascioliasis mostrando mayores prevalencias e intensidades se encuentran en zonas de gran altitud de los Países Andinos. En el Altiplano Norte de Bolivia, las prevalencias llegan a ser elevadísimas, de hasta incluso más del 72% por coprología y del 100% por serología y con intensidades de hasta más de 5000 huevos por g de heces, en determinadas localidades[12,13]. El solapamiento e incluso las asociaciones positivas estadísticamente significativas que la Fascioliasis muestra con otras parasitosis, varias de ellas de reconocida alta patogenicidad tanto protozoosis (por ejemplo *Giardia intestinalis, Cryptosporidium sp.* y *Balantidium coli*) como helmintiasis (por ejemplo *Taenia solium, Ascaris lumbricoides* y *Trichuris trichiura*), están en la base de una muy elevada mortalidad en niños de corta edad en el Altiplano Norte Boliviano[14]. En el Perú se han detectado situaciones epidemiológicas similares[15,16].

Formando parte de la iniciativa de la OMS, en los últimos años se han intensificado las prospecciones en otros países latinoamericanos, esencialmente aquellos que presentan zonas andinas, con el fin de verificar si existen zonas epidemiológicamente similares a las descritas en Bolivia y Perú. Estos estudios incluyen no únicamente encuestas en humanos, sino también prospecciones a nivel de animales domésticos reservorios y sobre los moluscos Lymnaeidae hospedadores intermediarios o vectores que intervienen en la transmisión de la enfermedad. Así es como en el año 2001, Roberto L. Mera y Sierra detecta la presencia de lymnaeidos en el sistema hidrográfico andino de la provincia de Mendoza, Argentina, y adscribe determinadas poblaciones de los moluscos recolectados a la especie *Galba truncatula* en base a estudios morfológicos[17], tras comparación con poblaciones de la misma especie en otros países[18-20]. El lymnaeido *Galba truncatula* es la especie reconocida como transmisor por excelencia de *Fasciola hepatica,* por lo que el hallazgo representaba un importante aporte a los estudios en cuestión.

Sin embargo, en los años subsiguientes, diferentes estudios han venido a demostrar que la diferenciación morfológica y morfométrica de determinadas especies de Lymnaeidae ofrece en realidad una enorme problemática[21]. En varios casos de especies próximas como algunas que también transmiten la Fascioliasis en América del Sur, esta cuestión llega hasta el extremo de ser totalmente indiferenciables y, consecuentemente, a tener que hablar de especies gemelas[22,23].

Estudios morfológicos más amplios sobre variabilidad de los lymnaeidos de la zona argentina de Mendoza permitieron concluir que allí también se daba un fenómeno del mismo tipo[24-26]. Por consiguiente, dada la trascendencia de la cuestión, se impone la verificación de la clasificación sistemática de la especie de lymnaeido de Mendoza. El presente trabajo tiene esta finalidad. Para ello, se ha

procedido a la obtención de la secuencia nucleotídica completa del segundo espaciador transcrito interno ITS-2 del ADN ribosomal nuclear y a su oportuno análisis por comparación con otras secuencias ya conocidas de *Galba truncatula* y otras especies próximas de Lymnaeidae.

Material y métodos

Material de lymnaeidos y descripción del área de estudio

El material de moluscos dulceaquícolas lymnaeidos procedente de la provincia de Mendoza, Argentina, y utilizado para los estudios moleculares fue recolectado en la localidad de El Salto, en cursos de agua y fijados vivos directamente en etanol al 70%. La provincia de Mendoza presenta planicies en el sector oriental ligadas al área montañosa del oeste a través de la cuenca del río Desaguadero, formado por el aporte de los ríos Mendoza, Tunuyan, Diamante y Atuel. Al oeste se encuentran los cordones montañosos de los Andes con una altitud media de 4500 m. El clima cordillerano es frío con precipitaciones nivales durante el invierno, y las llanuras presentan un clima templado con escasas precipitaciones durante el verano (aproximadamente 250 mm anuales).

De acuerdo con los datos del Censo Nacional de Población, Hogares y Viviendas efectuado en 2001 por el Instituto Nacional de Estadísticas y Censos de la Republica Argentina (INDEC), El Salto es una localidad de únicamente 361 habitantes, pero con una importante población flotante debido a su interés turístico. Esta localidad ve ostensiblemente aumentado el número de personas especialmente en fines de semana y días feriados, así como también en periodos vacacionales. Esta localidad forma parte del Departamento de Luján de Cuyo, de una superficie de 4.847 km^2 y una población de 104.470 habitantes, con una densidad poblacional de 16,5 habitantes por km^2. Este Departamento forma parte, a su vez, de la provincia de Mendoza, de 148.827 km^2 y una población de 1.579.651 habitantes, con una densidad poblacional de 10,6 habitantes por km^2. Para la provincia de Mendoza, el mismo INDEC, dentro de su Censo Nacional Agropecuario referente al año 2002, enumera las cifras siguientes sobre cabezas de ganado: 672.434 caprinos, 68.795 ovinos, 404.710 bovinos, 16.360 porcinos y 64.029 equinos.

Técnicas moleculares

La extracción del ADN genómico se realizó según protocolo y técnicas estandarizadas[27]. Para ello se utilizó una parte de tejido de la zona cefalopédea del molusco. Se utilizó más de un individuo por motivos de confirmación nucleotídica en caso de posibles secuencias sucias. Este tejido se disgregó con la ayuda de unas tijeras estériles en un pequeño volumen de una solución tampón de lisis (Tris-HCl 10 mM, pH=8,0; EDTA 100 mM; NaCl 100 mM), para posteriormente añadir: solución tampón de lisis, hasta completar 340 µl, 40 µl de dodecil sulfato de sodio al 10 %, y 20 µl de proteinasa K (500 µg/ml) (Promega, Madison, WI, USA). Se agitó suavemente y se incubó a 55° C durante 2 h, con agitación cada 15 min. La purificación del ADN total se realizó mediante una extracción en tres etapas, por el método de fenol-cloroformo. En la primera se adicionan 400 µl de fenol y se centrifuga durante 5 min a 13000 rpm. A continuación se recupera la fase acuosa que contiene el ADN. En la segunda etapa se utilizan 200 µl de fenol y 200 µl de cloroformo-alcohol isoamílico (49:1), se mezcla suavemente, se vuelve a centrifugar y se recupera nuevamente la fase acuosa. En la tercera etapa se añaden 400 µl de cloroformo-alcohol isoamílico (49:1), se mezcla, se centrifuga y se recupera nuevamente el sobrenadante. La precipitación del ADN se realizó con acetato de amonio 4 M y etanol absoluto y el precipitado resultante se resuspendió en 30 µl de tampón TE (Tris-HCl 10mM, pH=7,6; EDTA 0.1 mM, pH=8,0) y se conservó a -21°C hasta su uso.

La amplificación por PCR del fragmento correspondiente al segundo espaciador transcrito interno del ADN ribosomal (ITS-2) se realizó utilizando oligonucleótidos cebadores diseñados a partir de las regiones conservadas de los genes 5.8S y 28S que flanquean a este espaciador ribosomal[21,28]. La reacción de PCR se realizó en un termociclador MiniCycler™ PT-150 (MJ Research, Watertown, MA, USA) utilizando 4-6 µl de ADN genómico de cada lymnaeido por cada 50 µl de reacción de PCR y la siguiente programación: 94° C durante dos minutos; treinta ciclos repetitivos de 30 segundos a 94° C, 30 segundos a 50° C y 30 segundos a 72° C, seguidos de 2 minutos a 72° C y de una fase final de enfriamiento a 4° C. Se analizó 10 microlitros del producto de amplificación en geles de electroforesis al 1%, previamente teñidos con bromuro de etidio. En cada reacción de amplificación se incluyó un control negativo y un control positivo, así como un marcador de peso molecular en la electroforesis en gel de agarosa con el fin de identificar las bandas del fragmento del ITS-2 amplificado, correspondientes a las muestras analizadas.

La purificación del producto amplificado correspondiente a cada muestra se realizó con el kit UltraClean (MoBio Laboratories, CA, USA), en el cual los ácidos nucleicos son retenidos por una membrana recubierta de sílice en presencia de una alta concentración de sal. Las impurezas son eliminadas mediante lavados con etanol. Finalmente el ADN es recuperado eluyéndolo con agua o tampón Tris 10 mM. La concentración del ADN en las muestras se midió a través de su absorbancia a 260 y 280 nm en un espectrofotómetro Spectronic Genesis 5 (Spectronic, NY, USA).

La secuenciación de las regiones amplificadas por la reacción de PCR fue realizada utilizando el kit de secuenciación ABI Prism™ dRhodamine Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA, USA) en el secuenciador automático ABI Prism 377A (Perkin-Elmer) y con los mismos cebadores que para la PCR. El ADN marcado fue precipitado con acetato de sodio y etanol, y se analizó en un gel de poliacrilamida 5,25% y urea 6M.

Programas de software utilizados

Las secuencias directa e inversa correspondientes a cada muestra fueron alineadas entre si utilizando el programa Clustal W versión 1.8[29]. Los alineamien-

tos fueron realizados incluyendo los lymnaeidos argentinos analizados conjuntamente con las secuencias de otros haplotipos de *Galba truncatula* conocidos. Para el análisis comparado se utilizó las siguientes secuencias del ITS-2 del ADNr presentes en el GenBank: *Galba truncatula* H-1 de España, Portugal y Córcega (Accession No. AJ296271), *G. truncatula* H-2 de España, Portugal y Suiza (Accession No. AJ243017) y *G. truncatula* H-3 de Bolivia[21,28] (= *L. viatrix sensu* Ueno, *et al,* 1975; = *L. cubensis sensu* Ueno, *et al,* 1975; = *Lymnaea sp.* morph I y morph II *sensu* Oviedo, *et al,*, 1995)[18,30] (Accession No. AJ272051). La homología de las secuencias obtenidas se verificó también con el programa BLAST del National Center for Biotechnology information web site (http://www.ncbi.nlm.nih.gov/BLAST).

Resultados

Las secuencias del ITS-2 pertenecientes a las muestras de los lymnaeidos argentinos de El Salto presentaron todas ellas una longitud de 401 pares de bases (pb) y un contenido en GC del 58,6%. La secuencia nucleotídica completa fue siempre la misma y se muestra en la Tabla 1. La comparación con todas las secuencias del mismo marcador ITS-2 de Lymnaeidae disponibles en el GenBank mediante el programa BLAST mostró de inmediato la gran similitud con las secuencias de *Galba truncatula.*

Los lymnaeidos argentinos en cuestión presentan una secuencia del marcador ITS-2 similar en un 97% a la secuencia del ITS-2 perteneciente a *G. truncatula* del haplotipo H1 de Europa, respecto del cual se diferencia por presentar dos mutaciones: la transversión de T por G en la posición 55, y la transición de T por C en la posición 149. Respecto del haplotipo H2 de la misma especie, las diferencias nucleotídicas se restringen aún más, concretamente a una sola mutación: la transversión de T por G en la posición 55 (Tabla 2).

Tabla 1. **Secuencia nucleotídica completa del segundo espaciador transcrito interno ITS-2 del ADN ribosomal nuclear de los lymnaeidos de la población de El Salto, Mendoza, Argentina**

Secuencia Nucleotídica	
GCTAGTCACAAAGCATTCGTGTCCTTGCAGCTCTCGCAAAAACCGAAGCC	50
TTGCTGCGTGAGCTCTCACGCTGCTCGGCGATGGTTGGATACGCCCTGGA	100
CCCTCGCGGCCAAAGCTGTCGTTGCCTGCTCGGCGGCGACGGTGACGGTC	150
CCGTGGTCTTAAGCGCAAGCCGCGCCGTTGTCCGTTCATCTCGTAACGTC	200
TTCGACGCTGCCCTGCTCTTGGCGGCCTGTCCGTTTTCTCTACCGCCAGG	250
CAGGACCCGGCTCGCTTACTTTATTTATTATCGTGGCGTTCTCGGGCCTG	300
CAGTCCATGGCATCGCAGCTCGTGGGTGGAGAACAAGGGGCTCTAAGACG	350
CTACGTGGTCGGCGCCCGTCGTTGAATGAAACATTATTTGTTTCTTTTCTC	401

Tabla 2. **Longitud, composición y diferencias nucleotídicas encontradas en la comparación de las secuencias del segundo espaciador transcrito interno ITS-2 del ADN ribosomal nuclear de los lymnaeidos argentinos y los haplotipos de *Galba truncatula* de Europa y del Altiplano Norte Boliviano**

G. truncatula Haplotipos	Origen	Longitud	% GC	posiciones 55	posiciones 149	GenBank Accession No.
H1	España, Portugal, Córcega	401 pb	59,1	G	C	AJ296271
H2	España, Portugal, Suiza	401 pb	58,8	G	T	AJ243017
H3	Altiplano Norte Boliviano	401 pb	58,6	T	T	AJ272051
H3	El Salto, Mendoza, Argentina	401 pb	58,6	T	T	presente trabajo

Si se compara la secuencia del ITS-2 de estos mismos lymnaeidos argentinos con la del ITS-2 del haplotipo H3 de *G. truncatula* del Altiplano Norte Boliviano se obtuvo una similitud del 100%, no detectándose ni una sola diferencia nucleotídica entre ambas secuencias (Tabla 2).

Discusión

Los Lymnaeidae del denominado grupo Galba-Fossaria ostentan variabilidades intraespecíficas cuyas morfologías y morfometrías se solapan, dificultando cuando no impidiendo por completo la clasificación de los especímenes[21]. Son varias las técnicas de genotipaje y fenotipaje que se han propuesta para la clasificación definitiva de las especies en estos casos. De entre ellas, el ITS-2 ha demostrado ser el mejor marcador molecular, ya no únicamente a nivel de especie, sino incluso para la diferenciación de poblaciones mediante haplotipaje por SNPs (single nucleotide polymorphisms)[31].

Un ejemplo lo encontramos en lo sucedido en el Altiplano Norte Boliviano. Esta zona fue ampliamente prospectada hace ya tiempo desde el punto de vista veterinario, mostrándose que la problemática de fascioliasis animal en esa zona era muy importante y que la enfermedad del ganado estaba transmitida por dos especies de lymnaeidos americanos: *Lymnaea viatrix* y *Lymnaea cubensis*[30]. Sin embargo, los estudios sobre variabilidad intraespecífica demostraron que morfológicamente las poblaciones de ambas especies se solapaban ampliamente[18], además de mostrar una idéntica capacidad de transmitir *Fasciola hepatica* experimentalmente en el laboratoro, fenómeno desconocido en enfermedades parasitarias de transmisión vectorial, ya que cuando hay más de una especie vectora en una misma zona, el parásito siempre está más adaptado a una especie dada y consecuentemente es transmitido más por una especie que por la(s) otra(s). Todo ello vino a poner en duda esas clasificaciones sistemáticas. Los marcadores moleculares vinieron luego a demostrar que en realidad en el Altiplano Norte Boliviano no habían dos especies

diferentes sino una sola que mostraba una amplia variabilidad morfológica intraespecífica y que la especie presente no era ni una ni la otra de las dos americanas citadas inicialmente[30], sino *Galba truncatula* importada desde Europa, lo que representó el primer hallazgo de esta importantísima especie de vector no sólo en América del Sur sino en toda América Latina. De los diferentes marcadores moleculares (18S, ITS-2 e ITS-1) aplicados para la clasificación definitiva de los lymnaeidos altiplánicos en Bolivia, el ITS-2 demostró con creces ser el más útil[28,32,33].

Este marcador ITS-2 cuenta, además de (i) su característica de ser diferente según las diferentes especies (característica de especificidad), con las ventajas de que (ii) el operon del ADN ribosomal se encuentra altamente repetido y es, por tanto, de fácil detección y amplificación (característica de sensibilidad), y (iii) la evolución concertada de los genes y espaciadores del ADN ribosomal implica una tendencia a la uniformización de las secuencias dentro de una misma población, lo que permite la clasificación específica de una población simplemente con la secuenciación de un único individuo (característica de gran simplificación y reducción en tiempo y costes)[31].

Galba truncatula es una especie de origen europeo que se muestra genéticamente muy uniforme. En Europa se conocen únicamente los 2 haplotipos H1 y H2 (según la nomenclatura propuesta con anterioridad[31,34]), que se diferencian por solamente 1 mutación nucleotídica que está constituida por una transición en la posición 149: C en H1 y T en H2[28]. Esta uniformidad genética parece estar relacionada con la capacidad de autofecundación de esta especie de lymnaeido, que le confiere la habilidad de originar poblaciones monomórficas o clónicas[35]. Hasta la fecha se conoce únicamente un haplotipo del ITS-2 adicional a los dos europeos antes mencionados, que se designa con el código H3 y que fue hallado en el Altiplano Norte Boliviano. Este haplotipo H3 se caracteriza por presentar una transversión en la posición 55 (G en H1 y H2; T en H3), además de presentar una transición en la posición 149 que confiere un elemento diferencial adicional respecto del haplotipo europeo H1 (C en H1 y T en H3)[28].

La extracción y secuenciación del ITS-2 del ADN ribosomal del lymnaeido de Mendoza ha proporcionado una secuencia que se muestra idéntica, tanto en longitud (401 pb) como base a base (T en la posición 55 y T también en la posición 149), a la descrita para el mismo marcador en *Galba truncatula* procedente del Altiplano Norte Boliviano (Tabla 2) y adscrita al código AJ272051 en el GenBank[28]. La especie de lymnaeido originalmente detectada en Mendoza, Argentina, en el año 2001[17] es, pues, *Galba truncatula* H3.

Si se tiene en cuenta que el haplotipo H3 de *Galba truncatula* es precisamente el responsable de la zona de endemia humana con mayores prevalencias, intensidades y mortalidad infantil en el mundo, el resultado de la confirmación molecular de la clasificación del lymnaeido de la zona argentina de Mendoza viene a poner un punto serio de atención. En efecto, además de la gran capacidad de transmisión de *Fasciola hepatica* bien reconocida de esta especie de gasterópodo, hay otras cuatro características epidemiológicas muy importantes a considerar:

- *Galba truncatula* es una especie que va íntimamente ligada al ganado, con lo que su capacidad de ser transportada pasivamente por los animales es pronunciada y consiguientemente siempre muestra una gran capacidad de expansión geográfica.
- El área de Mendoza se encuentra en zona andina de altitud, lo que sugiere una aún mayor capacidad de transmisión por la longevidad de los individuos de esta especie de gasterópodo en altitud, mayor que en zonas bajas[28].
- La peculiar ecología de esta especie de molusco la acerca con frecuencia a núcleos poblacionales humanos, cuando no se encuentra totalmente dentro de ellos, incluso en aguas altamente polucionadas por la acción humana[12].
- Las adaptaciones de *Fasciola heptica* para su supervivencia en altitud, conducentes al incremento de la producción larvaria (mayor número de cercarias producidas por un individuo de molusco vector) y al aumento de la duración de la emisión cercariana en cada individuo de gasterópodo infectado pueden implicar una capacidad de transmisión en Mendoza semejante a la detectada en el Altiplano Norte Boliviano[28].

La presencia de un vector tan favorable para la transmisión puede muy probablemente estar relacionada con las elevadas prevalencias de Fascioliasis detectadas en ganado bovino en Mendoza. Así, estudios muy recientes han detectado una prevalencia del 34,0% en animales mendocinos abatidos en uno de los principales frigoríficos de la provincia[36] e incluso una altísima cifra de 67,5% en un frigorífico local que faena exclusivamente ganado del Departamento mendocino de Tupungato[37].

Dado el riesgo en salud pública de la existencia de *Galba truncatula* H3 y de su gran capacidad de transmisión de la Fascioliasis en Mendoza, es evidente que se impone un amplio estudio que permita establecer la distribución geográfica de esta especie ya no solamente en Mendoza, sino también en Argentina. El marcador molecular ITS-2 del ADNr se muestra como la mejor herramienta disponible hasta la fecha para poder efectuar dicho estudio. Los estudios posteriores habrán de dirigirse hacia el análisis de la Fascioliasis en humanos y animales y su paralelismo en distribución geográfica con *Galba truncatula*.

Agradecimientos

Investigaciones de laboratorio y secuenciación financiadas por los Proyectos No. BOS2002-01978 y No. SAF2006-09278 del Ministerio de Educación y Ciencia, Madrid, y por la Red de Investigación de Centros de Enfermedades Tropicales – RICET (Proyectos No. C03/04 y No. ISCIII2005-PI050574 del Programa de Redes Temáticas de Investigación Cooperativa) del Fondo de Investigación Sanitaria (FIS), Ministerio de Sanidad y Consumo, Madrid. Trabajos realizados en Argentina financiados por la Universidad Nacional de Cuyo. Soporte técnico para la secuenciación automática de los moluscos proporcionado por el Servicio Central de Secuenciación para la Investigación Experimental (SCSIE) de la Universidad de Valencia (Dra. A. Martínez).

Bibliografía

1. Mas-Coma S. Human fascioliasis. En: Cotruvo JA, Dufour A, Rees G, Bartram J, Carr R, Cliver DO, Craun GF, Fayer R, Gannon VPJ (ed). World Health Organization (WHO), *Waterborne Zoonoses: Identification, Causes and Control*. London, UK: IWA Publishing, 2004:305-22.
2. Mas-Coma S, Bargues MD, Valero MA. Fascioliasis and other plant-borne trematode zoonoses. *Int J Parasitol* 2005; 35:1255-78.
3. Mas-Coma S, Bargues MD. Human liver flukes: a review. *Res Rev Parasitol* 1997;57(3-4):145-218.
4. Chen MG, Mott KE. Progress in assessment of morbidity due to Fasciola hepatica infection: a review of recent literature. *Trop Dis Bull* 1990;87(4):R1-R38.
5. Mas-Coma S, Esteban JG, Bargues MD. Epidemiology of human fascioliasis: a review and proposed new classification. *Bull WHO* 1999;77(4):340-6.
6. Mas-Coma S, Bargues MD, Esteban JG. Human Fasciolosis. En: Dalton JP (ed). *Fasciolosis.* Wallingford, Oxon, UK: CAB International Publishing 1999:411-34.
7. Mas-Coma S. Epidemiology of fascioliasis in human endemic areas. *J Helminthol* 2005;79(3):207-16.
8. Mas-Coma S, Bargues MD, Marty AM, Neafie AM. Hepatic Trematodiases. En: Meyers WM, Neafie RC, Marty AM, Wear DJ (ed). *Pathology of Infectious Diseases, Vol. 1 Helminthiases.* Washington DC: Armed Forces Institute of Pathology and American Registry of Pathology 2000:69-92.
9. Valero MA, Santana M, Morales M, Hernández JL, Mas-Coma S. Risk of gallstone disease in advanced chronic phase of fascioliasis: an experimental study in a rat model. *J Inf Dis* 2003;188:787-93.
10. Hopkins DR. Homing in on helminths. *Amer J Trop Med Hyg* 1992;46:626-34.
11. Rim, HJ, Farag HF, Sornmani S, Cross JH. Food-borne trematodes: ignored or emerging? *Parasitol Today* 1994;10:207-9.
12. Mas-Coma S, Angles R, Esteban JG, Bargues MD, Buchon P, Franken M, Strauss W. The Northern Bolivian Altiplano: a region highly endemic for human fascioliasis. *Trop Med Int Health* 1999;4(6):454-67.
13. Esteban JG, Flores A, Angles R, Mas-Coma S. High endemicity of human fascioliasis between Lake Titicaca and La Paz valley, Bolivia. *Trans Roy Soc Trop Med Hyg* 1999;93(2):151-6.
14. Mas-Coma S, Angles R, Strauss W, Esteban JG, Oviedo JA, Buchon P. Human fasciolasis in Bolivia: a general analysis and a critical review of existing data. *Res Rev Parasitol* 1995;55(2):73-93.
15. Esteban JG, González C, Bargues MD, Angles R, Sánchez C, Naquira C, Mas-Coma S. High fascioliasis infection in children linked to a man-made irrigation zone in Peru. *Trop Med Int Health* 2002;7(4):339-48.
16. Marcos Raymundo LA, Maco Flores V, Terashima A, Samalvides F, Miranda E, Tantalean M, Espinoza JR, Gotuzzo E. Hiperendemicidad de Fasciolosis humana en el Valle del Mantaro, Perú: factores de riesgo de la infeccion por Fasciola hepatica. *Rev Gastroenterol Perú* 2004;24:158-64.
17. Mera y Sierra RL. *Distribución de Lymnaeidos en el sistema hidrográfico andino de la provincia de Mendoza, Argentina.* Tesis de Maestría, Master Internacional en Enfermedades Parasitarias Tropicales (XIV Edición). Tutor: M.D. Bargues. Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, 22 Junio 2001:1-46.
18. Oviedo JA, Bargues MD, Mas-Coma S. Lymnaeid snails in the human fascioliasis high endemic zone of the Northern Bolivian Altiplano. *Res Rev Parasitol* 1995;55(1):35-43.
19. Oviedo JA, Bargues MD, Mas-Coma S. The intermediate snail host of Fasciola hepatica on the Mediterranean island of Corsica. *Res Rev Parasitol* 1996;56(4):217-20.
20. Samadi S, Roumegoux A, Bargues MD, Mas-Coma S, Yong M, Pointier JP. Morphological studies of lymnaeid snails from the human fascioliasis endemic zone of Bolivia. *J Moll Stud* 2000;66:31-44.
21. Bargues MD, Vigo M, Horak P, Dvorak J, Patzner RA, Pointier JP, Jackiewicz M, Meier-Brook C, Mas-Coma S. European Lymnaeidae (Mollusca: Gastropoda), intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS-2 sequences. *Inf Gen Evol* 2001;1(2):85-107.
22. Durand P, Pointier JP, Escoubeyrou K, Arenas JA, Yong M, Amarista M, Bargues MD, Mas-Coma S, Renaud F. Occurrence of a sibling species complex within Neotropical lymnaeids, snail intermediate hosts of fasciolasis. *Acta Trop* 2002;83(3):233-40.
23. Pointier JP, Cazzaniga NJ, González-Salas C, Gutiérrez A, Arenas JA, Bargues MD, Mas-Coma S. Anatomical studies of sibling species within Neotropical lymnaeids snail intermediate hosts of fascioliasis. *Mem Inst Oswaldo Cruz* 2006;101(4):431-35.
24. Mera y Sierra RL, Artigas P, Abraham C, Hynes V, González M, Jiménez M, Khoubbane M, Bargues MD, Mas-Coma S. Conchiologic morphology of lymnaeid molluscs in the endemic zones of fasciolosis in Mendoza province, Argentina. *Acta Parasitol Port* 2005;12(1-2):313-4.
25. Artigas P, Mera y Sierra RL, Correa AP, Morales JR, Zuriaga MA, Jiménez M, Khoubbane M, Bargues MD, Mas-Coma S. Comparison of the conchiologic morphology of lymnaeid molluscs from eastern and western Andean slopes of the endemic zones of fasciolosis in Argentina and Chile. *Acta Parasitol Port* 2005;12(1-2):243-4.
26. Mera y Sierra RL, Artigas P, Mas-Coma S. Lymnaeid vectors of fascioliasis in endemic areas of Mendoza, Argentina. En: XI International Congress of Parasitology - ICOPA XI, Glasgow, UK, 6-11 August 2006: CD-ROM.
27. Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning. A Laboratory Manual.* 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA, Vols. I, II & III, 1989:1-1647.
28. Mas-Coma S, Funatsu IR, Bargues MD. Fasciola hepatica and lymnaeid snails occurring at very high altitude in South America. *Parasitology* 2001;123:S115-S127.
29. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity and progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucl Acids Res* 1994;22:4673-80.
30. Ueno H, Arandia R, Morales G, Medina G. Fascioliasis of livestock and snail host for Fasciola in the Altiplano region of Bolivia. *Nat Inst Animal Health Quart* 1975;15:61-7.
31. Bargues MD, Mas-Coma S. Reviewing lymnaeid vectors of fascioliasis by ribosomal DNA sequence analyses. *J Helminthol* 2005;79(3):257-67.
32. Bargues MD, Mas-Coma S. Phylogenetic analysis of lymnaeid snails based on 18S rDNA sequences. *Mol Biol Evol* 1997;14(5):569-77.
33. Bargues MD, Mangold AJ, Muñoz-Antoli C, Pointier JP, Mas-Coma S. SSU rDNA characterization of lymnaeid snails transmitting human fascioliasis in South and Central America. *J Parasitol* 1997;83(6):1086-92.
34. Bargues MD, Artigas P, Jackiewicz M, Pointier JP, Mas-Coma S. Ribosomal DNA ITS-1 sequence analysis of European stagnicoline Lymnaeidae (Gastropoda). En: Beiträge zur Süßwasser-Malakologie - Festschrift für Claus Meier-Brook und Hans D. Boeters (Glöer P, Falkner G edit.), Heldia (Münchner Malakologische Mitteilungen), München 2006;6(1/2):29-40.
35. Meunier C, Tirard C, Hurtrez-Bousses S, Durand P, Bargues MD, Mas-Coma S, Pointier JP, Jourdane J, Renaud F. Lack of molluscan host diversity and the transmission of an emerging parasitic disease in Bolivia. *Mol Ecol* 2001;10:1333-40.
36. Gonzalez MS, Di Nucci D, Sidoti L, Mera y Sierra RL. Decomiso de hígados de bovinos provenientes de Mendoza en frigorífico debido a Fasciola hepatica. En: XII Jornadas Argentinas de Microbiología, Mendoza, Argentina, Junio 2006:102.
37. Mera y Sierra R, Scibilia C, Pabst A, Irrazabal G, Senar M. Abattoir condemnation of bovine livers due to Fasciola hepatica in Tupungato, Mendoza. En: XXIII Reunión Científica Anual, Sociedad Biológica de Cuyo. *Biocell* 2005;29(3):371.

Veterinary Parasitology 166 (2009) 73–79

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Fascioliasis transmission by *Lymnaea neotropica* confirmed by nuclear rDNA and mtDNA sequencing in Argentina

Roberto Mera y Sierra [a,b], Patricio Artigas [c], Pablo Cuervo [a,b], Erika Deis [a,b], Laura Sidoti [b], Santiago Mas-Coma [c], Maria Dolores Bargues [c,*]

[a] *Área de Infectología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Av. Libertador 80, Mendoza, Argentina*
[b] *Cátedra de Parasitología y Enfermedades Parasitarias, Facultad de Ciencias Veterinarias, Universidad J.A. Maza, Av. de Acceso Este - Lateral Sur 2245, S. José, Guaymallén, Mendoza, Argentina*
[c] *Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Av. Vicente Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain*

ARTICLEINFO

Article history:
Received 10 April 2009
Received in revised form 20 July 2009
Accepted 4 August 2009

Keywords:
Fasciola hepatica
Lymnaea neotropica
Animal infection
Transmission
Molecular characterization
Argentina

ABSTRACT

Fascioliasis is widespread in livestock in Argentina. Among activities included in a long-term initiative to ascertain which are the fascioliasis areas of most concern, studies were performed in a recreational farm, including liver fluke infection in different domestic animal species, classification of the lymnaeid vector and verification of natural transmission of fascioliasis by identification of the intramolluscan trematode larval stages found in naturally infected snails. The high prevalences in the domestic animals appeared related to only one lymnaeid species present. Lymnaeid and trematode classification was verified by means of nuclear ribosomal DNA and mitochondrial DNA marker sequencing. Complete sequences of 18S rRNA gene and rDNA ITS-2 and ITS-1, and a fragment of the mtDNA *cox*1 gene demonstrate that the Argentinian lymnaeid belongs to the species *Lymnaea neotropica*. Redial larval stages found in a *L. neotropica* specimen were ascribed to *Fasciola hepatica* after analysis of the complete ITS-1 sequence. The finding of *L. neotropica* is the first of this lymnaeid species not only in Argentina but also in Southern Cone countries. The total absence of nucleotide differences between the sequences of specimens from Argentina and the specimens from the Peruvian type locality at the levels of rDNA 18S, ITS-2 and ITS-1, and the only one mutation at the mtDNA *cox*1 gene suggest a very recent spread. The ecological characteristics of this lymnaeid, living in small, superficial water collections frequented by livestock, suggest that it may be carried from one place to another by remaining in dried mud stuck to the feet of transported animals. The presence of *L. neotropica* adds pronounced complexity to the transmission and epidemiology of fascioliasis in Argentina, due to the great difficulties in distinguishing, by traditional malacological methods, between the three similar lymnaeid species of the controversial *Galba/Fossaria* group present in this country: *L. viatrix*, *Galba truncatula* and *L. neotropica*. It also poses a problem with regard to the use, for lymnaeid vector species discrimination, of several molecular techniques which do not show sufficient accuracy, as those relying on the 18S rRNA gene or parts of it, because both *L. neotropica* and *L. viatrix* present identical 18S sequence.

1. Introduction

Fascioliasis in the New World is only caused by *Fasciola hepatica* (Mas-Coma et al., 2009). This disease affects livestock throughout all countries of South America,

* Corresponding author. Tel.: +34 96 3544906; fax: +34 96 3544769.
E-mail address: M.D.Bargues@uv.es (M.D. Bargues).

0304-4017/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.vetpar.2009.08.001

causing great economic losses in the animal husbandry industry, similarly as everywhere (Torgerson and Claxton, 1999). Moreover, human infection has been described in most of the South American countries (Mas-Coma et al., 1999a, 2005), with important human endemic areas in Andean areas of countries such as Chile, Bolivia or Peru (Apt et al., 1993; Mas-Coma et al., 1999b; Esteban et al., 2002). As in all other vector-borne diseases, in fascioliasis the transmitting snails of the family Lymnaeidae are crucial with regard to the epidemiological situations and transmission patterns of the disease. Epidemiological and transmission heterogeneities in the different endemic areas are related to the lymnaeid vector species involved (Mas-Coma, 2005; Mas-Coma et al., 2009). The correct classification of the lymnaeid snail species acting as vector is therefore of great importance, in order to both determine risk zones and to achieve the appropriate control measures to reduce transmission, according to the different ecological characteristics of each lymnaeid species.

Unfortunately, lymnaeid species are freshwater snails which show a marked intraspecific variability in their shell characteristics and a surprising uniformity at the level of their anatomy. These features explain the great problems to distinguish between species within given controversial groups such as *Galba*/*Fossaria*, *Radix* and stagnicolines (Bargues et al., 2001, 2003, 2006a). For such groups in which not even expert malacologists are sometimes able to correctly classify specimens, sequences of molecular markers among the nuclear ribosomal DNA and the mitochondrial DNA appear to be the most useful tools for specimen classification purposes (Bargues and Mas-Coma, 2005).

In Argentina, production of mostly cattle, but also sheep and goats, is a main national economic activity, with wide areas mainly or totally dedicated to livestock management. Therefore, animal fascioliasis has always been a target of major concern in this country, due to its high pathogenicity, losses it causes, and economic efforts which shall be made for its control (Lombardero et al., 1979a; Pizzi et al., 1982; Olaechea, 1994). Moreover, human cases have been described in several provinces (see review in Chen and Mott, 1990) and the human infection situations in countries such as Chile and Bolivia are causes for concern in Argentina because of the close neighbourhood. Therefore, studies are being made since several years to assess the present situation of the disease throughout Argentina, taking into account the great climatic and physiographic heterogeneity of the different regions of this country.

Special efforts are being made in Mendoza province where prevalences in cattle are very high (Mera y Siera et al., 2005). The aim of the present study is to describe the results obtained in a recreational farm, including liver fluke infection in different domestic animal species, classification of the vector and verification of natural transmission of fascioliasis by identification of the intramolluscan trematode larval stages found in a naturally infected snail. The classification of the lymnaeid species and the trematode larval stages was confirmed by molecular characterization. Molecular markers were selected according to their usefulness for molluscs in general or lymnaeids in particular: small subunit or 18S rRNA gene (Bargues and Mas-Coma, 1997) and the second and first internal transcribed spacers ITS-2 and ITS-1 (Remigio and Blair, 1997b; Mas-Coma et al., 2001; Bargues et al., 2001, 2003, 2006a, 2007a) within nuclear ribosomal DNA (rDNA), and the cytochrome c oxidase subunit I *cox*1 (Remigio and Hebert, 2003; Bargues et al., 2007a) within mitochondrial DNA (mtDNA).

2. Materials and methods

2.1. Host studies

Study area: The recreational farm studied is situated in the locality of Perdriel (District Perdriel, Department of Lujan de Cuyo), at an altitude of 902 m, on Mendoza province, central-west Argentina. Climate data from this area was obtained using DIVA-GIS 5.2 software (WorldClim 1.4: climatic layers resolution 2.5 min) (Hijmans et al., 2005).

Animal infection: Several domestic animals of the farm were tested for *F. hepatica* infection by means of coprological examination. Faecal samples were obtained from cattle, goats, horses, donkeys and llama, and presence of liver fluke eggs was assessed by the Rapid Sedimentation Technique (Lumbreras et al., 1962).

Lymnaeid vectors: Lymnaeid snails were collected in the same area where definitive hosts showed liver fluke infection. Sampling of snails was carried out manually in December 2007, June 2008, July 2008 and August 2008, in water collections of an artificial pond and the overflow originated from an artificial irrigation channel. Snail population dynamics was assessed by 15-min manual collections by the same person within a reduced area of 10 m^2. Snails collected were transported alive to the laboratory for appropriate studies. The morphometric study of the intraspecific variability of the snail shell included shell length (SL), shell width (SW), last spire length (LSL), aperture length (AL), and aperture width (AW), as well as the ratios SL/SW, SL/AL and SL/LSL. Measurements were made under a calibrated stereomicroscope following traditional malacological methods (Oviedo et al., 1995; Samadi et al., 2000; Bargues et al., 2007a). Snail specimens for molecular analyses, as well as trematode larval stages found in one lymnaeid specimen, were fixed in 70% ethanol for DNA extraction procedures.

2.2. Molecular techniques

DNA extraction: The same procedure was performed for both lymnaeids and fluke larvae. The feet from 10 lymnaeid specimens and trematode rediae found were suspended in 400 μl of lysis buffer (10 mM Tris–HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate, SDS) containing 500 μg/ml proteinase K (Promega, Madison, WI, USA) and digested for 2 h at 55 °C with alternate shaking each 15 min. The procedure steps were performed according to methods outlined previously (Bargues and Mas-Coma, 1997; Bargues et al., 2001, 2007a). Total DNA was isolated according to the phenol–chloroform extraction and ethanol precipitation method

(Sambrook et al., 1989). The pellet was dried and resuspended in 30 ml sterile TE buffer (pH 8.0). This suspension was stored at —20 8 C until use.

DNA sequence amplification: DNA sequences were amplified by PCR using 4–6 ml of genomic DNA for each 50 ml PCR reaction, according to methods outlined previously (Bargues and Mas-Coma, 1997; Bargues et al., 2001, 2007a; Mas-Coma et al., 2001). A set of 8 conserved oligonucleotide primers were used for the amplification of five superimposed fragments of the 18S ribosomal RNA gene using specific primers and a standard protocol (Bargues et al., 1997) to amplify specific 18S rDNA regions. The rDNA spacers ITS-2 and ITS-1 were amplified using primers designed in conserved positions of 5.8S and 28S rRNA genes and 18S and 5.8S rRNA genes of several eukaryote Metazoa species, respectively (Bargues et al., 2001, 2006a, 2007a). A mitochondrial DNA *cox*1 gene fragment was amplified using universal primers (Folmer et al., 1994). The ITS-1 of the trematode rediae was amplified by PCR according to methods outlined previously (Mas-Coma et al., 2001). Amplifications were generated in a Mastercycle ep*gradient* (Eppendorf, Hamburg, Germany), by 30 cycles of 30 s at 94 8C, 30 s at 50 8C and 1 min at 72 8C, preceded by 30 s at 94 8C and followed by 7 min at 72 8C for ITS-2 and ITS-1, and by 40 cycles of 30 s at 90 8C, 1 min at 48 8C and 1 min at 72 8C, preceded by 2.5 min at 94 8C and followed by 10 min at 72 8C for *cox*1 Ten microlitre of each PCR product was checked by staining with ethidium bromide on 1% Nusieve ¹ GTG agarose (FMC) gel electrophoresis, using the Molecular Weight Marker VI (Boehringer Mannheim) at 0.1 mg DNA/ml as control.

Purification and quantification of PCR products: Primers and nucleotides were removed from PCR products by purification on Wizard™ PCR Preps DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's protocol and resuspended in 50 ml of 10 mM TE buffer (pH 7.6). The final DNA concentration was determined by measuring the absorbance at 260 and 280 nm.

DNA sequencing: The sequencing of the complete 18S rRNA gene, the complete rDNA ITS-2, and the fragment of the mtDNA *cox*1 gene was performed on both strands by the dideoxy chain-termination method (Sanger et al., 1977). It was carried out with the Taq dye-terminator chemistry kit for ABI 373A and ABI 3700 capillary system (PerkinElmer, Foster City, CA, USA), using PCR primers.

DNA haplotype nomenclature: The codes for the sequences obtained follow the standard nomenclature proposed for lymnaeid snails and fasciolids by Bargues and Mas-Coma (2005), Bargues et al. (2006a) and Mas-Coma et al. (2009).

Software programs used for sequence alignment: Sequences were aligned using CLUSTAL-W version 1.8 and MEGA 4.0 (Tamura et al., 2007), and assembly was made with the Staden Package (Staden et al., 2001). Subsequently, minor corrections were manually introduced for a better fit of nucleotide correspondences in microsatellite sequence regions. Homologies were performed using the BLASTN programme from the National Center for Biotechnology information web site (http://www.ncbi.nlm.nih.gov/BLAST). Genetic distances were measured using parameters provided by PAUP v.4.0b10 (Swofford, 2001).

Sequence comparisons: According to the shell morphology of the lymnaeids collected and to the results obtained with BLASTN, lymnaeid sequence comparisons were restricted to sequence data from species of the *Galba*/*Fossaria* group, such as *Lymnaea cubensis*, *L. viatrix* and *Lymnaea neotropica* from their respectives type localities in Central and South America, and to *G. truncatula* from Europe and South America. With regards to lymnaeids, the following sequences from GenBank-EMBL have been used for comparison analyses:

- 18S rRNA gene: *L. cubensis* (GenBank Accession No. Z83831) (Bargues et al., 1997, 2007a); *L. viatrix* and *L. neotropica* (both species with the same sequence AM412222), all three from respective type localities (Bargues et al., 2007a); *G. truncatula* (Z73985) (Bargues and Mas-Coma, 1997).
- rDNA ITS-2: *L. cubensis* H1 (AM412223), *L. viatrix* (AM412224) and *L. neotropica* (AM412225), all three from respective type localities (Bargues et al., 2007a); *G. truncatula* H1 (AJ296271), *G. truncatula* H2 (AJ243017) and *G. truncatula* H3 (=*L. viatrix sensu* Ueno et al., 1975; =*L. cubensis sensu* Ueno et al., 1975) (AJ272051) (Bargues et al., 2001; Mas-Coma et al., 2001; Bargues et al., 2007b).
- rDNA ITS-1: *L. cubensis* HA (AM412226), *L. viatrix* (AM412227), *L. neotropica* (AM412228), all three from respective type localities (Bargues et al., 2007a); *G. truncatula* HA (AJ243018), *G. truncatula* HB (AJ296270) and *G. truncatula* HC (=*L. viatrix sensu* Ueno et al., 1975; =*L. cubensis sensu* Ueno et al., 1975) (AJ272052) (Bargues et al., 2001, 2006a,b; Mas-Coma et al., 2001).
- mtDNA *cox*1 gene: *L. cubensis cox*1-a (AM494009), *L. viatrix* (AM494010), *L. neotropica* (AM494008), all three from respective type localities, and *G. truncatula* (AM494011) (Bargues et al., 2007a); *G. truncatula* (EU818799) (Albrecht et al., 2008).

The rDNA ITS-1 sequence of the trematode larval stages was compared with the following sequences from GenBank-EMBL:

- rDNA ITS-1: *F. hepatica* from Spain, France, Poland, Ireland, Iran, Japan, Korea, Vietnam, Australia, Egypt, Bolivia, Peru, Uruguay, Argentina, Chile, Venezuela, Ecuador and Mexico (AB207139, AB207140, AB207141, AB207145, AB211236, AB385611, AJ243016, EF612468, EF612469) (Mas-Coma et al., 2001, 2009).

3. Results

3.1. Mammal and snail hosts

Coprological studies showed that all mammal species of the farm were affected by *F. hepatica* infection: 3 infected among 4 cattle analyzed (75%), 3 among 4 goats (75%), 3 among 7 horses (42.8%), 4 among 4 donkeys (100%), and 1 out of 1 llama (100%).

A total of 5 lymnaeid specimens were collected in December 2007, and 50 and 12 specimens, respectively in

June and August 2008. In July 2008 only 1 living snail was found in the pond, when the artificial irrigation channel was dried for reparation. A month later, with overflow present, snails were there again and the population seemed to have quickly recovered. Except in cases of such artificial modifications, the fluctuations of the lymnaeid snail number appear to show a parallelism with the oscillation of temperature and rainfall, both with maximums in the November–March period. The main climatic characteristics of the endemic locality in question appear to be adequate for both lymnaeid vector population dynamics and *F. hepatica* intramolluscan larval development: annual mean temperature (15.0 8C), maximum temperature in the warmest month (30.9 8C), minimum temperature in the coldest month (—0.1 8C), mean temperature of wettest and driest quarters (21.0 and 7.8 8C), precipitation in wettest and driest quarters (103 and 23 mm), and precipitation in warmest and coldest quarters (98 and 23 mm).

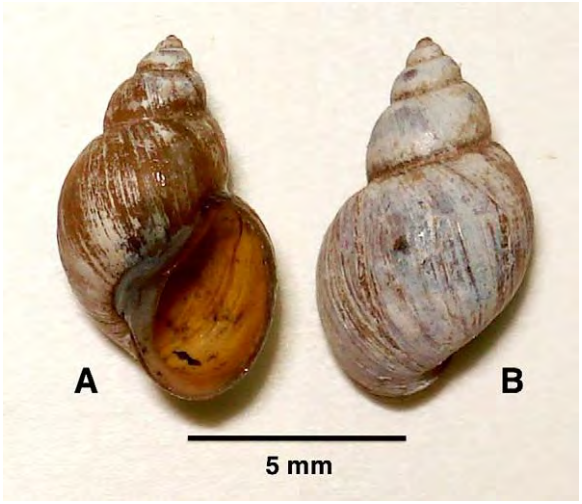


Fig. 1. Shell of *Lymnaea neotropica* from Argentina: (A) ventral view and (B) dorsal view.

3.2. Lymnaeid shell measurements

Specimens of the lymnaeid population sampled in the farm present morphological and morphometric characteristics which indicate that there is only one lymnaeid species present. The form of the shell is illustrated in Fig. 1 and its morphometric variability in Table 1. These characteristics fully agree with those of the species *L. neotropica* originally described from Peru. The morphometric shell comparison between *L. neotropica* from Argentina and *L. neotropica* from the type locality in Lima shows no significant differences in any of the parameters studied. The only small difference observed concerns shell length, which is indeed related to the age of the collected lymnaeids: specimens from the Peruvian type locality were described as presenting 5.5 deeply sutured whorls, whereas specimens from Perdriel are smaller, with 4 whorls in 28 of the specimens collected (84.8%) and 5 whorls in the remaining 5 specimens (15.1%) (Table 1).

3.3. Molecular characterization of lymnaeids

18S rRNA gene: Its sequence in the 10 lymnaeids analyzed is 1860 bp long, shows a GC content of 51.82%, and its base frequencies are: A = 0.236; G = 0.236; C = 0.282; and T = 0.245. This sequence is identical to that of the same gene in the species *L. neotropica* and *L. viatrix*, already deposited in the GenBank under the Accession No. AM412222.

rDNA ITS-2: All specimens from Perdriel furnished the same sequence of 417 bp long and 56.83% of GC content. When compared to the ITS-2 of close lymnaeid species of the *Galba/Fossaria* group present in South America (*L. cubensis*, *L. viatrix*, *L. neotropica* and *G. truncatula*), no one nucleotide difference could be found with *L. neotropica* ITS-2 haplotype H1 (AM412225).

rDNA ITS-1: Lymnaeid specimens from Perdriel furnished one identical sequence of 533 bp long and 56.66% of GC content. When compared to the ITS-1 of close lymnaeid species of the *Galba/Fossaria* group present in South America, no one nucleotide difference could be found with *L. neotropica* ITS-1 haplotype HA (AM412228).

*mtDNA cox*1: Lymnaeids studied from Perdriel showed only one 672-bp-long sequence which has been deposited in the GenBank (FRN356741) with the haplotype code *L. neotropica cox*1-b (this code is provisional because it only concerns a fragment and not the complete gene sequence). It differs by only 1 transition in position 468 from the *L. neotropica cox*1-a from the type locality of Rio Rimac, Lima, Peru (AM494008). This mutation (C/T in Ha/Hb, respectively) gives rise to no difference in the amino acid

Table 1
Lymnaeid shell measurement comparison between *Lymnaea neotropica* from Argentina and *L. neotropica* from the type locality in Peru. Range include minimum and maximum extremes; mean and standard deviation SD in parentheses. Measurements in mm.

Shell parameters	*L. neotropica* Perdriel, Mendoza, Argentina (*n* = 33)	*L. neotropica* Rio Rimac, Lima-Callao type locality, Peru (*n* = 33)
Shell length (SL)	5.24–7.90 (6.28 ▼0.67)	5.89–8.74 (7.13 ▼1.20)
Shell width (SW)	2.95–4.76 (3.76 ▼0.44)	3.29–4.56 (3.95 ▼0.54)
Last spire length (LSL)	4.19–6.19 (5.02 ▼0.54)	4.47–6.78 (5.58 ▼0.91)
Aperture length (AL)	2.29–4.38 (3.19 ▼0.50)	2.90–4.53 (3.75 ▼0.64)
Aperture width (AW)	1.52–3.14 (2.16 ▼0.36)	1.97–2.99 (2.46 ▼0.44)
SL/SW ratio	1.50–1.86 (1.67 ▼0.08)	1.69–1.92 1.80 ▼0.07
SL/AL ratio	1.44–2.42 (1.99 ▼0.20)	1.76–2.03 (1.91 ▼0.09)
SL/LSL ratio	1.19–1.49 (1.25 ▼0.05)	1.21–1.38 (1.28 ▼0.05)

Table 2
Pairwise distances between mtDNA *cox*1 sequences of the lymnaeid species analyzed according to PAUP. Below diagonal = total character differences; above diagonal = mean character differences (adjusted for missing data).

		1	2	3	4	5	6
1 *L.neotropica cox*1-b	Argentina	–	0.00149	0.02232	0.04464	0.10119	0.10833
2 *L.neotropica cox*1-a	Peru	1	–	0.02083	0.04315	0.09970	0.10667
3 *L.cubensis cox*1-a	Cuba	15	14	–	0.05655	0.11161	0.12000
4 *L.viatrix cox*1-a	Argentina	30	29	38	–	0.10119	0.10500
5 *G.truncatula cox*1-a	Spain	68	67	75	68	–	0.00333
6 *G.truncatula*	Germany	65	64	72	63	2	–

sequence between both haplotypes. Genetic pairwise distances at the level of *cox*1 between these *L. neotropica* haplotypes and other lymnaeids of the *Galba/Fossaria* group are noted in Table 2.

3.4. Molecular characterization of trematode larval stages

rDNA ITS-1: Its complete sequence in the trematode rediae found in a *L. neotropica* specimen from Perdriel is 432 bp long and with a 51.85% GC content. It showed no one nucleotide difference with that spacer in *F. hepatica* from the Northern Bolivian Altiplano and Spain (AJ243016), and thus corresponds to the haplotype code Fh ITS-1-HA.

4. Discussion

Studies performed demonstrate that the high prevalences of infection by *F. hepatica* in the animals inhabiting the recreative farm of Perdriel are related to the presence of a snail population of only one lymnaeid species with vectorial capacity. Climatic conditions appear to be appropriate for fascioliasis transmission, with temperatures surpassing the *F. hepatica* minimum threshold of 10 8C (Mas-Coma and Bargues, 1997; Malone et al., 1998) throughout most of the months. However, temperatures and precipitation rates in the May–August period are so low that fascioliasis transmission cannot occur. Hence, in this area the disease shall follow a seasonality, similarly as in the Northern Hemisphere (Mas-Coma and Bargues, 1997).

Sequences of rDNA 18S, ITS-2 and ITS-1 and mtDNA *cox*1 prove that the lymnaeids present in the farm belong to the combined haplotype 1A of the species *L. neotropica* (Bargues et al., 2007a). This is the first finding of this lymnaeid vector species in Argentina. The morphological characteristics of the shell of the Argentinian specimens (Table 1) agree with those of this species in the type locality of Peru (Bargues et al., 2007a). Up to the present, only six or seven lymnaeid species were considered valid among the numerous species described in Argentina: *L. columella*, *L. viatrix*, *L. diaphana*, *L. peculiaris*, *L. pictonica* and *L.plicata* (Hubendick, 1951; Paraense, 1976, 1982a,b, 1983, 1984; Castellanos and Landoni, 1981). Maleck (1985), in the most recent general review on South American lymnaeids, listed the same species mentioned by Hubendick (1951), but accepting *L. patagonica* as valid and not including *L. peculiaris*.

The report in Argentina of an European stagnicoline species as *L. palustris* by N.J. Evans of the British Natural History Museum (in Lombardero et al., 1979b) is a little bit surprising, as it would indeed be the only stagnicoline known in the Neotropical Region. However, this report should be confirmed, as it is well known that lymnaeids are able to be transported from one continent to another (Mas-Coma et al., 2005). Similarly, the quotation of the South American species *L. cousini* by Olaechea (1994) should also be confirmed. Anyway, there may be a misunderstanding on this species because, contrary to what is noted in Olaechea (1994), no report of the presence of *L. cousini* in Argentina is included in the review of Castellanos and Landoni (1981).

More recently, the finding of *G. truncatula* in the province of Mendoza (Mera y Siera, 2001), has represented both a new lymnaeid species for the Argentinian fauna and an additional concern from the point of view of the disease. Sequencing of the rDNA ITS-2 and ITS-1 proved that Argentinian specimens belong to *G. truncatula* combined haplotype Gt CH-3C, the same as the one responsible for the fascioliasis transmission in the human hyperendemic area with the highest human prevalences and intensities known, the Northern Bolivian Altiplano (Bargues et al., 2006b, 2007b). No additional lymnaeid species was added in the most recent review of freshwater molluscs in Argentina (Rumi et al., 2008).

The discovery of *L. neotropica* in Argentina represents moreover the first finding of this vector species in countries of the Southern Cone. Up to the present, this species was only known from its type locality at the Rimac river near the cities of Lima and Callao in Peru (Bargues et al., 2007a). The total absence of nucleotide differences between the sequences of specimens from Argentina and the specimens from the Peruvian type locality at the levels of rDNA 18S, ITS-2 and ITS-1, and the only one mutation at the mtDNA *cox*1 gene suggest that such a great geographical separation between the two localities in Peru and Argentina is the consequence of a very recent spread of *L. neotropica*. The ecological characteristics of this lymnaeid, living in small, superficial water collections frequented by livestock, suggest that it may be carried from one place to another by remaining in dried mud stuck to the feet of transported animals, similarly as *G. truncatula* (Mas-Coma et al., 2009). This passive spreading capacity suggests, moreover, that this lymnaeid species may be more widespread, not only in Argentina but also in other South American countries.

The ITS-1 sequence proves that the trematode rediae found in one *L. neotropica* specimen was *F. hepatica*. The sequence from the Argentinian farm does not show any nucleotide difference with the ITS-1 sequence of the liver

fluke, which appears to be identical throughout the world and hence an excellent species marker (Mas-Coma et al., 2009).

The presence of *L. neotropica* in Argentina adds pronounced complexity to the transmission and epidemiology of fascioliasis in this country. The problem in Argentina does not only lie on the different lymnaeid vector species which are present, such as *L. viatrix*, *L. diaphana*, *Pseudosuccinea columella*, *G. truncatula* and now *L. neotropica*, but also to the fact that three of them belong to the controversial *Galba/Fossaria* group of species. Thus, *L. viatrix*, *G. truncatula* and *L. neotropica* are lymnaeids which are very similar from the phenotypic point of view, including: (i) shell characteristics and snail anatomy (Samadi et al., 2000; Pointier et al., 2006) and (ii) ecological characteristics (Euzeby, 1971; Mera y Siera, 2001; Ciocco and Scheibler, 2008). The close systematic relationships between these lymnaeid vector species has recently been confirmed by DNA marker sequences and corresponding phylogenetic reconstructions (Bargues et al., 2007a). This similarity poses a great problem, as there is no way to distinguish between one and other species of these three lymnaeids when working on the field (even expert malacologists may misclassify specimens) and it is evident that the need to obtain DNA sequences each time a lymnaeid vector population is found does not appear to be feasible.

Moreover, this problem also concerns several molecular techniques which do not show sufficient accuracy. For instance, molecular techniques which rely on the 18S rRNA gene or parts of it, as the helix E10-1 of the variable region V2 which is useful for the differentiation of European lymnaeids (Bargues and Mas-Coma, 1997), are evidently not useful in Argentina because of the existence of species as *L. viatrix* and *L. neotropica* which present identical 18S sequence (Bargues et al., 2007a). Consequently, the recently proposed, 18S-based, real-time PCR strategy for the rapid discrimination among main lymnaeid species from Argentina (Duffy et al., 2009) cannot be applied because it does not distinguish between these two vector species.

All suggests that vector misclassifications may probably be included in several of the numerous fascioliasis studies made in Argentina in which *L. viatrix* was the species noted to be involved in disease transmission. Many of these studies probably included *L. neotropica* and/or even *G. truncatula*. Therefore, appropriate molecular studies are needed to assess the geographical distribution of each one of these three lymnaeid vector species in Argentina, for which ITS-2, ITS-1 and *cox*1 appear to be the most useful markers.

Acknowledgements

Studies in Argentina funded by Project No. 06-J266 of the Programa de Proyectos Bienales, Secretarı´a de Ciencia, Técnica y Posgrado, Universidad Nacional de Cuyo, Mendoza, Argentina. Spanish collaboration supported by Project No. SAF2006-09278 of the Spanish Ministry of Science and Technology, Madrid, Spain, and the Red de Investigación de Centros de Enfermedades Tropicales–RICET (Grants No. C03/04, No. PI030545 and No. RD06/0021/0017 of the Program of Redes Temáticas de Investigación Cooperativa), FIS, Spanish Ministry of Health, Madrid, Spain. Joint coordination activities carried out within Project No. RLA5049 of the International Atomic Energy Agency (Animal Production and Health Section, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Department of Nuclear Sciences and Applications, IAEA Headquaters Vienna, Austria). Technical support provided by the Servicio Central de Secuenciación para la Investigación Experimental (SCSIE) of the Universidad de Valencia (Dr. A. Martı´nez).

References

Albrecht, C., Wolf, C., Gloeer, P., Wilke, T., 2008. Concurrent evolution of ancient sister lakes and sister species: the freshwater gastropod genus *Radix* in lakes Ohrid and Prespa. Hydrobiologia 615, 157–167. Apt, W., Aguilera, X., Vega, F., Alcaino, H., Zulantay, I., Apt, P., Gonzalez, V., Retamal, C., Rodriguez, J., Sandoval, J., 1993. Prevalencia de Fascio- liasis en humanos, caballos, cerdos y conejos silvestres en tres provincias de Chile. Bol. Of. Sanit. Panam. 115, 405–414.

Bargues, M.D., Mas-Coma, S., 1997. Phylogenetic analysis of lymnaeid snails based on 18S rDNA sequences. Mol. Biol. Evol. 14, 569–577.

Bargues, M.D., Mas-Coma, S., 2005. Reviewing lymnaeid vectors of fascioliasis by ribosomal DNA sequence analyses. J. Helminthol. 79, 257–267.

Bargues, M.D., Mangold, A.J., Muñoz-Antoli, C., Pointier, J.P., Mas-Coma, S., 1997. SSU rDNA characterization of lymnaeid snails transmitting human fascioliasis in South and Central America. J. Parasitol. 83, 1086–1092.

Bargues, M.D., Vigo, M., Horak, P., Dvorak, J., Patzner, R.A., Pointier, J.P., Jackiewicz, M., Meier-Brook, C., Mas-Coma, S., 2001. European Lymnaeidae (Mollusca: Gastropoda), intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS-2 sequences. Infect. Genet. Evol. 1, 85–107.

Bargues, M.D., Horak, P., Patzner, R.A., Pointier, J.P., Jackiewicz, M., Meier-Brook, C., Mas-Coma, S., 2003. Insights into the relationships of Palaearctic and Nearctic lymnaeids (Mollusca: Gastropoda) by rDNA ITS-2 sequencing and phylogeny of stagnicoline intermediate host species of *Fasciola hepatica*. Parasite 10, 243–255.

Bargues, M.D., Artigas, P., Jackiewicz, M., Pointier, J.P., Mas-Coma, S., 2006a. Ribosomal DNA ITS-1 sequence analysis of European stagnicoline Lymnaeidae (Gastropoda), Heldia, München, 6, 29–40.

Bargues, M.D., Mera y Sierra, R.L., Gómez, H.G., Artigas, P., Mas-Coma, S., 2006b. Ribosomal DNA ITS-1 sequencing of *Galba truncatula* (Gastropoda: Lymnaeidae) and its potential impact on fascioliasis transmission in Mendoza, Argentina. Anim. Biodivers. Conserv. 29, 191–194.

Bargues, M.D., Artigas, P., Mera y Sierra, R.L., Pointier, J.P., Mas-Coma, S., 2007a. Characterisation of *Lymnaea cubensis*, *L. viatrix* and *L. neotro- pica* n. sp., the main vectors of *Fasciola hepatica* in Latin America, by analysis of their ribosomal and mitochondrial DNA. Ann. Trop. Med. Parasitol. 101, 621–641.

Bargues, M.D., Mera y Sierra, R.L., Gómez, H.G., Artigas, P., Mas-Coma, S., 2007b. Caracterización molecular de *Galba truncatula*, vector principal de la Fascioliasis, en Argentina. Implicaciones en salud pública. Enf. Emerg. Barcelona 9, 77–82.

Castellanos, Z.J.A., de Landoni, N.A., 1981. Mollusca, Gastropoda. La familia Lymnaeidae Rafinesque, 1815 en la Argentina. In: Ringuelet, R.A.(Dir.) (Ed.), Fauna de Agua Dulce de la República Argentina, vol. 15. FECIC, Buenos Aires, pp. 55–82.

Chen, M.C., Mott, M.G., 1990. Progress in assessment of morbidity due to *Fasciola hepatica* infection: a review of recent literature. Trop. Dis. Bull. 87, R1–R38.

Ciocco, N.F., Scheibler, E., 2008. Malacofauna of the littoral benthos of a saline lake in southern Mendoza, Argentina. Fundam. Appl. Limnol. (Arch. Hydrobiol.) 172, 87–98.

Duffy, T., Kleiman, F., Pietrokovsky, S., Issia, L., Schijman, A.G., Wisnivesky-Colli, C., 2009. Real-time PCR strategy for rapid discrimination among main lymnaeid species from Argentina. Acta Trop. 109, 1–4.

Esteban, J.G., Gonzalez, C., Bargues, M.D., Angles, R., Sanchez, C., Naquira, C., Mas-Coma, S., 2002. High fascioliasis infection in children linked to a man-made irrigation zone in Peru. Trop. Med. Int. Health 7, 339–348.

Euzeby, J., 1971. Ecologie et biologie de *Lymnea truncatula* en Europe. Cahiers Méd. Vét. 40, 283–289.

Folmer, O., Black, M., Hoch, W., Lutz, R., Vrijenhoeck, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3, 294–299.

Hijmans, R.J., Cameron, S.E., Parra, J.L., Jones, P.G., Jarvis, A., 2005. Very high resolution interpolated climate sufaces for global land areas. Int. J. Climatol. 25, 1965–1978.

Hubendick, B., 1951. Recent Lymnaeidae. Their Variation, Morphology, Taxonomy, Nomenclature, and Distribution. Kungliga Svenska Vetenskapsakademiens Handlingar, vol. 3, Fjä rde Serien, pp. 1–223 (+5 pl).

Lombardero, O.J., Moriena, R.A., Racioppi, O., Coppo, J.A., Schiffo, H.P., 1979a. Distomatosis hepática de los bovinos en la Provincia de Corrientes. Vet. Rev. Fac. Cienc. Vet., Univ. Nac. NE, Corrientes, Argentina 2, 21–29.

Lombardero, O.J., Moriena, R.A., Racioppi, O., Quiroz, L., 1979b. Bionomía de *Limnaea* sp. en la Provincia de Corrientes. Vet. Rev. Fac. Cienc. Vet., Univ. Nac. NE, Corrientes, Argentina 2, 5–19.

Lumbreras, H., Cantella, R., Burga, R., 1962. Acerca de un procedimiento de sedimentación rápida para investigar huevos de *Fasciola hepatica* en las heces, su evaluación y uso en el campo. Rev. Méd. Peruana 31, 167–174.

Maleck, E.A., 1985. Snail Hosts of Schistosomiasis and Other Snail-Transmitted Diseases in Tropical America: A Manual. PAHO, Washington, Scientific Publication No. 478, 325 pp.

Malone, J.B., Gommes, R., Hansen, J., Yilma, J.M., Slingenberg, J., Snijders, F., Nachtergaele, F., Ataman, E., 1998. A geographic information system on the potential distribution and abundance of *Fasciola hepatica* and *F. gigantica* in east Africa based on Food and Agriculture Organization databases. Vet. Parasitol. 78, 87–101.

Mas-Coma, S., 2005. Epidemiology of fascioliasis in human endemic areas. J. Helminthol. 79, 207–216.

Mas-Coma, S., Bargues, M.D., 1997. Human liver flukes: a review. Res. Rev. Parasitol. 57, 145–218.

Mas-Coma, S., Bargues, M.D., Valero, M.A., 2005. Fascioliasis and other plant-borne trematode zoonoses. Int. J. Parasitol. 35, 1255–1278.

Mas-Coma, S., Esteban, J.G., Bargues, M.D., 1999a. Epidemiology of human fascioliasis: a review and proposed new classification. Bull. WHO 77, 340–346.

Mas-Coma, S., Funatsu, I.R., Bargues, M.D., 2001. *Fasciola hepatica* and lymnaeid snails occurring at very high altitude in South America. Parasitology 123, s115–127.

Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv. Parasitol. 69, 41–146.

Mas-Coma, S., Angles, R., Esteban, J.G., Buchon, P., Franken, M., Strauss, W., 1999b. The Northern Bolivian Altiplano: a region highly endemic for human fascioliasis. Trop. Med. Int. Health 4, 454–467.

Mera y Siera, R.L., 2001. Distribución de Lymnaeidos en el sistema hidrográfico andino de la provincia de Mendoza, Argentina. Tesis de Maestría, Master Internacional en Enfermedades Parasitarias Tropicales (XIV Edición). Dep. Parasitol. Fac. Farmacia, Univ. Valencia, 46 pp.

Mera y Siera, R.L., Scibilia, C., Pabst, A., Irrazabal, G., Senar, M., 2005. Abattoir condemnation of bovine livers due to *Fasciola hepatica* in Tupungato, Mendoza. In: XXIII Reunión Científica Anual, Sociedad Biológica de Cuyo, Biocell 29, 371.

Olaechea, F.V., 1994. Epidemiologia y control de *Fasciola hepatica* en la Argentina. In: Nari, A., Fiel, C. (Eds.), Enfermedades Parasitarias de Importancia Económica en Bovinos. Editorial Agropecuaria. Hemisferio Sur SRL, Montevideo, Uruguay, pp. 213–232.

Oviedo, J.A., Bargues, M.D., Mas-Coma, S., 1995. Lymnaeid snails in the human fascioliasis high endemic zone of the Northern Bolivian Altiplano. Res. Rev. Parasitol. 55, 35–43.

Paraense, W.L., 1976. *Lymnaea viatrix*: a study of topotypic specimens (Mollusca: Lymnaeidae). Rev. Brasil. Biol. 36, 419–428.

Paraense, W.L., 1982a. *Lymnaea viatrix* and *Lymnaea columella* in the Neotropical region: a distributional outline. Mem. Inst. Oswaldo Cruz 77, 181–188.

Paraense, W.L., 1982b. *Lymnaea rupestris* sp. n. from southern Brazil (Pulmonata: Lymnaeidae). Mem. Inst. Oswaldo Cruz 77, 437–443.

Paraense, W.L., 1983. *Lymnaea columella* in northern Brazil. Mem. Inst. Oswaldo Cruz 78, 477–482.

Paraense, W.L., 1984. *Lymnaea diaphana*: a study of topotypic specimens (Pulmonata: Lymnaeidae). Mem. Inst. Oswaldo Cruz 79, 75–81.

Pizzi, H., Navarro, Z., Pizzi, D., Benvissuto, G., 1982. Estudio epidemiológico e implicaciones económicas de la distomatosis hepatica en la provincia de Córdoba, Argentina. Gaz. Vet. 44, 944–947.

Pointier, J.P., Cazzaniga, N.J., Gonzalez-Salas, C., Gutierrez, A., Arenas, J.A., Bargues, M.D., Mas-Coma, S., 2006. Anatomical studies of sibling species within Neotropical lymnaeids snail intermediate hosts of fascioliasis. Mem. Inst. Oswaldo Cruz 101, 431–435.

Remigio, E.A., Blair, D., 1997b. Relationships among problematic North American stagnicoline snails (Pulmonata: Lymnaeidae) reinvestigated using nuclear ribosomal DNA internal transcribed spacer sequences. Can. J. Zool. 75, 1540–1545.

Remigio, E.A., Hebert, P.D., 2003. Testing the utility of partial COI sequences for phylogenetic estimates of gastropod relationships. Mol. Phylogenet. Evol. 29, 641–647.

Rumi, A., Gregoric, D.E., Nuñez, V., Darrigran, G.A., 2008. Malacologia latinoamericana. Moluscos dulceacuícolas de Argentina. Rev. Biol. Trop. 56, 77–111.

Samadi, S., Roumegoux, A., Bargues, M.D., Mas-Coma, S., Yong, M., Pointier, J.P., 2000. Morphological studies of lymnaeid snails from the human fascioliasis endemic zone of Bolivia. J. Moll. Stud. 66, 31–44. Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning. A Laboratory Manual, 2nd ed., vols. I–III. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1647 pp.

Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Nat. Acad. Sci. U.S.A. 74, 5463–5467.

Staden, R., Judge, D.P., Bonfield, J.K., 2001. Sequence assembly and finishing methods. Met. Biochem. Anal. 43, 302–322.

Swofford, D.L., 2001. PAUP*: Phylogenetic Analysis Using Parsimony (*and other Methods). Version 4. Computer Program Distributed by the Smithsonian Institution. Sinauer Associates, Inc. Publishers, Sunderland, MA.

Tamura, K., Dudley, J., Nei, M.B., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetic Analysis (MEGA) sofware version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Torgerson, P., Claxton, J., 1999. Epidemiology and control. In: Dalton, J.P. (Ed.), Fasciolosis. CAB International Publishing, UK, pp. 113–149.

Ueno, H., Arandia, R., Morales, G., Medina, G., 1975. Fascioliasis of livestock and snail host for *Fasciola* in the Altiplano region of Bolivia. Nat. Inst. Animal Health Quart. 15, 61–67.

6th INTERNATIONAL SYMPOSIUM

LIMNOLOGY AND AQUATIC BIRDS

Monitoring, Modelling and Management

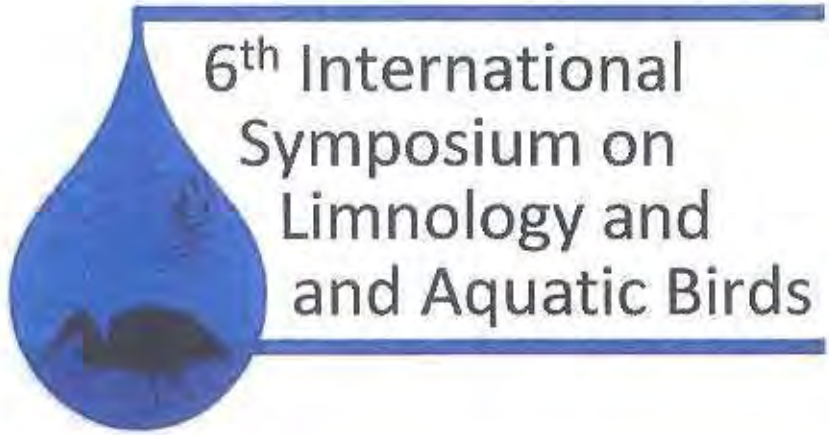
HUESCA, Spain, 27-30 October 2009

6th INTERNATIONAL SYMPOSIUM

HUESCA, Spain, 27-30 October 2009

LIMNOLOGY AND AQUATIC BIRDS

Monitoring, Modelling and Management



SIL Working Group on Limnology and Aquatic Birds

6th International Symposium on Limnology and Aquatic Birds: Monitoring, Modelling and Management

Huesca, Spain.
26-31 October 2009

PROCEEDINGS

Scolopacidae and other Aquatic Migratory Birds as possible dispersal agents of Lymnaeid Snails, in Mendoza Province, Argentina

Artigas P.[a], Mera y Sierra R.[b,c], Cuervo P.[b,c], Bargues M.D.[a] & Mas-Coma S.[a]

[a]Department of Parasitology, University of Valencia, Av. V.A. Estellés s/n, 46100 Burjassot, Valencia, Spain (Patricio.Artigas@uv.es)
[b]Fac. Cs. Vet. Amb., UJA-Maza. Av. Este-Lat. Sur 2245, Guaymallén, Mendoza, Argentina
[c]Fac. Cs Méd., UNCuyo, Av. Libertador 80 C. Univ., P.O. Box 33,5500 Mendoza, Argentina

INTRODUCTION

The role of migratory birds as dispersal agents of snails was hypothesized by Darwin as an efficient long-distance transportation[1] (except of human interference), since a migratory bird can cover hundreds km in few days. *Galba truncatula* is the most efficient vector of *Fasciola hepatica*. This freshwater snail, of European origin, was introduced to the Bolivian Altiplano within the last 500-year period[2-3]. Recently, this species has also been described in Andean sites of Mendoza province, Argentina, as far as 2,000 km south [4-6]. This poses the question about how the considered "most pedestrian of creatures" reached so far southward. According to Darwin's hypothesis, migratory aquatic birds may be counted as one possibility amongst the probable spreading ways.

METHODS

Around 26 wetlands lay in southern and western Mendoza province, with a combined population of around 240,000 aquatic birds, distributed among 90 species. Various populations of *G. truncatula* and other lymnaeid species have been identified in the surrounding areas[7].

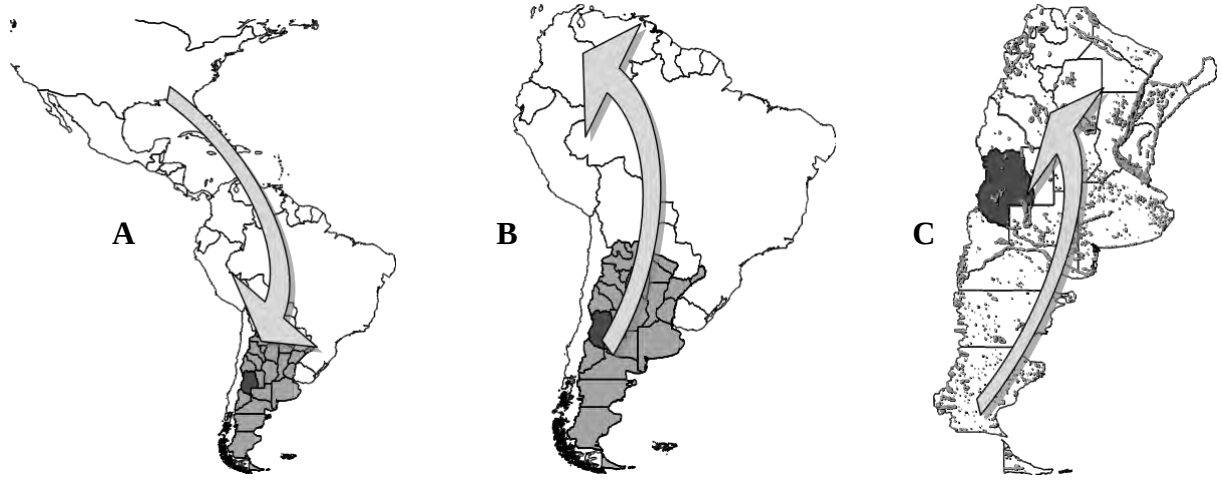


Fig. 1. Maps of main migratory categories of the waterfowl across Mendoza province (A, B, C).

RESULTS AND DISCUSSION

At least 25 bird species follow the 3 known migratory routes communicating Mendoza with Bolivia[8-12]. Table 1 shows the main migratory categories of the waterfowl across Mendoza province (A, B, C)[13]. Whether resident or in the case of species that may be in continuous exchange with Chile. Migratory category A: Birds that nest in the Northern Hemisphere and then fly to Argentina, they are found in our province in spring-summer; Migratory category B: Birds that nest in Argentina, during spring-summer and migrate to the north in winter; Migratory category C: Birds that nest in Patagonia during the spring and appear in the centre of the country during their migration to the north in winter (Fig. 1).

Table 1. Classification of the migratory categories of the waterfowl across Mendoza province by type (A, B, C)*

Migrating water birds Inventorys	Migrator A	Migrator B	Migrator C	Exchange with Chile	Resident
Podicipedidae					X
Phalacrocoracidae					X
Ardeidae					X
Ciconiidae					X
Threskiornithidae					X
Phoenicopteridae				X	X
Anatidae	X		X	X	X
Rallidae					X
Rostratulidae					X
Recurvirostridae					X
Charadriidae	X		X		X
Scolopacidae	X				X
Phalaropodidae	X				
Thinocoridae					X
Laridae	X			X	X
Sternidae	X				X
Rynchopidae	X				

*Adapted of Narosky & Izurieta (1988)

Scolopacidae is the most plausible family involved, since 11 species traverse these routes. Meanwhile, only 2 Charadriidae and 1 Anatidae may be involved. Laridae, Sternidae and Rynchopidae are not considered of main concern due to ecological features[14-17].

CONCLUSIONS

The snails could be transported attached to birds' feathers or feet, surviving the passage. The possibility of aquatic migratory birds as the original reasons for the presence and dispersal of *G. truncatula* and other lymnaeid snails in Mendoza province should be taken into account. Further studies are required to reach confirmation and clarify the situation.

ACKNOWLEDGEMENTS

Funded by the UNCuyo, Argentina & Projects Nos. BOS2002-01978 (Ministry of Science and Technology), and C03/04 and ISCIII2005-PI050574 (Red RICET, FIS, Ministry of Health), Spain.

REFERENCES

1. Darwin C.R. (1859). *On the origin of species by means of natural selection*, *or the preservation of favoured races in the struggle for life*. London: J. Murray. 1st Edition.
2. Mas-Coma S., Funatsu I.R., Bargues M.D. (2001). *Fasciola hepatica* and lymnaeid snails occurring at very high altitude in South America. *Parasitology*; 123:S115-S127.
3. Mas-Coma S., Angles R., Esteban J.G., Bargues M.D., Buchon P., Franken M., Strauss W. (1999). The Northern Bolivian Altiplano: a region highly endemic for human fascioliasis. *Trop Med Int Health* 4:454-467.
4. Mera y Sierra R.L. (2001). Tesis de Maestría, MIEPT. Depto. Parasitología, U. Valencia, 22.06.2001:1-46.
5. Artigas P., Mera y Sierra R.L., Khoubbane M., Mas-Coma S. & Bargues M.D. (2009). Detection of H3C comb. haplotype rDNA ITS1-ITS2 of *Galba truncatula* (Gastropoda, Lymnaeidae) and its potential impact on fascioliasis transmission in Argentina. *Acta Parasitol Port*; 16(1-2):122-123.
6. Artigas P., Sidoti L., Deis E., Cuervo P., Mera y Sierra R.L. (2009). Physical-Chemical parameters of water where *Galba truncatula* is found in Mendoza, Argentina. *Biocell*, 33 (1): a58.
7. Bargues M.D., Artigas P., Mera y Sierra R.L., Pointier J.P. & Mas-Coma S. (2007). Characterisation of *Lymnaea cubensis*, *L. viatrix* and *L. neotropica* n.sp., the main vectors of *Fasciola hepatica* in Latin America, by analysis of their ribosomal and mitochondrial DNA. *Annals of Tropical Medicine and Parasitology* 101(7): 621-641.
8. Cowardin L.M., Carter V., Golet F. & Laroe E. (1977). *Clasification of wetlands and deep-water habitats of the United States*, U.S.Fish Wildl. Serv. 100p.
9. Canevari P., Blanco D., Bucher E., Castro G. & Davidson I. (1999). Los Humedales de la Argentina. Clasificación, situación actual, conservación y legislación. *Wetlands Internacional* -Nº46, Secretaría de Recursos Naturales y Desarrollo Sustentable de la Nación. Argentina.
10. Contreras J. & Fernández A. (1980). Biología de la Avifauna de la Laguna el Viborón, Departamento de Maipú. Prov. Mendoza. *Rev. Del Museo de Hist. Nat. De San Rafael* 7(1): 3-14.
11. Darrieu C., Soave G. & Martínez M. (1985). Estudio de la avifauna de la Laguna Llancanelo II Presencia de *Larus pipixcan, Larus serranus, Sterna trudeaui* y *Chlidonias niger* (Aves=Laridae), *Historia Natural* 5(3): 25-28.
12. Martinez M., Darrieu C. & Soave G. (1997). The Avifauna of Lag. Llancanelo (Mendoza Argentina) A South American Wetland of international Importance. *Freshwater Forum* Vol. 9: 35-44 Freshwater Biological Association.
13. Narosky T. & Izurieta D. (1988). *Guía para la identificación de las Aves de la Argentina y Uruguay*. Vazquez Mazzini Ed. 144p.
14. Olrog C. & Pescetti E. (1991). *Las Aves del Gran Cuyo:Mendoza, San juan, San Luis y La Rioja*. Guía de Campo. CRICYT, Gobierno de Mendoza, INCA Ed, 160p.
15. Scott D. & Carbonell M. (1986). *Inventory of Neotropical Wetlands*. IWRB y IUCN. Slimbridge & Cambridge.
16. Sosa H. (1992). Censo estival de aves acuáticas en: Laguna Llancanelo, Mendoza. In: Carp E. (1992). *Censo Neotropical de aves acuáticas* 1991. Programa de Ambientes Acuáticos Neotropicales (NWP) Bs. As. Arg. 62p.
17. Sosa H. (1995). Actualización de la lista de avifauna de la reserva provincial Laguna Llancanelo Malargüe Mendoza. Presencia estacional, preferencia de hábitats y nidificación. *Rev. Multequina* 4: 65-75, Direc. De Rec. Nat. Renov. Mendoza.

Parasites &Vectors

REVIEW Open Access

Human fascioliasis in Argentina: retrospective overview, critical analysis and baseline for future research

Roberto Mera y Sierra[1,2], Veronica H Agramunt[3], Pablo Cuervo[1] and Santiago Mas-Coma[3*]

Abstract

In Argentina, human fascioliasis has never been adequately analysed, although having a physiography, climate, animal prevalences and lymnaeids similar to those of countries where the disease is endemic such as Bolivia, Peru and Chile. We performed a literature search identifying 58 reports accounting for 619 cases, involving 13 provinces, their majority (97.7%) from high altitudes, in central mountainous areas and Andean valleys, concentrated in Cordoba (430 cases), Catamarca (73), San Luis (29) and Mendoza (28), the remaining provinces being rarely affected. This distribution does not fit that of animal fascioliasis. Certain aspects (higher prevalence in females in a local survey, although a trend non-significant throughout Argentina) but not others (patient's age 3-95 years, mean 37.1 years) resemble human endemics in Andean countries, although the lack of intensity studies and surveys in rural areas does not allow for an adequate evaluation. Human infection occurs mainly in January-April, when higher precipitation and temperatures interact with field activities during summer holidays. A second June peak may be related to Easter holidays. The main risk factor appears to be wild watercress ingestion (214) during recreational, weekend outings or holiday activities, explaining numerous family outbreaks involving 63 people and infection far away from their homes. Diagnosis mainly relied on egg finding (288), followed by serology (82), intradermal reaction (63), surgery (43), and erratic fluke observation (6). The number of fascioliasis-hydatidosis co-infected patients (14) is outstanding. Emetine appears as the drug most used (186), replaced by triclabendazole in recent years (21). Surgery reports are numerous (27.0%). A long delay in diagnosis (average almost 3.5 years) and high lithiasis proportion suggest that many patients are frequently overlooked and pose a question mark about fascioliasis detection in the country. High seroprevalences found in recent random surveys suggest human endemic situations. This analysis highlights that human fascioliasis may have been overlooked in the past and its real epidemiological situation in high risk rural, mainly altitudinal areas, may currently be underestimated. Results provide a valuable baseline on which to design appropriate multidisciplinary studies on humans, animals and lymnaeids to assess up to which level and in which areas, human fascioliasis may represent a health problem in Argentina.

Background

Fascioliasis, a major veterinary problem worldwide due to the economic losses it causes in animal husbandry, has recently become increasingly important in public health, with human reports increasing in number and the description of human endemic areas, comprising hypo- to hyperendemic situations in many countries of Latin America, Africa, Europe and Asia [1-4]. This emergence appears to be partly related to climate change, global warming and the so-called global change, among which mainly anthropogenic modifications of the environment and increasing short- and long-distance travel and import/export facilities available nowadays. All these phenomena have shown to have a great impact on snail-borne zoonotic diseases, as is the case of a trematodiasis very dependent on climate and environment characteristics such as fascioliasis [5-7].

The magnitude of fascioliasis impact on communities of human endemic areas, mainly on children and females [3], is due to its chronic, debilitating, and poverty-promoting characteristics, with a pathogenicity until recently considered restricted mainly to the acute phase

* Correspondence: S.Mas.Coma@uv.es
[3]Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Av. Vicente Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain
Full list of author information is available at the end of the article

[2,8], but which has recently proved to constitute a health problem during the very long chronic phase [9-12]. Impact and wide emergence prompted the World Health Organization (WHO) to include human fascioliasis on its list of priorities among neglected tropi- cal diseases (NTDs) [13].

In the Americas, this helminthic disease is caused by the liver fluke *Fasciola hepatica* [4], transmitted by many different freshwater snail vectors belonging to the family Lymnaeidae, mainly species included within the *Galba/Fossaria* group [14,15]. In South America, human endemic areas have been described in Andean regions, mainly in higher altitude areas of countries such as Bolivia, Peru and Chile, and secondarily in Ecuador and Venezuela [3,16-22].

In Argentina, the situation of human fascioliasis has never been the subject of an adequate analysis. Only short reports within large worldwide reviews may be specifically acknowledged [2,8]. This is surprising when taking into account that (i) neighbouring countries such as Bolivia and Chile reported hyperendemic areas of human fascioliasis long ago [16-21], (ii) the country presents a very widely distributed veterinary problem of fascioliasis in livestock [23], (iii) it includes Andean environmental characteristics appropriate for fascioliasis transmission to humans [3,24], and (iv) recent studies have reported the discovery of lymnaeid vector species of well-known transmission capacity to humans to be the same combined haplotype of *Galba truncatula* responsible for the human hyperendemic area presenting the highest prevalences and intensities known [25,26] and *Lymnaea neotropica* [27]. Argentina is a country of high livestock production, where sheep and cattle but also equines constitute important economic sources. All these different domestic species are important reservoirs of fascioliasis and represent similar sources of infection for humans, given the results obtained in experimental studies which have demonstrated that snail-borne infective metacercarial stages originating from different animal species do not significantly differ in their infection capacity [28,29].

The purpose of the present ten-year research work is to provide an in-depth analysis of the results obtained in a thorough bibliographical search of human fascioliasis cases in Argentina. In that country, even though there are national data on animal fascioliasis, where slaughterhouse reports have been submitted to the authorities for practically a hundred years, there are, however, no official reports on human fascioliasis, because human infection by the liver fluke is not of obligatory declaration. Thus, published and unpublished written reports are the only source of information, whether they may be articles in scientific journals, books, university theses, communications at scientific meetings, or internal reports of agencies, ministries, hospitals, health centres, etc. [30].

Review

Description of dataset

The first case published in Argentina concerned an Arab immigrant who had just arrived in Argentina [31]. The onset of symptoms was upon arrival, indicating that the disease was most probably acquired in his homeland. The patient died and the diagnosis was made during autopsy when numerous flukes were found in the liver. Since this case was not autochthonous, it is not considered in this review.

This overview begins from the first autochthonous case diagnosed by coprology in 1924 [32]. More cases followed soon, such as a coprologically diagnosed patient from San Luis province [33], another diagnosed during surgery [34] and yet another one from San Luis province [35]. Fifty three cases had already been published prior to 1960.

The first WHO review [8] refers to only 13 human reported cases in Argentina for the 1970-1990 period, namely only those reported by Carena et al. [36]. This number of human cases was increased to 85 in the following extensive WHO initiative [2]. The present review offers a completely new picture of human fascioliasis in Argentina, including a total of 619 authochthonous cases in 58 reports of different kinds analysed up to 2010 (Table 1), which means that the number of human cases published is more than seven times greater than previously noted. Such a pronounced difference seems to be due to the great amount of overlooked local publications (and also communications at scientific meetings with abstract books of restricted dissemination). When considering that human fascioliasis infection is in Argentina of non-obligatory declaration, similarly to the rest of the world, one may conclude that the number of infected patients should be even greater than that. Interestingly, the need for Argentinian health authorities to warn about this disease was already noted long ago when fascioliasis was cited in animals in the country for the first time [37]. Both report and case numbers follow a parallel evolu- tion with quite important fluctuations (Figure 1). This result is most likely to be linked to particular circum- stances encouraging physicians to publish their diag- nosed cases rather than a real reflection of the annual evolution of the epidemiological situation. For instance, only two authors, C. Rodriguez and C. Siciliano [38-42], are responsible for 51% of the cases reported in Argen- tina, the decades when they were active publishing appearing as those decades with the greatest amount of cases (1960's and 1980's).

Characteristics of the infected population

In only 267 cases did the authors specify the gender of the patients: 120 (44.94%) were male and 147 (55.06%) were female. A somewhat higher preference for females

Table 1 Human fascioliasis reports in Argentina, arranged chronologically, including details on infection according to number of cases, gender, province, diagnostic method, treatment, and clinical data

YEAR	AUTHOR	Ref. No.	No. cases	GENDER	PROVINCE	DIAGNOSTIC METHOD	TREATMENT	CLINICAL DATA
1924	Greenway	[32]	1	$NS_{(1)}$	$NS_{(1)}$	$Ec_{(1)}$	$NS_{(1)}$	$NS_{(1)}$
1927	Bengolea et al.	[33]	1	$F_{(1)}$	$SL_{(1)}$	$Ec_{(1)}$ $Es_{(1)}$	$NS_{(1)}$	$AP_{(1)}$
1928	Del Valle & Donovan	[34]	1	$F_{(1)}$	$NS_{(1)}$	$Surg_{(1)}$	$NS_{(1)}$	$AP_{(1)}$ $Nau_{(1)}$ $Ic_{(1)}$ $HA_{(1)}$
1930	Bacigalupo et al.	[35]	1	$F_{(1)}$	$SL_{(1)}$	$Ec_{(1)}$ $Es_{(1)}$	$NS_{(1)}$	$NS_{(1)}$
1933	Mascheroni	[108]	1	$F_{(1)}$	$BA_{(1)}$	$Ec_{(1)}$ $Es_{(1)}$	$NS_{(1)}$	$NS_{(1)}$
1933	Scrimaglio (In Bacigalupo et al. 1943)	[93]	1	$M_{(1)}$	$SFe_{(1)}$	$Ec_{(1)}$ $Es_{(1)}$	$NS_{(1)}$	$NS_{(1)}$
1937	Castex & Greenway	[91]	1	$M_{(1)}$	$Cba_{(1)}$	$Ec_{(1)}$ $Ect_{(1)}$	$NS_{(1)}$	$AP_{(1)}$ $Lks_{(1)}$
1939	Boto	[69]	1	$F_{(1)}$	$Tuc_{(1)}$	$Es_{(1)}$	$Em_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$ $Fev_{(1)}$ $Lks_{(1)}$ $Urt_{(1)}$
1939	Paladino & Galarce	[92]	1	$F_{(1)}$	$BAcity_{(1)}$	$Surg_{(1)}$ $Ect_{(1)}$	$NS_{(1)}$	$AP_{(1)}$
1940	Cames	[76]	2	$M_{(2)}$	$Cba_{(1)}$ $SFe_{(1)}$	$Es_{(1)}$ $Surg_{(1)}$	$Em_{(2)}$ $MFE_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$ $Fev_{(2)}$ $WL_{(1)}$ $Ic_{(1)}$
1942	Bacigalupo	[52]	$5_{(fo1)}$	$F_{(2)}$ $M_{(3)}$	$Cba_{(4)}$ $BA_{(1)}$	$Ec_{(2)}$ $Es_{(1)}$ $CE_{(2)}$	$Em_{(4)}$ $NS_{(1)}$	$Eo_{(4)}$ $AP_{(4)}$ $Fev_{(3)}$ $WL_{(1)}$
1943	Solari & Canepa	[77]	1	$F_{(1)}$	$Cba_{(1)}$	$Es_{(1)}$	$Em_{(1)}$	$AP_{(1)}$
1943	Bacigalupo et al.	[93]	1	$M_{(1)}$	$Cba_{(1)}$	$Ect_{(1)}$	$Em_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$
1944	Cuenya	[109]	3	$F_{(1)}$ $M_{(2)}$	$Tuc_{(1)}$ $Ju_{(1)}$ $Sal_{(1)}$	$Ec_{(3)}$ $Es_{(1)}$	$NS_{(3)}$	$AP_{(1)}$
1947	Cid Cames et al.	[79] [80]	1	$F_{(1)}$	$Tuc_{(1)}$	$Surg_{(1)}$ $Ect_{(1)}$	$Em_{(1)}$	$AP_{(1)}$ $WL_{(1)}$ $Lith_{(1)}$ $Ic_{(1)}$ $HA_{(1)}$
1952	Rodríguez	[38]	16	$F_{(3)}$ $M_{(1)}$ $NS_{(12)}$	$Cbsa_{(15)}$ $Cat_{(1)}$	$Ec_{(16)}$	$NS_{(16)}$	$Eo_{(5)}$ $Ap_{(1)}$ $Lks_{(4)}$ $Ic_{(1)}$
1953	Longo & Daraio	[89]	1	$F_{(1)}$	$Sal_{(1)}$	$Surg_{(1)}$ $NI_{(1)}$	$Em_{(1)}$	$AP_{(1)}$ $Urt_{(1)}$ $Nau_{(1)}$ $Lith_{(1)}$ $Vo_{(1)}$
1954	Petraglia	[98]	1	$M_{(1)}$	$Cha_{(1)}$	$Ec_{(1)}$	$Em_{(1)}$	$AP_{(1)}$ $Eo_{(1)}$ $Fev_{(1)}$
1954	Rodríguez	[39]	10	$F_{(1)}$ $M_{(2)}$ $NS_{(7)}$	$Cba_{(9)}$ $Cat_{(1)}$	$Ec_{(10)}$	$NS_{(10)}$	$Eo_{(4)}$ $Lks_{(1)}$
1955	Cáceres	[55]	1	$F_{(1)}$	$BAcity_{(1)}$	$Surg_{(1)}$	$Em_{(1)}$	$AP_{(1)}$ $Fev_{(1)}$ $Ic_{(1)}$ $Lith_{(1)}$ $Vo_{(1)}$
1955	Logaldo	[101]	1	$F_{(1)}$	$Mza_{(1)}$	$Surg_{(1)}$	$Em_{(1)}$	$Ap_{(1)}$ $Nau_{(1)}$ $Lith_{(1)}$
1961	Ahualli & Arias	[110]	1	$F_{(1)}$	$Tuc_{(1)}$	$Es_{(1)}$	$Em_{(1)}$	$AP_{(1)}$ $WL_{(1)}$ $Nau_{(1)}$ $Vo_{(1)}$ $HA_{(1)}$ $Dia_{(1)}$
1961	Rodriguez	[40]	23	$NS_{(23)}$	$Cba_{(22)}$ $Cat_{(1)}$	$Ec_{(23)}$	$NS_{(23)}$	$NS_{(23)}$
1961	"Other colleagues" cited in Rodriguez 1961	[40]	150	$NS_{(150)}$	$Cba_{(150)}$	$NS_{(150)}$	$NS_{(150)}$	$NS_{(150)}$
1961	Strada	[72]	19	$F_{(1)}$ $M_{(1)}$ $NS_{(17)}$	$Cba_{(19)}$	$Ec_{(1)}$ $Es_{(2)}$ $Surg_{(1)}$ $NS_{(15)}$	$Em_{(2)}$ $NS_{(17)}$	$Eo_{(2)}$ $AP_{(3)}$ $Fev_{(3)}$ $Lks_{(1)}$ $WL_{(1)}$ $Vo_{(1)}$ $HA_{(1)}$
1962	Urrutia & Ferraris	[111]	1	$NS_{(1)}$	$NS_{(1)}$	$Surg_{(1)}$	$NS_{(1)}$	$NS_{(1)}$
1964	Cornejo & Castillo	[112]	1	$M_{(1)}$	$Sal_{(1)}$	$Ec_{(1)}$ $Es_{(1)}$	$Em_{(1)}$	$Eo_{(1)}$ $Lks_{(1)}$
1964	Simon et al	[86]	1	$M_{(1)}$	$Mza_{(1)}$	$Es_{(1)}$ $ID_{(1)}$	$Em_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$ $Fev_{(1)}$ $Lks_{(1)}$ $WL_{(1)}$ $Vo_{(1)}$
1964	Cañas et al	[113]	1	$M_{(1)}$	$Ju_{(1)}$	$Es_{(1)}$	$Em_{(1)}$	$AP_{(1)}$ $Asth_{(1)}$ $Nau_{(1)}$ $Dia_{(1)}$ $Cst_{(1)}$
1965	Niño	[107]	4	$NS_{(4)}$	$BAcity_{(2)}$ $NS_{(2)}$	$Ec_{(2)}$ $Es_{(2)}$	$NS_{(4)}$	$NS_{(4)}$
1967	Sosa & Romero	[114]	2	$F_{(1)}$ $M_{(1)}$	$Cba_{(2)}$	$Es_{(2)}$	$Em_{(2)}$	$Eo_{(2)}$ $AP_{(1)}$ $Fev_{(2)}$ $WL_{(1)}$ $Nau_{(1)}$ $Vo_{(1)}$ $Dia_{(1)}$
1967 1969	Ruggieri et al. Correa et al.	[94] [95]	1	$F_{(1)}$	$Cba_{(1)}$	$Surg_{(1)}$ $Ect_{(1)}$	$NS_{(1)}$	$AP_{(1)}$ $Fev_{(1)}$ $Urt_{(1)}$ $Nau_{(1)}$ $Ic_{(1)}$ $Vo_{(1)}$ $HA_{(1)}$
1969	Peiretti et al.	[87]	17	$NS_{(17)}$	$Mza_{(15)}$ $SL_{(2)}$	$Es_{(5)}$ $ID_{(17)}$	$NS_{(17)}$	$NS_{(17)}$
1969	Trossero & Nocetti	[103]	1	$F_{(1)}$	$SFe_{(1)}$	$Surg_{(1)}$	$Em_{(1)}$	$AP_{(1)}$ $Fev_{(1)}$ $Ic_{(1)}$ $Vo_{(1)}$
1970	Padilla Antoni et al.	[99]	1	$NS_{(1)}$	$Tuc_{(1)}$	$Surg_{(1)}$	$NS_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$ $Vo_{(1)}$ $HA_{(1)}$
1972	Carena et al.	[36]	13	$F_{(4)}$ $M_{(9)}$	$Cba_{(11)}$ $Cat_{(1)}$ $Tuc_{(1)}$	$Es_{(13)}$	$Em_{(13)}$	$Eo_{(13)}$ $AP_{(8)}$ $Fev_{(1)}$ $Asth_{(3)}$ $Urt_{(2)}$ $HA_{(2)}$ $Cst_{(1)}$ $Dia_{(1)}$
1972	Ossola et al.	[49]	$12_{(fo3)}$	$F_{(7)}$ $M_{(5)}$	$Cba_{(12)}$	$Ec_{(2)}$ $Es_{(2)}$ $ID_{(12)}$	$NS_{(12)}$	$Eo_{(11)}$ $AP_{(12)}$ $Fev_{(9)}$ $WL_{(8)}$ $Asth_{(10)}$ $Urt_{(4)}$ $Ic_{(3)}$ $Cst_{(3)}$ $Dia_{(2)}$
1973	Sonzini Astudillo et al.	[104]	5	$F_{(4)}$ $M_{(1)}$	$NS_{(5)}$	$Es_{(1)}$ $Surg_{(4)}$	$Em_{(1)}$ $NS_{(4)}$	$Eo_{(1)}$ $AP_{(4)}$ $Asth_{(1)}$ $Lith_{(3)}$ $Ic_{(4)}$ $HA_{(1)}$
1973	Peiretti & Morales	[50]	$4_{(fo1)}$	$F_{(1)}$ $M_{(3)}$	$SL_{(4)}$	$ID_{(4)}$	$Em_{(4)}$	$Eo_{(4)}$ $AP_{(4)}$ $Fev_{(2)}$ $Lks_{(2)}$ $WL_{(1)}$ $Ic_{(1)}$ $Cst_{(1)}$ $Vo_{(1)}$
1981	Majul et al.	[102]	6	$NS_{(6)}$	$NS_{(6)}$	$Es_{(2)}$ $Surg_{(6)}$	$Em_{(6)}$	$Eo_{(2)}$ $AP_{(6)}$ $Lith_{(6)}$

Table 1 Human fascioliasis reports in Argentina, arranged chronologically, including details on infection according to number of cases, gender, province, diagnostic method, treatment, and clinical data *(Continued)*

1981	Alaggia (in Andrada et al., 1983 [90])	[100]	16	$NS_{(16)}$	$NS_{(16)}$	$Surg_{(16)}$	$NS_{(16)}$	$NS_{(16)}$
1982	Pizzi et al.	[106]	54	$NS_{(54)}$	$Cba_{(54)}$	$Ec_{(54)}$	$NS_{(54)}$	$NS_{(54)}$
1982	Siciliano	[41]	101	$F_{(50)}$ M (51)	$Cba_{(101)}$	Ec (61) $Es_{(16)}$ ID (29)	$Em_{(97)}$ $Clq_{(4)}$	$Eo_{(87)}$ $AP_{(78)}$ $Fev_{(58)}$ $Lks_{(69)}$ $WL_{(56)}$ $Anrx_{(53)}$ $Asth_{(67)}$ $Urt_{(37)}$ $Nau_{(31)}$
1983	Andrada et al.	[90]	5	$F_{(5)}$	$Cat_{(5)}$	$Surg_{(4)}$ $NI_{(5)}$	$Em_{(5)}$	$Eo_{(3)}$ $AP_{(5)}$ $Urt_{(1)}$ $Ic_{(2)}$ $Lith_{(2)}$
1983	Giffoniello et al.	[78]	2	$F_{(1)}$ $M_{(1)}$	$Cba_{(2)}$	$Es_{(1)}$ $Surg_{(1)}$ NI (1)	$Em_{(2)}$	$Eo_{(2)}$ $AP_{(2)}$ $Fev_{(2)}$ $WL_{(1)}$ $Asth_{(1)}$ $Lith_{(1)}$ Vo (1) $HA_{(1)}$
1985	Miguel et al.	[97]	5	$NS_{(5)}$	$BA_{(3)}$ $Mza_{(1)}$ $For_{(1)}$	$Es_{(5)}$	$Em_{(5)}$	$Eo_{(1)}$ $AP_{(5)}$ $Lks_{(4)}$ $Ic_{(2)}$
1989	Siciliano et al	[42]	15 (fo2)	$F_{(9)}$ $M_{(5)}$ NS(1)	$Cba_{(15)}$	$Ec_{(12)}$ $Es_{(1)}$ $Surg_{(2)}$	$Em_{(15)}$	$Eo_{(14)}$ $AP_{(15)}$ $Fev_{(15)}$ $Lks_{(10)}$ $Urt_{(8)}$ $Ic_{(2)}$
1991	Melero et al.	[88]	1	$M_{(1)}$	$SL_{(1)}$	$Es_{(1)}$ $NI_{(1)}$	$Tcl_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$ $Fev_{(1)}$ $Lks_{(1)}$ $WL_{(1)}$
1995	Minoprio et al.	[51]	5(fo1)	$F_{(3)}$ $M_{(2)}$	$Mza_{(5)}$	$Ect_{(1)}$ $CE_{(4)}$ $NI_{(3)}$	$Tcl_{(5)}$ $Pzq_{(1)}$	$Eo_{(3)}$ $AP_{(2)}$ $Fev_{(2)}$ $Lks_{(1)}$ $Asth_{(2)}$ $Urt_{(3)}$ $Ic_{(1)}$
2000	Ale et al.	[115]	1	$F_{(1)}$	$SL_{(1)}$	$Ec_{(1)}$	$NS_{(1)}$	$Eo_{(1)}$ $Lks_{(1)}$
2005	Carnevale (in Rubel et al. 2005)	[61]	4	$NS_{(4)}$	$SL_{(4)}$	$Ser_{(4)}$	$NS_{(4)}$	$NS_{(4)}$
2005	Rubel et al.	[61]	1	$F_{(1)}$	$Neu_{(1)}$	$Ser_{(1)}$	$Tcl_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$ $Fev_{(1)}$ $Lks_{(1)}$
2005	Lloret et al.	[54]	5(fo1)	$M_{(5)}$	$Mza_{(5)}$	$Ec_{(2)}$ $Es_{(1)}$ $Ser_{(2)}$ $CE_{(2)}$	$Tcl_{(5)}$	$Eo_{(5)}$ $AP_{(5)}$ $Lks_{(5)}$ $WL_{(2)}$ $Fev_{(5)}$ $Dia_{(2)}$ $Urt_{(3)}$
2006	Salomon et al.	[48]						
2006	Corti et al.	[85]	1	$F_{(1)}$	$Cba_{(1)}$	$Ser_{(1)}$ $NI_{(1)}$	$Tcl_{(1)}$	$Eo_{(1)}$ $Fev_{(1)}$
2008	Rios et al.	[62]	1	$M_{(1)}$	$Neu_{(1)}$	$Ec_{(1)}$ $Ser_{(1)}$	$Alb_{(1)}$	$Eo_{(1)}$ $AP_{(1)}$ $Fev_{(1)}$ $Lks_{(1)}$ $Asth_{(1)}$ $Urt_{(1)}$
2008	Nieto Sosa et al.	[47]	23 (fo2)	$NS_{(23)}$	$Cba_{(8)}$ $SL_{(15)}$	$Ec_{(23)}$ $Ser_{(9)}$	$Em_{(15)}$ $Tcl_{(8)}$	$Eo_{(23)}$ $AP_{(23)}$ $Fev_{(23)}$ $Lks_{(23)}$
2009	Malandrini et al.	[43]	54	$F_{(37)}$ M (17)	$Cat_{(54)}$	$Ser_{(54)}$	$NS_{(54)}$	$NS_{(54)}$
2009	Malandrini et al.	[53]	10	$NS_{(10)}$	$Cat_{(10)}$	$Ser_{(10)}$	$NS_{(10)}$	$NS_{(10)}$

Abbreviations: F: female; M: male; NS: not specified; Fo: family/group outbreaks.

Provinces: BA: Buenos Aires; BAcity: Buenos Aires city; Cat: Catamarca; Cha: Chaco; Cba: Córdoba; For: Formosa; Ju: Jujuy; Mza: Mendoza; Neu: Neuquén; Sal: Salta; SL: San Luís; SFe: Santa Fé; Tuc: Tucumán.

Treatment: Em: emetine; Tcl: triclabendazole; Alb: albendazole; MFE: male fern extract; Pzq: praziquantel; Clq: Cloroquine.

Diagnostic Method: Ec: egg observation in coprological sample; Es: egg observation in sample obtained by sondage; ID: intradermal test; Surg: surgical; CE: clinical/epidemiological; Ect: ectopic presentation; NI: non invasive image-based diagnosis; Ser: serology.

Clinical Data: AP: abdominal pain; Anrx: anorexia; Asth: asthenia; Cst: Constipation; Dia: diarrhea; Eo: eosinophilia; Fev: fever; HA: headache; Ic: ictericia; Lks: leucocitosis; Lith: lithiasis; Nau: nausea; Urt: urticaria; Vo: vomiting; WL: weight loss.

Note: number of cases noted in parenthesis

(68.52% out of 54 subjects) was also found in the serological study performed with ELISA by Malandrini and collaborators [43] in the locality of Taton, Tinogasta, Catamarca, the only randomized survey carried out in Argentina so far. Unfortunately, no studies on eggs per gram of faeces (epg) have been perfomed in Argentinian patients, so that a gender relationship with intensity could not be assessed. Although that apparent prefer- ence for females is not statistically significant in Argen- tina, this trend is consistent with what has been described in human endemic areas: significantly higher intensities in females in Bolivia and Peru [19-22], and significantly higher prevalences in Chile [17] and Egypt [44]. However, such a female preference rule does not always appear to be significant at prevalence level [19-22,45,46].

Age in years was specified in 219 (35.38%) patients and noted for guidance only (e.g., child, adult) in another 12. The range was from 3 [41] to 95 years of age [43] (average 37.09 ± 17.07 years) (Figure 2). In Argentina, the only random survey detected positivity from small children to old individuals, without age preference [43]. This absence of age correlation contrasts with other countries, where prevalences and intensities peak in the 9-11 age group, although adults and old age groups may also show high infection rates, as is the case of Bolivia [19-21], Peru [22] and Egypt [44]. However, no differences between age groups were found in Chile [17] or Iran [45].

In Argentina, several outbreaks presenting typical foodborne characteristics appear related to the most common risk factor: ingestion of watercress naturally growing along the river- and stream-beds picked during recreational, weekend or vacation activities. Many of these field excursions are undertaken by a family or as a group activity. This explains why family outbreaks have

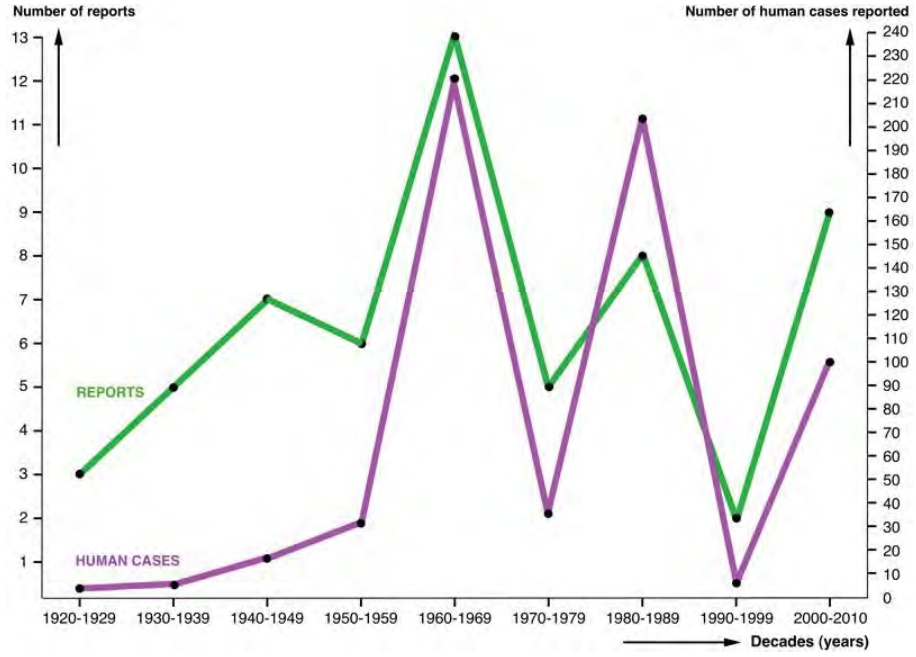


Figure 1 Reports on human fascioliasis in Argentina. Evolution of the number of reports on human fascioliasis and number of human infection cases in Argentina according to decades.

been noted to be common, whereas isolated cases seem to be rare in the country [47]. Outbreaks described in eleven families involving a total of 63 people [42,47-52], including a maximum of up to 15 family members affected at once [47], are good examples. However, results obtained in a recent serological survey of a local resident population in Catamarca [43] show that not all situations including several infected subjects are in fact family outbreaks.

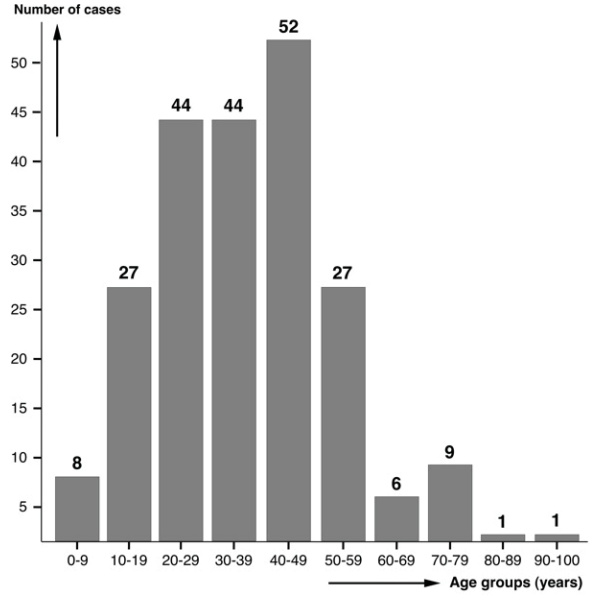


Figure 2 Age of fascioliasis patients. Age distribution in 219 patients in whom fascioliasis was diagnosed.

Biogeographical aspects

Geographical distribution according to provinces

The geographical origin of the patients was specified in 587 patients (94.83%), whereas in 32 cases (5.17%) not even the province of origin was noted. Sometimes only the province where the infection occurred was mentioned, in other instances the specific locality was also added.

Human cases have been found in 13 out of the total of 23 provinces plus Buenos Aires capital, covering more than half (51.48%) of the total surface of continental Argentina. Cordoba, Catamarca, San Luis and Mendoza include the largest number of patients detected, the remaining provinces being only rarely affected (Table 2 Figure 3). Human infection has most probably been completely overlooked in La Rioja and San Juan provinces, with no case reported despite being surrounded by the aforementioned provinces. Adequate studies are needed to assess whether human infection occurs in La Rioja and San Juan given their physiographic and climatic characteristics which suggest infection risks similar to those in the aforementioned neighbouring ones.

The pronounced concentration of cases in the Cordoba and Andean areas is worth mentioning, although not in absolute numbers, at least with regard to the

Table 2 Evolution of human fascioliasis infection reports in Argentina, according to provinces where infection was presumed to have occurred, number of cases and respective articles furnishing the information

Province	Total No. of cases	No. of cases per individual references (ordered according to year of publication)	Observations
Cordoba	430	1 case published in 1937 [91]; 1 in 1940 [76]; 4 in 1942 [52]; 1 in 1943 [93]; 1 in 1943 [77]; 1 in 1944 [109]; 5 in 1952 [38]; 9 in 1954 [39]; 22 in 1961 [40]; 150 in 1961 [unpublished by other colleagues, cited in 40]; 19 in 1961 [72]; 2 in 1967 [114]; 1 in 1967 & 1969 [94,95]; 11 in 1972 [36]; 12 in 1972 [49]; 54 in 1982 [106]; 101 in 1982 [41]; 2 in 1983 [78]; 15 in 1989 [42]; 1 in 2006 [85]; 8 in 2008 [47]	Two papers deal with the same case [94,95]
Catamarca	73	1 case published in 1952 [38]; 1 in 1954 [39]; 1 in 1961 [40]; 1 in 1972 [36]; 5 in 1983 [90]; 54 in 2009 [43]; 10 in 2009 [53]	these 73 cases do not include 10 serologically suspicious patients who could not be confirmed due to absence of eggs in stools [116]
San Luis	29	1 case published in 1927 [33]; 1 in 1930 [35]; 2 in 1969 [87]; 4 in 1973 [50]; 1 in 1991 [88]; 1 in 2000 [115]; 4 in 2005 [Carnevale in 61]; 15 in 2008 [47]	these 29 cases include an 11% seropositivity found in 34 samples obtained randomly in the population by Carnevale [unpublished data in 61]
Mendoza	28	1 case published in 1955 [101]; 1 in 1964 [86]; 15 in 1969 [87]; 1 in 1985 [97]; 5 in 1995 [51]; 5 in 2005 & 2006 [48,54]	Two papers describe the same outbreak [48,54]
Tucuman	6	1 case published in 1939 [69]; 1 in 1944 [109]; 1 in 1947 [79,80]; 1 in 1961 [110]; 1 in 1970 [99]; 1 in 1972 [36]	Two papers refer to the same case [79,80]
Buenos Aires	5	1 case published in 1933 [108]; 1 in 1942 [52]; 3 in 1985 [97]	
City of Buenos Aires	4	1 case published in 1939 [92]; 1 in 1955 [55]; 2 in 1965 [107]	
Salta	3	1 case published in 1944 [109]; 1 in 1953 [89]; 1 in 1964 [112]	
Santa Fe	3	1 case published in 1940 [76]; 1 in 1933 [Scrimaglio in 93]; 1 in 1969 [103]	
Neuquen	2	1 case published in 2005 [61]; 1 in 2008 [62]	
Jujuy	2	1 case published in 1944 [109]; 1 in 1964 [113]	
Chaco	1	1 case published in 1954 [98]	
Formosa	1	1 case published in 1985 [97]	
Province not specified	32	1 case published in 1924 [32]; 1 in 1928 [34]; 1 in 1962 [111]; 2 in 1965 [107]; 5 in 1973 [104]; 16 in 1981 [100]; 6 in 1981 [102]	
Total: 13 provinces	619 cases	58 reports	

proportion of human case distribution. Unfortunately, human community surveys (active detection) have not been undertaken. All reports concern symptomatological subjects who voluntarily seek medical assistance (passive detection), except the only survey performed [43,53] whose results increased the human case number for Catamarca province from 9 to 73.

The very high case number in Cordoba results from patients diagnosed by different physicians throughout many decades (see Table 2), mainly at the end of the 50s and beginning of the 60s as noted in the compilation, made by one active Cordobese author, of the many patients diagnosed by himself plus the 150 patients diagnosed by other Cordobese colleagues, and which he presented at the Primer Congreso Médico Sanitario de la Provincia de Cordoba, held in La Falda on August 1957 [see [40]. Although of course such a pronounced case number difference when comparing Cordoba with other provinces may in part be the consequence of a bias due to the absence of similarly actively publishing authors in the other provinces, the very high case number in Cordoba merits an analysis. The literature review suggests an explanation related to the very large number of villages and towns playing an important role in recreational, weekend or holiday activities. These recreational areas attended by thousands of tourists, campers or weekend visitors overlap with areas in which lymnaeids and animal fascioliasis are present (Figure 4) [27]. In such places, the infection risk for a large amount of people is greater than in scarcely inhabited Andean areas. Moreover, the social, cultural and economic level of people spending holidays in such areas is high and the likelihood for them to seek appropriate medical assistance and obtain a correct diagnosis is greater than for people living in rural areas.

This highlights that people may become infected in a place different, sometimes even far or very far away,

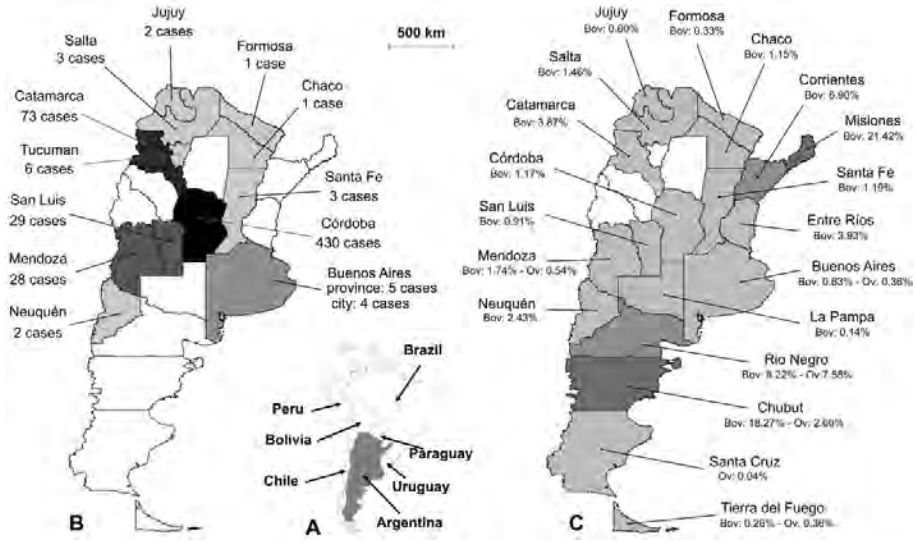


Figure 3 Geographical distribution of fascioliasis in Argentina. A) country location in the southern cone of South America; B) distribution of human fascioliasis infection reports (number of cases), according to provinces where infection was presumed to have occurred; C) distribution of fascioliasis in livestock including province prevalences according to slaughterhouse condemnation data for the 2006-2009 period provided by the Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA) (bov = bovines; ov = ovines).

from the place where they live. Several family outbreaks described in Cordoba province support this assumption: the first such outbreak in La Calera [52], another family infected during a picnic, another during a few days camping, another family buying uninspected watercress sold by a street vendor [49], and finally an eight-member family in La Punilla [47]. In San Luis province, an outbreak involved two families that camped together in El Volcan [50], and another affected 15 family members in Merlo [47]. Similar family outbreaks occurred in Mendoza province, one involving five members [51] and another with five members infected during a trip to the Andean region of San Carlos [48,54].

Distribution according to altitude

Even though human infection risk is present in many geographical regions of the country, data indicate higher probabilities in given high altitude areas. Indeed, the great majority of cases including information on infection place (574: 97.79%), are from hilly or mountainous areas. Human reports appear concentrated in: (i) the central mountainous areas of Cordoba and San Luis, and (ii) the Andes mountains, mainly in Andean valleys.

Cordoba, the province with the greatest amount of cases, is a good example, with practically all human cases coming from its western mountainous areas (Sierras de Cordoba), despite higher precipitation rates and livestock abundance in its eastern lowland plains. Nevertheless, a possible sample bias cannot be ruled out in this case concentration due to the extensive patient-record publica- tion by two local authors [38-42]. Similar situations are found in San Luis and Mendoza provinces, both with all human cases from their western mountainous areas, instead from their eastern plains.

Only 13 cases (2.21%) originated from areas near sea level and flat terrain, namely from Buenos Aires province and City, Sante Fe, Chaco and Formosa. These few reports were all related to important rivers. One interesting case was a patient from Buenos Aires city who declared not having left the city in the previous 17 years [55].

Such a case concentration in mountainous areas is not unlike Bolivia, Peru and Chile, where human endemic areas are linked to altitude areas, as a consequence of both (i) geographical distribution of the main lymnaeid vectors involved in transmission to humans restricted to or preferring such altitude areas [56], and (ii) the greater liver fluke transmission capacity in high altitude areas [24]. This suggests the appropriateness of verifying whether human fascioliasis endemic situations may also exist in high altitude areas of Argentina.

Links to livestock infection

The distribution of human infection does not appear to fit the one of animal fascioliasis, which covers the whole country according to official slaughterhouse records (Figure 3). In this respect certain areas are worthy of note, such as Corrientes province with absolutely no human case reported in the literature but high prevalences in livestock [57-60], and Neuquen province with only two human cases reported [61,62] despite a very high prevalence in cattle [63].

A similar lack of geographical fit between human and animal fascioliasis has already been seen in other

Figure 4 Human infection risk. A recreational farm in the locality of Perdriel (902 m altitude), Mendoza province, well known because of its great touristic attraction, mainly during holiday periods and weekend outings: A) water collections of an artifical pond, and B) the overflow originated from an artificial irrigation channel, where the snail vector species *Lymnaea neotropica* was collected and cattle, goats, horses, donkeys and llamas proved to be infected [27].

countries [3,4]. Unfortunately, in Argentina it becomes impossible to ascertain whether this is the real situation or only a distorted picture due to incomplete data. Lack of appropriate human community surveys in areas with high prevalences in animals, absence of human-case reporting due to non-obligatory declaration, and overlooked human infection related to misdiagnosis or to inhabitants of rural areas not attending health centres for diagnosis may explain such situations.

However, the lack of geographical fit in question may also be due to an altitude factor, in its turn related to both (i) altitudinal selection of lymnaeid vector species with ecological characteristics adequate for transmission to humans and (ii) altitudinal climatic factors enhancing *F. hepatica* life cycle development, as already demonstrated in other Andean countries [24,56]. Concentration of human cases in hilly or mountainous areas support this altitude explanation.

Seasonality

Both fasciolid life cycle and lymnaeid population dynamics are markedly dependent on climate, mainly temperature and rainfall [5-7,64]. This climatic influence is evidenced by three different transmission patterns which define human and animal infection characteristics [3,65]: monoseasonal, biseasonal and annual, permanent depending on the existence of one rainfall concentration period per year, two of them, or appropriate water body availability throughout the year [5,56]. Sometimes seasonality is related to the ingestion of contaminated plants, with most human cases occurring during the watercress season [2].

To estimate the moment when metacercariae were ingested, a prepatent period of 2-4 months should be considered. This overlaps with the acute phase, egg appearance in faeces marking the beginning of the chronic phase [66]. For instance, in Europe, human infection takes place in summer and autumn and symptoms appear in winter, and a prolonged and wet summer has often been followed by an outbreak [2].

In Argentina, a total of 110 case reports were found in which the month of the first appearance of symptoms was noted. Most of them (97) corresponded to reports from Cordoba province, of which 93 had already been analysed from that point of view [41].

A first pronounced January-April peak appears in the monthly distribution of these 110 cases. When comparing case distribution with the annual distributions of mean monthly data of precipitation and humidity and with mean monthly data of maximum and minimum temperatures for Cordoba province concerning the decades 1961-1970 and 1971-1980 during which the patients were infected (Figure 5), significant correlations with monthly precipitation, monthy maximum temperature and monthly minimum temperature appeared when time lags of 2 months (p values of 0.013, 0.008 and 0.0069, respectively), 3 months (p values of 0.003, 0.0049 and 0.0035, respectively) and 4 months (p values of 0.0389, 0.0293 and 0.0358, respectively) were considered. This fits with the logical delay between infection moment and symptom appearance and diagnosis. However, this largest peak of January-April may not only be due to higher precipitation and temperature data. Many people enjoy summer holidays in January and February in Cordoba province, so that the increase of recreational field activities may also interact with rainfall and temperature increases in inducing this monthly January-April incidence peak.

There is a second peak in June which poses a question mark, as no such climatic correlation appears. Other factors may be involved, such as the second yearly increase of recreational field activities during the Easter holidays. Metacercariae are known to keep their

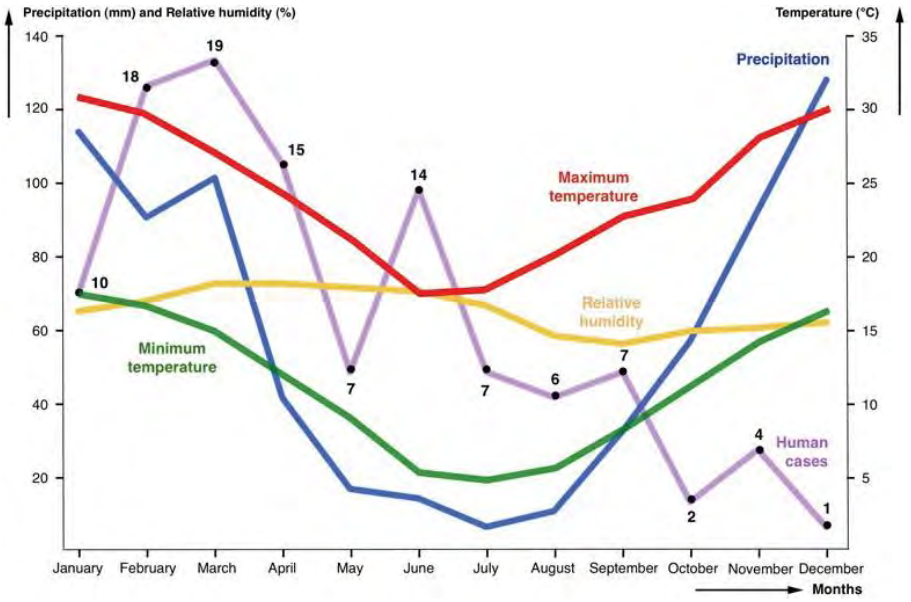


Figure 5 Human fascioliasis monthly incidence. Distribution of human fascioliasis incidence according to the month in which symptoms appear, compared with the annual distributions of mean monthly data of precipitation, humidity, and maximum and minimum temperatures. Data concerns the province of Cordoba in the 1960's and 1970's when the patients were infected.

infectivity for months (even up to two years) under adequate environmental conditions [28], so that remaining metacercariae, namely those already available but not having been ingested from January, may be the cause of that delayed incidence peak.

Human infection mainly during the first months of the year is also suggested in another 15 case reports from Cordoba, in which the first month with symptoms was unfortunately not specified (therefore not included in the aforementioned statistical analysis) but the season of autumn (i.e., 21 March to 21 June) was noted [42].

However, in Argentina other transmission patterns and human infection risk seasonalities/periodicities may not be ruled out according to the large physiographical heterogeneity of the different endemic areas of such a vast country.

Relationships with annual climate changes

The climatic dependence of fascioliasis is also known to modify the interannual distribution of human case detection, with increases in years with heavy rainfall [2,66].

When analysing the evolution of the human case number in the whole country according to decades, one peak appears in the 1960s and another one in the 1980s (Figure 1). The annual human case number for a more detailed study was, however, only sufficient when restricted to Cordoba province during the 1960's and 1970's. The comparative analysis with annual precipitation (Figure 6) shows that the 1972 outbreak may have been caused by the sudden increase of rainfall in that year in Cordoba province, as already highlighted a long time ago [41]. However, we could not find any signifi- cant correlation.

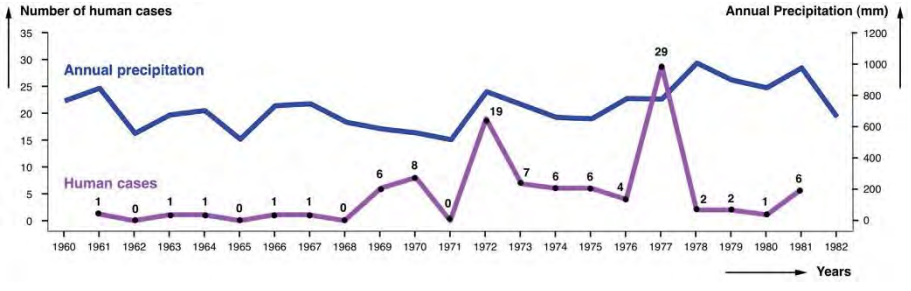


Figure 6 Human fascioliasis annual incidence. Distribution of human fascioliasis incidence according to the year in which symptoms appear, compared with annual precipitation data. Data concerns the province of Cordoba in the 1960-1982 period during which the patients were infected.

The most pronounced outbreak in 1977 does not appear to have any apparent climatic causal origin, although it was argued that this year was the one with the largest amount of precipitation during the 1973-1981 period [41]. We have confirmed that the rainiest year in the Cordoba area in question was 1978 and not 1977. Thus, this second human report increase may have been linked to a passing fad or temporary professional trend for reporting.

Sources of human infection

Ingestion of freshwater plants carrying attached infective metacercariae, among which preferentially watercress, is known to constitute the main fascioliasis infection source in humans worldwide [3,67]. The consumption of aquatic plants other than watercress, as described in the hyperendemic area of Bolivia [30], is not a common practice in Argentina. Watercress is almost the only aquatic wild plant regularly consumed. In this country, with the highest beef consumption in the world, it is traditional to accompany barbecue meat ("asado") with salad. When camping or doing outdoor activities, it is very common to go to a stream and collect watercress to cover for the absence of salads.

Previous watercress ingestion has been described in 214 patients. A relationship between watercress and fascioliasis was described already long ago [33,35] and has recently been highlighted again in an editorial article [68]. Among a total of 101 cases reported from Cordoba, 94 (93,07%) had a history of watercress consumption [41].

Besides watercress, there are a very few reports which relate fascioliasis to other plants. In two cases from Cordoba province, ingestion of dandelion (*Taraxacum officinale*) was noted as a possible infection source [41]. Two other patients are mentioned to have chewed on grass that grew on a riverbank [69].

Water consumption has also been involved as a human infection source [58]. In Argentina, natural water was already presumed to be an infection source for livestock as early as two centuries ago [37]. With regard to humans, contaminated water as a possible infection source was noted in only two patients who mentioned having drunk natural water from mountain streams [51,69].

Diagnostic aspects

Methods and techniques used

The diagnosis of human fascioliasis poses well-known methodological and technical problems [3,70,71]. In Argentina, the diagnostic method used was specified in the majority of the human patient reports analysed (454 cases: 73.34%) (Table 1). In several cases, even more than one method/technique was used. The diagnostic method was not specified when referring to 150 unpublished cases detected by other colleagues [40], and it was insufficiently clarified in 15 other cases mentioned in an endnote as "patients having been diagnosed while in press" [72].

Fascioliasis diagnosis in Argentina has usually relied on traditional techniques, mainly egg detection (288 positive patients), followed by common serological techniques (82), intradermal reaction (63), fluke finding during surgery (45), and erratic fluke observation (6). Sensitivity and specificity of these techniques are far from the standards in more modern ones [46,73,74]. Additionally, the absence of the use of quantitative methods, such as the standardized Kato-Katz technique to assess both pathogenicity and intensity for adequate treatment dose selection [75], is worth mentioning.

An historical account (see Table 1) shows that diagnosis by egg identification was the method used from the very beginning and, although still used in the last decade, surgical findings were common during the 1969-1989 period and serology from 2005 onwards. The intradermal test appears to have been used only during the 60's and at the beginning of the 70's and abandoned after 1982. Other methods based on clinical/epidemiological observations, non-invasive techniques, and ectopic presentations have been only sporadically applied.

The long delay with which many patients were diagnosed should be emphasized. In given reports on 26 patients, the time elapsed between the appearance of symptoms and confirmation of infection by appropriate diagnosis is noted in days or sometimes years. Calculated delay average is very high, of 1262 days, nearly three and a half years, and there are references about patients having suffered from symptoms for ten or more years without diagnosis [33,34,76-80]. This suggests either infected subjects not looking for professional diagnosis due to mild symptoms of low fluke burdens and/or misdiagnosis of patients due to the non-pathognomonic clinical picture, easily confused with other diseases when the patient attends a health centre not used to dealing with fascioliasis [81].

Egg finding

Coprological analyses were performed in 278 patients, including positive egg finding in 221 (79.50%). Details about the coprological technique used were very rarely described. Where noted, the Charles Barthelemy sedimentation technique, M.I.F., flotation, Baerman and multiple concentrations were mentioned, although without giving further specifications [40]. Other techniques, such as the veterinary Lumbreras Rapid Sedimentation technique [42] and those of Faust and Sheather [48], have been also used in Argentina.

Duodenal sondage was performed in 90 patients, yielding egg detection in 67 (74.44%). Interestingly, nine cases appeared negative in a coprological examination, while positive in duodenal sondage.

Despite egg finding still being the gold standard today, problematic situations giving rise to overlooking an infection may be taken into account, such as (i) absence of egg shedding during the acute phase, (ii) no egg production by given fluke strains when in humans, and (iii) lack of sufficient sensitivity in light infections. These are common in human sporadic infections in animal endemic areas, as appears to be the case in many reports from Argentina, e.g. in travellers, weekend family outings, and tourists. Thus, the widespread use of an egg finding technique as the single method for patient diagnosis may have been the reason for overlooking human infection in Argentina in the past.

Unfortunately, no study on egg size variability in human stools has been performed in the country. Con- sequently, given the absence of *F. gigantica*, the recently corrected egg size ranges to be henceforth used in Argentina are 100.6-162.2/65.9-104.6 μm in humans and 73.8-156.8/58.1-98.1 μm in animals [82], which are pronouncedly different from the range of 130-150/63-90 μm previously used both for humans and animals worldwide.

Serology

Its use in Argentina has been reported only recently, despite serological techniques having been available for this disease for a long time [71]. However, an ELISA developed locally by means of recombinant procathepsin L cystein proteinase was successfully assayed in test serum samples from 16 coprologically positive patients [83], and a Micro-ELISA method showed a sensitivity of 100% and a specificity of 97% when applied to 22 test serum samples from patients with fascioliasis infection previously verified by coprology, surgical observation, or retrograde cholangiopancreatography, thus proving to be highly useful, mainly for the previous screening of a large amount of samples [84]. The technique specified was such an ELISA in 69 cases [43,48,53,61,62]. In another case, an ELISA without further details specified was employed [85].

In 13 patients the serological technique applied was not mentioned [Carnevale, unpublished data in 47,62]. In three patients diagnosed by coprology or duodenal sondage, serology appeared positive, being negative in two other patients in whom eggs were not found [48]. Surprisingly, when applying serology to 11 patients shedding eggs in their stools, only nine of them gave a positive serological result [47]. The two coprologically positive although serologically negative patients suggest either spurious cases (fluke eggs in transit after infected livestock liver ingestion) or lack of sufficient sensitivity of the serological test applied. The opposite, that is negative coprology and positive serology, was found in another case [85]. In many human case reports, serology was the sole diagnostic method used [43,53,61].

Serological diagnosis was mentioned to have been used in the only article dealing with a random human survey performed in Argentina up to the present. In 148 randomly selected subjects from Tinogasta, province of Catamarca, ELISA was positive in 54 of whom (36.48%), as well as in ten among other 14 patients from the same locality [43,53].

Intradermal tests

This diagnostic method, today known to be insufficiently specific [70] and considered obsolete for the diagnosis of individual patients although still potentially useful as a quick indicator within broad field screening, seems to have been quite commonly used in Argentina in the sixties, seventies and eighties (in chronological order): 1 case [86], 17 cases [87], 12 cases [49], 4 cases [50], and 29 cases [41]. Since there has never been a commercial or standardized test, details on antigen and correlation with other diagnostic tests noted in the aforementioned articles need to be taken into account.

Diagnosis by non-invasive techniques

Many image-based diagnostic techniques are useful for fascioliasis [2,8,70] and have also been applied in Argentina since the eighties, although mainly for initial detection followed by confirmation by another more specific diagnostic technique [51,78,85,88-90].

Ectopic cases

In a 25-year-old male patient, a fluke was eliminated through the urethra [91]. In a female patient undergoing surgery for appendicitis, a fluke was removed from the appendix [92]. A *F. hepatica* specimen was found when surgically opening a tumor at the level of the last rib in a 32-year-old man who lived in the endemic zones of Cordoba, San Luis and Mendoza [93]. In a 35-year-old woman from Tucuman, *F. hepatica* eggs were found in peritoneal granulomas adhered to the gall bladder and near to the transverse colon [79,80]. Another case of intracranial fascioliasis was reported in a 44-year-old female from Cordoba [94,95]. More recently, a case of cutaneous fascioliasis was described in a 26-year-old male from Mendoza [51].

Clinical/epidemiological diagnosis

In some cases, patients were diagnosed based on clinical and epidemiological characteristics compatible with fascioliasis even though coprological or serological analyses yielded negative results.

In an outbreak, four family members living in the same dwelling had shared meals including watercress and presented compatible symptoms, although eggs were only found in two of them [52,93]. One of the coprologically negative patients had eosinophilia, fever and generalized pain. Symptoms disappeared after emetine treatment and in two months the patient had gained 7 kg weight. The other negative patient also showed eosinophilia (52%) and suffered generalized pain but no temperature. After

treatment, pain disappeared and eosinophilia diminished to 14%.

In another family outbreak [51], the wife of a male patient showing a cutaneous fluke, had pruritic skin lesions, eosinophilia and compatible images in the gall bladder during ecography later confirmed by CATScan; her symptoms disappeared after triclabendazol treatment. The mother had asthenia, urticaria, and multiple gall-stones that did not allow for the visualization of parasites through ecography; her symptoms also subsided after treatment. The brother-in-law had eosinophilia and fever, and became asymptomatic after triclabendazole selftreatment. A fifth patient, a 14-year-old girl who participated in the same family outing, had hepatomegalia and urticaria, compatible images upon ecography, and also recovered after triclabenzadole medication.

After consuming wild watercress during an outing, five persons showed compatible symptoms, including eosinophilia and leucocytosis. In three of them diagnosis was confirmed by coprology and/or serology. In the remaining two patients, analyses yielded negative results but they were considered as having been infected when recovering after fascioliasis treatment [48].

Medical aspects

Clinical findings

Symptoms, laboratory results and their frequency do not appear to differ from those described elsewhere. Articles reviewed are very diverse in nature. Some include detailed clinical descriptions, but many are merely an enumeration of human cases with very vague or sometimes even no accompanying clinical data at all. Thus, information has to be treated with caution. Establishing prevalences of symptoms in the total population of patients becomes impossible, and assumptions may only be obtained from the few articles in which the symptomatology was sufficiently described (Table 1).

Of 225 patients in whom eosinophil counts where performed, 198 (88.00%) had eosinophilia. In 143 patients, authors did not note the eosinophil level. Among those in whom it was quantified, the mean was 28.00% (± 21.33%). Very high counts were found in some patients, with a maximum of 84% [69].

Leucocytosis was found in 128 of 167 patients (76.65%) in whom leucocyte counts were analysed, with a mean of 12478.72 (± 7917.89), the lowest value of 4,400, and the maximum of 52,600 found in the aforementioned patient in whom eosinophilia of 84% was also detected [69].

Fever was described in 138 among 153 patients (90.20%) in whom temperature was analysed. In the great majority it was just referred to as simply presenting fever. In the only 10 patients in whom the exact temperature was described, the mean was 39 °C (± 0.62 °C). In the article in which a higher number (69) of patients was recorded in whom temperature analysis was performed, 58 (84.06%) had fever [41].

Abdominal pain, noted in 200 patients, appears to be the most frequent finding. Unfortunately, it appears not always adequately described. Thus, in most instances only terms as "diffuse", "right hypocondrial pain", "epigastrial", or similar are mentioned.

Weight loss due to the disease was found in 76 of 91 patients (83.52%) in whom a weight control follow-up was made. In the majority of cases only presence or absence of weight loss is stated. It was quantified in only 8 patients, with a range from 4 to 21 kg lost (mean 13.00 ± 5.48 kg). In two articles, the weight loss was classified according to its degree [41,49]: in 20 patients it was slight, in 18 moderate, and in 29 intense or very intense.

Anorexia was described in 53 out of 74 patients (71.6%) in whom this symptom was evaluated in a study [41]. In the literature analysed, anorexia appeared described only in one other patient [69]. Asthenia was described in only 86 patients in Argentina, whereas weakness is commonly associated with mild to moderate anaemia during the acute phase elsewhere [2]. Urticaria was only found in 62 Argentinian patients, although this symptom is considered a distinctive feature in the early stage of fluke invasion [2]. Other less diagnosed symp- toms in the country include nauseas in 38 patients, ictericia 23, lithiasis 17, vomiting 11, headache 9, diarrhoea 8, and constipation 6.

Co-infection with other parasites

Co-infections of fascioliasis with other protozoan and helminth species have recenly proved to be the usual rule in human fascioliasis endemic areas [3,19-22,44]. Clinical synergistic associations of fascioliasis with other pathogens are believed to be at the base of high morbidity and mortality rates in children [66], immunological responses being markedly suppressed and concomitant infection being exacerbated following liver fluke infection [12,96].

In Argentina, unfortunately patient analyses do not appear to have particularly focused on co-infections. The most frequently reported parasite co-infecting with *F. hepatica* appears to be *Echinococcus granulosus*, including a total of 14 patients reported to simultaneously present both parasites. All of these cases were from an hydatidosis endemic zone in Mendoza province [87,97]. This relative high number of fascioliasis-hydatidosis co-infected patients is outstanding, as such a co-infection has never been detected in human endemic areas of other countries so far.

Other parasite species found in the coprological diagnosis of fascioliasis patients were: *Entamoeba coli* (6 patients), *Giardia intestinalis* (5), *Blastocystis hominis*

(4) and *Entamoeba histolytica* (2) among protozoans, and *Enterobius vermicularis* (1), *Strongyloides stercolaris* (1) and *Hymenolepis nana* (1) among helminths. Only two patients have been described to present more than one parasite species additional to *F. hepatica*, namely *G. intestinalis* and *E. vermicularis* in one case [98], and *E. coli* and *G. intestinalis* in another patient [72]. In total, only 30 patients had co-infections with other parasites, of whom only 16 had intestinal parasites. This low amount of co-infection reports may perhaps partly be explained by the geographical origin of the fascioliasis patients, usually dry regions where other helminth diseases are relatively difficult to find, and also by the age of the patients, since most were adult subjects in whom intestinal parasitic diseases are not so prevalent.

Treatment

This was specified in 212 patients. Drugs mentioned to have been used were emetine (in 186 patients), triclabendazole (21), cloroquine (4), praziquantel (1), albendazole (1) and male fern extract (1). In two patients, more than one drug was used. A historical analysis shows that emetine was the only drug used up to the end of the 80's, except for the sporadic use of chloroquine [41]. From the beginning of the 90's, triclabendazole became the drug of choice, although praziquantel, albendazole and again emetine were applied in given cases (Table 1).

The first successful treatment report was with emetine intravenous administration in a 38-year-old female patient. Fever and abdominal pain subsided shortly after treatment and coprological analyses showed the disappearance of eggs [33]. Emetine was also sucessfully used a few years later [35] and in another patient to solve the lack of effectiveness of male fern extract [76]. In Argentina, only a few treatment failures with emetine have been reported [39]. An efficacy of 92.4% was obtained in 65 patients in whom *F. hepatica* eggs ceased to be found after treatment [39]. Emetine was even recently used in a family outbreak involving 15 patients, amongst whom two presented severe hypotension. Coprological analyses became negative 60 days after a 10-day-long treatment [47].

Emetine derivatives were the classic drugs and continue to be used today, administered intramuscularly or subcutaneously, at doses of 1-10 mg/kg emetine/day for ten days [8,42,99]. Worldwide, dehydroemetine, at a dose of 1 mg/kg daily for 10-14 days, was considered the therapy of choice a few decades ago [2,8,70]. How- ever, emetine derivatives cause a variety of toxic manifestations involving the heart, liver and digestive tract. A cardiac counter-indication led to the ineffective treatment with chloroquine of four patients to whom eme- tine could not be prescribed: all of these patients continued to shed fluke eggs after cloroquine adminis- tration [41].

Even though there was no triclabendazole formulation for humans available in Argentina, it was, nevertheless, given to patients in its veterinary form, usually with prior consent. The first report of triclabendazole treatment concerned a dose of 10 mg/kg in a 40-year-old male patient with prolonged high temperature and eosinophilia, including a repeated dose 9 weeks later. Clinical symptoms and eggs in stools disappeared thereafter [88].

Another report of triclabendazole use concerned a family outbreak. First, a 26-year-old male patient with fever, eosinophilia, hepatic abscesses, and an ectopic subcutaneous fluke in the abdominal region, was treated with 900 mg/day praziquantel for three consecutive days, with initial disappearance of symptoms. After relapsing two weeks later, the patient was re-treated with 750 mg praziquantel every 8 hours for two days. As symptoms persisted, 10 mg/kg triclabendazole were applied three weeks later. Symptoms disappeared within 48 hours, and normalization of clinical and laboratory parameters was obtained. Four other patients of the same outbreak were also successfully treated with triclabendazole [51]. Triclabendazole was also used at a dose of 400 mg/day for two days in a 20-year-old female [61], as well as with a single 10 mg/kg dose in a 58-year-old woman [85], both with remission of symptoms. In an eight-person family outbreak, six patients received triclabendazole (dosage and protocol not specified) with elimination of egg shedding. Another patient had to receive a second dose to stop egg shedding, and three triclabendazole doses were needed for the last patient [47].

Albendazole at a dose of 400 mg/12 hours was used in a 54-year-old man with remission of clinical manifestations within days. This report stated that albendazole was used for it was impossible to obtain triclabendazole [62]. Although Egaten® (triclabendazole, the drug of choice for human treatment at present) has been recently available from WHO [13,75] it has been still never applied in Argentina. Nitazoxanide has been approved for human use in Argentina for fascioliasis, but no reports have been found in the literature.

Surgical cases

Even though the number of patients in whom surgery was involved is small (6.9%), publications dealing with surgical cases appear to be proportionally important (15; 26.7%). Surgical description was indeed the main objective of several articles. In the first surgical case reported, actually the third autochthonous case of the country [34], the intervention was described in great detail, but no epidemiological information was provided, not even the province of origin of the patient.

In 45 cases, a surgical procedure contributed to the diagnosis when flukes were unexpectedly found upon liver exploration. The largest number of surgical cases

described within one article is 16 [100]. Unfortunately, sometimes no details were given and cases were merely referred to as surgical [72].

In the majority, surgery was indicated due to abdominal pain and biliary obstruction suggestive of lithiasis. Indeed, gallstone disease has recently proved to be one of the effects of advanced chronic fascioliasis, since *F. hepatica* is able to survive up to 9-13.5 years within a human host [9]. Hence, such a high lithiasis proportion suggests that long-term infected patients having been overlooked for a long time has been a relatively frequent situation in Argentina. This agrees with the not unusual long delay in diagnosis already emphasized before, and both observations together pose a question mark about human fascioliasis detection in the country.

In most of these surgical cases with lithiasis suspicion, the patient inhabited a large city (Buenos Aires, Cordoba, Mendoza, Tucuman) as opposed to a rural area where attending a health centre is less usual due to economic reasons or at least complicated due to the long journey that has to be made. This additionally suggests a far greater underestimation of the problem in rural areas.

The importance of intraoperative cholangiography was highlighted in cases in which, even though gallstones were removed, evidence of obstruction observed during the cholangiography led to the finding of flukes [55,89,101]. Gallstones were found and concomitant fascioliasis diagnosis made while performing choledoctomy in another six cases [102]. Two more patients with lithiasis in whom *F. hepatica* was diagnosed upon surgery were described later [42].

In a patient in whom a gallstone was suspected, intraoperative cholangiography showed that in fact, instead of a lithiasis problem, a *F. hepatica* specimen was involved [78]. In another patient operated due to lithiasis suspicion and to resolve a hiatus hernia, stones were neither found at cholecystectomy but flukes were after undertaking an intraoperative cholangiography that indicated estenosis altering normal bile flow to the duodenum [103]. Similarly, no stones were observed but parasites found when performing choledoctomy in another patient diagnosed shortly after [99].

Among four operated patients, fluke infection was detected in two only after a second surgical intervention. In the first operation, cholecistectomy was performed to remove stones but flukes were not detected since intraoperative cholangiography had not been applied. Upon re-operation, fascioliasis was diagnosed when *F. hepatica* was found in the common bile duct [104].

The usefulness of intraoperatory cholangiography for fascioliasis diagnosis was higlighted for cases in which preoperatory diagnosis was difficult [90]. This was concluded when operating five cases due to severe abdominal pain and lithiasis suspicion in only three of them.

In an interesting case, diagnosis was made after surgery to the brain when an expansive parasagital process was diagnosed by means of a carotid arteriogram performed in a patient with memory loss, nominal aphasia and discrete right facial paresia [95]. Two cysts containing *F. hepatica* eggs were extracted from the cortex. The patient died 24 hours after surgery. This is one of the few fatal cases known to be due to fascioliasis worldwide [2]. In Argentina, the other reported fatal case was the first ever to be diagnosed in the country [31], but since it was an imported case it is not accounted for in the present study.

Present situation and future perspectives

All aforementioned aspects suggest that, in Argentina, human fascioliasis may have been overlooked in the past and its real epidemiological situation may be underestimated in the present, mainly in high risk rural high altitude areas. The recent detection of lymnaeid vector species such as *G. truncatula* [25,26] and *L. neotropica* [15,27], well-known to be linked to high prevalences and intensities of human fascioliasis in neighbouring and close countries such as Bolivia and Peru, add concern to this question. The very high prevalences recently obtained in a survey in Catamarca province [43], of the level of a human hyperendemic situation [1], also point in the same direction.

Thus, the need for appropriate epidemiological studies in the field, in selected areas where lymnaeid vector species of high liver fluke transmission are present, has to be emphasized. Health centres in these areas sould be informed about human infection probabilities, main clinical picture characteristics, adequate diagnosis techniques and the need for Egaten® (triclabendazole for human use) availability. Triclabendazole resistance, recently detected in Argentinian cattle in the province of Neuquen [105], where human infection has already been reported twice [61,62], and its capacity to spread to other areas of the country poses a serious question mark on human treatment in Argentina in the future.

The results of this retrospective overview provide a valuable baseline on which to design adequate multidisciplinary studies on fascioliasis in humans, animal reservoirs and lymnaeid vectors to assess up to which level and in which areas of this very large and environmen- tally heterogeneous country, human fascioliasis may represent a public health problem in Argentina.

Methods

Information sources and review methods

Sources of the literature reviewed include databases, national and multinational web-entries and free collections, multititle packages or web platforms, libraries, and personal e-mail requests when appropriate. Different key

words were used when searching in digital sources. Due to the fact that most of the references originate from local publications, the majority of them are not to be found in electronic databases. Special efforts were made to obtain old references published in local journals or very secondary, non-digitalised journals, unpublished reports, abstracts of meetings, symposia, congresses or similar (usually produced by simple photocopying and in very reduced number of copies), and Master's and PhD theses.

The scope was in need to be widened to non-medical journals, as this disease in humans was so neglected in the past that obtaining acceptance for an article dealing with human reports in a medical journal was sometimes difficult. Articles including a high number of human reports but published in veterinary journals as that of Pizzi et al. [106] are good examples.

Human fascioliasis case reports were obtained from the following sources: a) local medical and veterinary articles published in Argentina: 41 references; b) local medical and biological publications from Uruguay: 2 references [35,52]; c) a publication in a medical journal from Spain: 1 reference [36]; d) a publication in an international journal: 1 reference [94]; e) scientific com- munications at medical and veterinary congresses and meetings (abstract books): 7 references; f) a parasitology book published in Argentina: 1 reference [107]; and g) doctoral theses made at Argentinan universities: 2 refer- ences [41,69]. More than half of the references were more than 40 years old.

Great care was taken not to repeat any case, since in many instances duplications could be ascertained and the same patient accounted for in successive publica- tions. Additionally, most of the old articles were pub- lished in local, non-peer-reviewed journals, and several were made by non-specialists in fascioliasis. Conse- quently, data were in many cases only considered at informative or suggestive level. However, information furnished by old published reports proved to be very useful to assess areas where fascioliasis transmission may follow characteristics enabling human infection. In many of these endemic areas, the absence of additional human reports or the very low number of patients diag- nosed may be due to inhabitants not attending health centres for different reasons, misdiagnosis of other patients, and/or lack of appropriate surveys.

The problem of being a neglected, usually non-fatal, clinically mild, and non-reportable infection explains why many human cases are never published or reported to national health authorities, nor reported anywhere else. Liver fluke-infected patients described in university theses and afterwards never published anywhere else clearly show this problem.

Climatic data

For the analysis of the seasonal and annual distributions of human cases with regard to climatic characteristics, only Cordoba province was selected due to its relatively high number of human reports. No other province presented a sufficiently large enough number of cases as to allow for a significant comparative analysis.

Mean monthly data of maximum and minimum temperatures, precipitation and humidity was obtained from the Servicio Nacional de Meteorologia, Buenos Aires. Data analysed only concerned the 1960's and 1970's, when the patients were infected. The aforementioned climatic variables were furnished by ten different meteorological stations throughout the province of Cordoba, strategically selected according to the completeness of the data per station and the appropriate coverage of the geographical distribution of human cases. Geographical coordenates of the ten stations are as follows: Cordoba Aero (31° 19' S, 64° 13'W); Cordoba Observatorio (31° 24' S, 64° 11' W); Dique Cruz del Eje (30° 45' S, 64° 45' W); Dique La Viña (31° 53' S, 65° 02' W); Dique Pisco Huasi (30° 20' S, 64° 00' W); Embalse (32° 11' S, 64° 23' W); Huerta Grande (31° 05' S, 64° 29' W); Rio Cuarto Aero (33° 05' S, 64° 16' W); Rio Tercero (32° 10' S, 64° 08' W); and Villa Dolores Aero (31° 57' S, 65° 08 W).

To investigate the correlation between the moment of diagnosis of the human cases in Cordoba province between the years 1961 and 1981, and different climatic parameters (precipitation, relative humidity, maximum temperature, and minimum temperature), Pearson's correlation coefficient (r) was used by means of Infostat 2008 software. The human cases where correlated with the climatic parameters on a month per month basis and considering time lags of 1, 2, 3, 4, and 5 months considering the incubation period of fascioliasis which can be of up to two to four months. A correlation analysis was also applied to assess the potential relationship between annual precipitation and the number of cases per year in the aforementioned years.

Acknowledgements and funding

Studies in Argentina funded by Programa de Proyectos Bienales, Secretaría de Ciencia, Técnica y Posgrado, Universidad Nacional de Cuyo, and Area de Investigación, Universidad Juan Agustin Maza, both Mendoza, Argentina. Spanish collaboration supported by Projects No. SAF2006-09278 and No. SAF2010-20805 of the Spanish Ministry of Science and Technology, Madrid, Spain, and the Red de Investigación de Centros de Enfermedades Tropicales - RICET (Grants No. C03/04, No. PI030545 and No. RD06/0021/0017 of the Program of Redes Temáticas de Investigación Cooperativa), FIS, Spanish Ministry of Health, Madrid, Spain. Joint coordination activities carried out within Project No. RLA5049 of the International Atomic Energy Agency (Animal Production and Health Section, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Department of Nuclear Sciences and Applications, IAEA Headquaters Vienna, Austria). Literature search and review partially funded by the Global Burden of Foodborne Disease Initiative of the Department of Food Safety Zoonoses and Foodborne Diseases (FOS) at the World Health Organization (WHO Headquarters Geneva) in relationship with the Foodborne Disease Burden Epidemiology Reference Group (FERG).

Official livestock prevalence data furnished by the Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA), Buenos Aires.

Author details
[1]Cátedra de Parasitología y Enfermedades Parasitarias, Facultad de Ciencias Veterinarias y Ambientales, Universidad J.A. Maza, Av. de Acceso Este - Lateral Sur 2245, S. José, Guaymallén, Mendoza, Argentina. [2]Área de Parasitología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Av. Libertador 80, Mendoza, Argentina. [3]Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Av. Vicente Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain.

Authors' contributions
RMS performed the bibliographical search in the country, analysed the data and contributed to article drafting; VHA performed the bibliographical search on the internet and contributed to article preparation; PC performed the climatic data search and analyses; SMC conceived and designed the study and wrote the paper. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 15 November 2010 Accepted: 11 June 2011
Published: 11 June 2011

References

1. Mas-Coma S, Esteban JG, Bargues MD: Epidemiology of human fascioliasis: a review and proposed new classification. *Bull WHO* 1999, 77:340-346.
2. Mas-Coma S, Bargues MD, Esteban JG: Human Fasciolosis. In *Fasciolosis.* Edited by: Dalton JP. Wallingford, Oxon, UK: CAB International Publishing; 1999:411-434.
3. Mas-Coma S, Bargues MD, Valero MA: Fascioliasis and other plant-borne trematode zoonoses. *Int J Parasitol* 2005, 35:1255-1278.
4. Mas-Coma S, Valero MA, Bargues MD: *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Adv Parasitol* 2009, 69:41-146.
5. Fuentes MV, Valero MA, Bargues MD, Esteban JG, Angles R, Mas-Coma S: Analysis of climatic data and forecast indices for human fascioliasis at very high altitude. *Ann Trop Med Parasitol* 1999, 93:835-850.
6. Mas-Coma S, Valero MA, Bargues MD: Effects of climate change on animal and zoonotic helminthiases. *Rev Sci Techn Off Int Epiz* 2008, 27:443-457.
7. Mas-Coma S, Valero MA, Bargues MD: Climate change effects on trematodiases, with emphasis on zoonotic fascioliasis and schistosomiasis. *Vet Parasitol* 2009, 163:264-280.
8. Chen MG, Mott KE: Progress in assessment of morbidity due to *Fasciola hepatica* infection: a review of recent literature. *Trop Dis Bull* 1990, 87: R1-R38.
9. Valero MA, Santana M, Morales M, Hernandez JL, Mas-Coma S: Risk of gallstone disease in advanced chronic phase of fascioliasis: an experimental study in a rat model. *J Inf Dis* 2003, 188:787-793.
10. Valero MA, Navarro M, Garcia-Bodelon MA, Marcilla A, Morales M, Garcia JE, Hernandez JL, Mas-Coma S: High risk of bacterobilia in advanced experimental chronic fasciolosis. *Acta Trop* 2006, 100:17-23.
11. Valero MA, Girones N, Garcia-Bodelon MA, Periago MV, Chico-Calero I, Khoubbane M, Fresno M, Mas-Coma S: Anemia in advanced chronic fasciolosis. *Acta Trop* 2008, 108:35-43.
12. Girones N, Valero MA, Garcia-Bodelon MA, Chico-Calero MI, Punzon C, Fresno M, Mas-Coma S: Immune supression in advanced chronic fascioliasis: an experimental study in a rat model. *J inf Dis* 2007, 195:1504-1512.
13. World Health Organization: Fact sheet on fasciolíasis. *Action Against Worms* Geneva, Switzerland: World Health Organization, Headquarters; 2008, , 10: 1-8.
14. Bargues MD, Vigo M, Horak P, Dvorak J, Patzner RA, Pointier JP, Jackiewicz M, Meier-Brook C, Mas-Coma S: European Lymnaeidae (Mollusca: Gastropoda), intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS-2 sequences. *Inf Gen Evol* 2001, 1:85-107.
15. Bargues MD, Artigas P, Mera y Sierra R, Pointier JP, Mas-Coma S: Characterisation of *Lymnaea cubensis, L. viatrix* and *L. neotropica* n. sp., the main vectors of *Fasciola hepatica* in Latin America, by analysis of their ribosomal and mitochondrial DNA. *Ann Trop Med Parasitol* 2007, 101:621-641.
16. Apt W, Aguilera X, Vega F, Zulantay I, Retamal C, Apt P, Sandoval J: Fascioliasis en la población rural de las provincias de Curico, Talca y Linares. *Rev Méd Chile* 1992, 120:621-626.
17. Apt W, Aguilera X, Vega F, Alcaino H, Zulantay I, Apt P, Gonzalez V, Retamal C, Rodriguez J, Sandoval J: Prevalencia de fascioliasis en humanos, caballos, cerdos y conejos silvestres en tres provincias de Chile. *Bol Of Sanit Panam* 1993, 115:405-414.
18. Hillyer GV, Soler de Galanes M, Rodriguez-Perez J, Bjorland J, Silva de Lagrava M, Guzman SR, Bryan RT: Use of the Falcon™ assay screening test-enzyme-linked immunosorbent assay (FAST-ELISA) and the enzyme-linked immunoelectrotransfer blot (EITB) to determine the prevalence of human fascioliasis in the Bolivian altiplano. *Am J Trop Med Hyg* 1992, 46:603-609.
19. Esteban JG, Flores A, Aguirre C, Strauss W, Angles R, Mas-Coma S: Presence of very high prevalence and intensity of infection with *Fasciola hepatica* among Aymara children from the Northern Bolivian Altiplano. *Acta Trop* 1997, 66:1-14.
20. Esteban JG, Flores A, Angles R, Strauss W, Aguirre C, Mas-Coma S: A population-based coprological study of human fascioliasis in a hyperendemic area of the Bolivian Altiplano. *Trop Med Int Health* 1997, 2:695-699.
21. Esteban JG, Flores A, Angles R, Mas-Coma S: High endemicity of human fascioliasis between Lake Titicaca and La Paz valley, Bolivia. *Trans Roy Soc Trop Med Hyg* 1999, 93:151-156.
22. Esteban JG, Gonzalez C, Bargues MD, Angles R, Sanchez C, Naquira C, Mas-Coma S: High fascioliasis infection in children linked to a man-made irrigation zone in Peru. *Trop Med Int Health* 2002, 7:339-348.
23. Olaechea FV: Epidemiologia y control de *Fasciola hepatica* en la Argentina. In *Enfermedades Parasitarias de Importancia Económica en Bovinos.* Edited by: Nari A, Fiel C. Montevideo, Uruguay: Editorial Agropecuaria, Hemisferio Sur SRL; 1994:213-232.
24. Mas-Coma S, Funatsu IR, Bargues MD: *Fasciola hepatica* and lymnaeid snails occurring at very high altitude in South America. *Parasitology* 2001, 123:S115-S127.
25. Bargues MD, Mera y Sierra R, Gomez HG, Artigas P, Mas-Coma S: Ribosomal DNA ITS-1 sequencing of *Galba truncatula* (Gastropoda: Lymnaeidae) and its potential impact on fascioliasis transmission in Mendoza, Argentina. *Anim Biodiv Cons* 2006, 29:191-194.
26. Bargues MD, Mera y Sierra R, Gomez HG, Artigas P, Mas-Coma S: Caracterización molecular de *Galba truncatula*, vector principal de la Fascioliasis, en Argentina. Implicaciones en salud pública. *Enf Emerg, Barcelona* 2007, 9:77-82.
27. Mera y Sierra R, Artigas P, Cuervo P, Deis E, Sidoti L, Mas-Coma S, Bargues MD: Fascioliasis transmission by *Lymnaea neotropica* confirmed by nuclear rDNA and mtDNA sequencing in Argentina. *Vet Parasitol* 2009, 166:73-79.
28. Valero MA, Mas-Coma S: Comparative infectivity of *Fasciola hepatica* metacercariae from isolates of the main and secondary reservoir animal host species in the Bolivian Altiplano high human endemic region. *Folia Parasitol* 2000, 47:17-22.
29. Valero MA, Darce NA, Panova M, Mas-Coma S: Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the Northern Bolivian Altiplano hyperendemic region. *Vet Parasitol* 2001, 102:85-100.
30. Mas-Coma S, Angles R, Strauss W, Esteban JG, Oviedo JA, Buchon P: Human fasciolasis in Bolivia: a general analysis and a critical review of existing data. *Res Rev Parasitol* 1995, 55:73-93.
31. Roffo AH: *Distomatosis humana, primer caso observado en el país* Buenos Aires, Argentina: Imprenta y Editora Coni Hermanos; 1913, 1-38.
32. Greenway D: Distomatosis humana por *Fasciola hepatica* en la Argentina. Primer diagnóstico coproscópico. *Prensa Méd Arg* 1924, 11:369.
33. Bengolea J, Bacigalupo J, Velazco Suarez G: Distomatosis por *Fasciola hepatica*. *Arch Arg Enf Apar Dig Nutr* 1927, 3(1):151.
34. Del Valle D, Donovan R: Síndrome de cólico hepático provocado por *Fasciola hepatica*. *Arch Arg Enf Apar Dig Nutr* 1928, 4:697-710.
35. Bacigalupo J, Bengolea J, Velazco Suarez G: Algunas consideraciones biológicas sobre distomatosis por *Fasciola hepatica* en el hombre. A propósito de dos casos encontrados en la Argentina. *Arch Soc Biol Montevideo* 1930, 5:1211.

36. Carena EJ, Trakal E, Ortiz GA, Butti AL, Carena FL, Robin de Augier MR: Infestación humana por *Fasciola hepatica* (nuestra experiencia en 13 casos). *Rev Esp Enf Apar Dig* 1972, 36:531-542.
37. Durand Savoyat M: El saguaipé. Epizootía causada por el Saguaipé vulg. Duva o *Fasciola* de Linnéo. *An Soc Rural Arg* 1867, 173-176.
38. Rodriguez C: Parasitosis por *Fasciola hepatica,* Consideraciones. Experiencia Personal. *Rev Méd Córdoba* 1952, 40:9-12.
39. Rodriguez C: *Fasciola hepatica*. Biología del Parásito. *Rev Méd Córdoba* 1954, 42:213-216.
40. Rodriguez C: Parasitosis intestinales en Córdoba. Estadística sobre diez mil casos. *Rev Méd Córdoba* 1961, 49:37-40.
41. Siciliano C: Distomatosis hepática, perfil clínico. *Tesis Doctoral* Universidad Nacional de Córdoba, Argentina; 1982, 1-118.
42. Siciliano C, Chalub E, Sosa L, Barnes A, Shicton G: Distomatosis hepática, nuevos casos en Córdoba, Argentina. *Prensa Méd Arg* 1989, 76:106-109.
43. Malandrini JB, Carnevale S, Velazquez J, Soria CC: Diagnóstico de *Fasciola hepatica* con la técnica de ELISA en el Departamento de Tinogasta. *Ciencia* 2009, 4:143-151.
44. Esteban JG, Gonzalez C, Curtale F, Muñoz-Antoli C, Valero MA, Bargues MD, El Sayed M, El Wakeel A, Abdel-Wahab Y, Montresor A, Engels D, Savioli L, Mas-Coma S: Hyperendemic fascioliasis associated with schistosomiasis in villages in the Nile Delta of Egypt. *Am J Trop Med Hyg* 2003, 69:429-437.
45. Moghaddam AS, Massoud J, Mahmoodi M, Mahvi AH, Periago MV, Artigas P, Fuentes MV, Bargues MD, Mas-Coma S: Human and animal fascioliasis in Mazandaran province, northern Iran. *Parasitol Res* 2004, 94:61-69.
46. Espinoza JR, Maco V, Marcos L, Saez S, Gonzalez C, Neyra V, Terashima A, Samalvides F, Gotuzzo E, Chavarry E, Huaman C, Bargues MD, Valero MA, Mas-Coma S: Evaluation of Fas2-ELISA for the serological detection of *Fasciola hepatica* infection in humans. *Am J Trop Med Hyg* 2007, 76:977-982.
47. Nieto Sosa L, Barnes A, Jimenez G, Cicaerlli G: Fasciolosis: evaluación clínica y terapéutica. *III Congreso Latinoamericano de Zoonosis y VI Congreso Argentino de Zoonosis* Buenos Aires, Argentina, Resúmenes de las Comunicaciones; Junio 2008, 35.
48. Salomon MC, De Jong L, Tonelli RL, Borremans CG, Bertello D, Jofre CA, Pagella H, Lloret L, Carnevale S, Carena J: Confirmación etiológica de un brote de distomatosis hepática en Mendoza. *XII Jornadas Argentinas de Microbiología: 14-17 Junio 2006* Mendoza, Argentina: Resúmenes de las Comunicaciones; 2006, 108.
49. Ossola A, Siciliano C, Garcia Faure J: Distomatosis hepática, tres epidemias familiares en Córdoba, Argentina. *Rev Asoc Bioq Arg* 1972, 37(201):100-106.
50. Peiretti JA, Morales JR: Epidemia familiar de distomatosis por *Fasciola hepatica*. *Prensa Méd Arg* 1973, 60:262-264.
51. Minoprio JE, Donna RA, Borremans CG, Passardi AH, Glatstein de Wolf T, Olindo AL, Jorg ME: Epidemia familiar de distomatosis humana por *Fasciola hepatica*. Localización errática (cutánea). *Prensa Méd Arg* 1995, 82:869-877.
52. Bacigalupo J: *Fasciola hepatica,* su ciclo evolutivo en la República Argentina. *An Fac Vet Montevideo* 1942, 4:7-134.
53. Malandrini JB, Soria CC, Carnevale S, Velazquez J, Pizarro MC: Estudio de la Fascioliasis desde una perspectiva multidisciplinaria. *XXVIII Jornadas de Actualización en Ciencias Veterinarias: 25-27 September 2009* Villa Giardino, Córdoba; 2009.
54. Lloret L, de Jong L, Salomon S, Carena J: Hipereosinofilia en un brote urbano de distomatosis hepática. *XIV Congreso Nacional de Medicina: 21-24 November 2005; Buenos Aires, Argentina Libro de Resúmenes* 2005, P-08-02.
55. Caceres MA: Distomatosis hepática - Síndrome coledociana por *Fasciola hepatica*. *Soc Arg Cirujanos, Boletines y Trabajos* 1955, 19:528-534.
56. Mas-Coma S, Angles R, Esteban JG, Bargues MD, Buchon P, Franken M, Strauss W: The Northern Bolivian Altiplano: a region highly endemic for human fascioliasis. *Trop Med Int Health* 1999, 4:454-467.
57. Moriena RA, Racioppi O, Alvarez JD, Lombardero OJ: *Fasciola hepatica* y otros trematodos de bovinos. Prevalencia según decomisos en plantas faenadoras en la provincia de Corrientes (Argentina). *Veterinaria Argentina* 2001, 17(177):493-498.
58. Moriena RA, Racioppi O, Alvarez JD, Lombardero OJ: Distomatosis hepática (*Fasciola hepatica*) y otros trematodos de los rumiantes. Distribución en la provincia de Corrientes. *Com Cient Tecnol Univ Nac Nordeste, Cienc Vet, Corrientes, Argentina* 2002 [http://www.unne.edu.ar/Web/cyt/cyt/2002/04-Veterinarias/V-055.pdf].
59. Lombardero OJ, Moriena RA, Racioppi O, Quiroz L: Bionomía de *Limnaea* sp. en la Provincia de Corrientes. *Veterinaria, Rev Fac Cienc Vet, Univ Nac Nordeste, Corrientes, Argentina* 1979, 2:5-19.
60. Lombardero OJ, Moriena RA, Racioppi O, Coppo JA, Schiffo HP: Distomatosis hepática de los bovinos en la Provincia de Corrientes. *Veterinaria, Rev Fac Cienc Vet, Univ Nac Nordeste, Corrientes, Argentina* 1979, 2:21-29.
61. Rubel D, Prepelitchi L, Kleiman F, Carnevale S, Wisnivesky-Colli C: Estudio del foco en un caso de fasciolosis humana en Neuquén. *Medicina Buenos Aires* 2005, 65:207-212.
62. Rios N, Pavia M, Meana M, Serrano G, Navarro R: Un caso de fascioliasis hepática. *III Congreso Latinoamericano de Zoonosis y VI Congreso Argentino de Zoonosis: Junio 2008* Buenos Aires, Argentina: Resúmenes de Comunicaciones; 2008, 35.
63. Kaczorkiewicz AJ: Distomatosis en la provincia de Neuquén. *Rev Med Vet* 1983, 64:354-356.
64. Ollerenshaw CB: The influence of climate on the life cycle of *Fasciola hepatica* in Britain with some observations on the relationship between climate and the incidence of fascioliasis in the Netherlands. *Facts and Reflections, Symposium Proceedings: 3 June 1971* Lelystad: Parasitology Department of the Centraal Diergeneeskundig Institut; 1971, 41-63.
65. Mas-Coma S: Epidemiology of fascioliasis in human endemic areas. *J Helminthol* 2005, 79:207-216.
66. Mas-Coma S: Human fascioliasis. In *Waterborne Zoonoses: Identification, Causes and Control.* Edited by: Cotruvo JA, Dufour A, Rees G, Bartram J, Carr R, Cliver DO, Craun GF, Fayer R, Gannon VPJ. London, UK: World Health Organization and IWA Publishing; 2004:305-322.
67. Ashrafi K, Valero MA, Massoud J, Sobhani AR, Solaymani-Mohammadi S, Conde P, Khoubbane M, Bargues MD, Mas-Coma S: Plant-borne human contamination by fascioliasis. *Am J Trop Med Hyg* 2006, 75:295-302.
68. Barcat JA: El berro y otras comidas peligrosas. *Medicina, Buenos Aires* 2005, 65:277-279.
69. Boto D: Distomatosis humana por *Fasciola hepatica*. *Tesis de Doctorado* Universidad de Buenos Aires; 1939, , 5193: 1-46.
70. Esteban JG, Bargues MD, Mas-Coma S: Geographical distribution, diagnosis and treatment of human fascioliasis: a review. *Res Rev Parasitol* 1998, 58:13-42.
71. Hillyer GV: Immunodiagnosis of human and animal fasciolosis. In *Fasciolosis.* Edited by: Dalton JP. Wallingford, Oxon, UK: CAB International Publishing; 1999:435-447.
72. Strada L: Fascioliasis hepática humana. *Prensa Méd Arg* 1961, 48:2985-2992.
73. O'Neill SM, Parkinson SM, Strauss W, Angles R, Dalton JP: Immunodiagnosis of *Fasciola hepatica* (Fascioliasis) in a human population in the Bolivian Altiplano using purified cathepsin L cysteine proteinase. *Am J Trop Med Hyg* 1998, 58:417-423.
74. Ubeira FM, Muiño L, Valero MA, Periago MV, Perez-Crespo I, Mezo M, Gonzalez-Warleta M, Romaris F, Paniagua E, Cortizo S, Llovo J, Mas-Coma S: MM3-ELISA detection of *Fasciola hepatica* coproantigens in preserved human stool samples. *Am J Trop Med Hyg* 2009, 81:156-162.
75. World Health Organization: Report of the WHO Informal Meeting on use of triclabendazole in fascioliasis control. Meeting: 17-18 October 2006. Geneva, Switzerland: World Health Organization, Headquarters, 2007;, WHO/CDS/NTD/PCT/2007.1.
76. Cames OJ: Distomatosis por *Fasciola hepatica*. *An Cirugía Rosario* 1940, 6:143-147.
77. Solari AV, Canepa L: Consideraciones sobre distomatosis hepática por *Fasciola hepatica,* un nuevo caso de parasitismo humano. *Rev Med Cienc Afines* 1943, 55:795-799.
78. Giffoniello AH, Miravet SV, D'Angelo JC, Nogaro E: Distomatosis por *Fasciola hepatica*. *Prensa Med Arg* 1983, 70:70-73.
79. Cid JM: Granuloma tuberculoide y gomoide peritoneal por Fasciola errática. *Arch Soc Arg Anat Norm y Pat* 1947, 9:389-401.
80. Cames OJ, Cid JM, Alvarez A: Parasitosis errática peritoneal por fasciola hepática. *Boletin de la Sociedad de Cirugia de Rosario* 1947, 8:306-313.
81. La Pook JD, Magun AM, Nickerson KG, Meltzer JI: Sheep, watercress, and the Internet. *Lancet* 2000, 356:218.
82. Valero MA, Perez-Crespo I, Periago MV, Khoubbane M, Mas-Coma S: Fluke egg characteristics for the diagnosis of human and animal fascioliasis by *Fasciola hepatica* and *F. gigantica*. *Acta Trop* 2009, 111:150-159.

83. Carnevale S, Rodriguez MI, Guarnera EA, Carmona C, Tanos T, Angel SO: Immunodiagnosis of fasciolosis using recombinant procathepsin L cysteine proteinase. *Diagn Microbiol Inf Disease* 2001, 41:43-49.
84. Carnevale S, Rodriguez MI, Santillan G, Labbe JH, Cabrera MG, Belegarde EJ, Velasquez JN, Trgovcic JE, Guarnera EA: Immunodiagnosis of human fascioliasis by an Enzyme-Linked Immunosorbent Assay (ELISA) and a Micro-ELISA. *Clin Diagn Lab Immunol* 2001, 8:174-177.
85. Corti ME, Palmieri OJ, Mendez N, Gancedo E, Sawicki M, Millet S: Diagnóstico por imágenes en la fasciolosis hepática. *Rev Arg Radiol* 2006, 70:197-200.
86. Simon MP, Bustos Araujo A, Sosa Lara E: Colecistitis crónica alitiásica (cólico hepático) por distomatosis hepática. *VII Jornada Quirúrgica de Cuyo: 11 Julio 1964* Hospital Central, Mendoza, Argentina: Resúmenes de las Comunicaciones; 1964, 1-15.
87. Peiretti JA: Distomatosis hepática, contribucion al diagnóstico precoz. *Día Médico* 1969, 10:248.
88. Melero M, Rigou RC, Lloveras J, Gennaro O: Fascioliasis hepática causa infrecuente de síndrome febril prolongado con hipereosinofilia e imágenes hipodensas en la tomografía computada de hígado. *Medicina Buenos Aires* 1991, 51:244-248.
89. Longo OF, Daraio M: Distoma del colédoco. Importancia de la colangiografía operatoria. *Prensa Méd Arg* 1953, 43:2910-2911.
90. Andrada JG, Avalos E, Eguez A, Bosch Andrada JP: Aspectos quirúrgicos de la distomatosis biliar. *Rev Argentina Cirugía* 1983, 46:44-49.
91. Castex M, Greenway D: Distomatosis hepática conejemplar errático eliminado por uretra. *Bol Acad Med Buenos Aires* 1937, 461-464.
92. Paladino JE, Galarce J: Parasitosis apendicular. *La Semana Médica* 1939, 2:689-690.
93. Bacigalupo J, Zavaleta DE, Laurito JO: *Fasciola hepatica* en la Argentina. *Semana Méd* 1943, 4:5-9.
94. Ruggieri F, Correa AJE, Martinez E: Cerebral distomiasis. Case report. *J Neurosurg* 1967, 27:268-271.
95. Correa AJ, Ruggieri F, Martinez E: *Fasciola hepatica* de localización intracraneal. *Rev Méd Santa Fé* 1969, 3:38-41.
96. Brady MT, O'Neill SM, Dalton JP, Mills KH: *Fasciola hepatica* supresses a protective Th1 response against *Bordetella pertussis*. *Infection Immunity* 1999, 67:5372-5378.
97. Miguel CM, Mallea Gil MS, Basile MA, Mauro EL: Distomatosis por *Fasciola hepatica*. *Prensa Méd Arg* 1985, 72:192-195.
98. Petraglia AA: Parasitosis humana por *Fasciola hepatica*: primer caso que se describe en el Noreste Argentino. *Act Trab Asoc Arg Est Enf Transm* 1954, 3:47-48.
99. Padilla Antoni F, Saleme A, Jorratt M: *Fasciola hepatica*. A propósito de una observación. *Prensa Méd Arg* 1970, 57:521-525.
100. Alaggia LE: Patología hepatobiliar por distomatosis. *XXIII Congreso Cuyano de Cirugía* San Luis, Argentina; 1981.
101. Logaldo E: Distomatosis de las vias biliares, litiasis vesicular. *Rev Asoc Méd Arg* 1955, 260-261.
102. Majul EJ, Salich JC, Bender AL: Distomatosis hepatobiliar por *Fasciola hepatica*. *Prensa Méd Arg* 1981, 68:81-83.
103. Trossero AI, Nocetti VM: Distomatosis por *Fasciola hepatica*. *Semana Méd* Septiembre 1969, 879-882.
104. Sonzini Astudillo C, Cuenca Perez M, Tiglio M, Candizano H: Distomatosis hepática *Fasciola hepatica*. *Semana Méd* 1973, 143(17):507-510.
105. Olaechea FV, Lovera V, Larroza M, Raffo F, Cabrera R: Triclabendazole-resistant liver fluke in Patagonian (Argentina) cattle. *XII International Congress of Parasitology ICOPA XII: 16-20 August 2010* Melbourne, Australia. Conference Abstracts; 2010, No. 1783.
106. Pizzi H, Navarro Z, Pizzi D, Benvissuto G: Estudio epidemiológico e implicaciones económicas de la distomatosis hepatica en la provincia de Córdoba, Argentina. *Gaceta Vet* 1982, 44:944-947.
107. Niño F: *Parasitología. Zooparásitos y Patología de las Zooparasitosis Humanas* Buenos Aires, Argentina: Editorial Beta; 1965, 1-656.
108. Mascheroni HA: Colecistitis parasitaria: sobre un caso de distomatosis por *Fasciola hepatica*. *Rev Méd Quir Patol Fem* 1933, 2:710.
109. Cuenya C: Parasitosis raras en el *Norte Argentino: Fasciola hepatica* (L.). *Arch Farm. Bioquím Tucumán* 1944, 1:235-239.
110. Ahualli A, Arias E: *Fasciola hepatica*. *Rev Fac Medicina Tucumán* 1961, 3:105-118.
111. Urrutia JM, Ferraris LV: Distomatosis del hepatocoledoco. Importancia de la colangiografia operatoria. *Primera Conferencia Nacional de Anatomia Normal y Patologica, Histologia, Embriologia y Tecnica Quirurgica* Cordoba; 1962.
112. Cornejo JA, Castillo E: Mioglobinemia secundaria a distomatosis por *Fasciola*. *Rev Fac Med Tucumán* 1964, 7:241-257.
113. Cañas RF, Escola MA, Rossini AP: Distomatosis hepatobiliar por *Fasciola hepatica*. *Prensa Méd Arg* 1964, 51:590-595.
114. Sosa MA, Romero AE: Distomatosis hepática. *Acta Méd Córdoba* 1967, 12(2):78-83.
115. Ale D, Merciai G, Gil Echevarria M: *Fasciola hepatica*, intensificación de su búsqueda en pacientes con eosinofilia y epidemiología positiva. *III Congreso Argentino de Parasitología: 1-4 November 2000* Mar del Plata, Argentina, Libro de Resúmenes; 2000, 421.
116. Malandrini JB, Soria CC, Foresi I, Correa S: Fasciolosis en Catamarca. Primeros resultados. *La Investigación Cientifica en la Facultad de Ciencias de la Salud, en los Umbrales del Siglo XXI* Catamarca: Editorial Universitaria; 2002, 287-293.

doi:10.1186/1756-3305-4-104
Cite this article as: Mera y Sierra *et al.*: Human fascioliasis in Argentina: retrospective overview, critical analysis and baseline for future research. *Parasites & Vectors* 2011 4:104.

Acta Tropica 120 (2011) 245–257

Contents lists available at SciVerse ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Characterisation of fascioliasis lymnaeid intermediate hosts from Chile by DNA sequencing, with emphasis on *Lymnaea viator* and *Galba truncatula*

Patricio Artigas[a], M. Dolores Bargues[a], Roberto L. Mera y Sierra[b], Veronica H. Agramunt[a], Santiago Mas-Coma[a,*]

[a] *Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Av. Vicente Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain*
[b] *Cátedra de Parasitología y Enfermedades Parasitarias, Facultad de Ciencias Veterinarias y Ambientales, Universidad J.A. Maza, Av. de Acceso Este - Lateral Sur 2245, S. José, Guaymallén, Mendoza, Argentina*

a r t i c l e i n f o

Article history:
Received 4 June 2011
Received in revised form 5 September 2011
Accepted 7 September 2011
Available online 14 September 2011

Keywords:
Lymnaeidae
Intermediate hosts
rDNA
mtDNA
Fascioliasis
Chile

a b s t r a c t

In South America, *Fasciola hepatica* infection poses serious health problems in both humans and livestock. In Chile, the medical impact appears yearly stable and mainly concentrated in central regions, where the veterinary problem is highlighted by higher animal prevalences. Studies were undertaken by rDNA ITS-2 and ITS-1 and mtDNA *cox*1 sequencing to clarify the specific status of the lymnaeids, their geographical distribution and fascioliasis transmission capacity in Chile, by comparison with other American countries and continents. Results change the lymnaeid scenario known so far. The lymnaeid fauna of mainland Chile shows to be poor, including only two authochthonous species, *Lymnaea viator* and *Pectinidens diaphana*, and a third introduced species of Palaearctic origin *Galba truncatula*. Both *Lymnaea lebruni* and *Lymnaea patagonica* proved to be synonyms of *P. diaphana*. *G. truncatula* appears to have always been confused with *L. viator* and seems distributed from Región VI to Región IX, overlapping with human endemic areas. DNA sequencing results suggest that the absence of correlation between remote sensing data and disease prevalences could be due to transmission capacity differences between *L. viator* and *G. truncatula*. Results furnish a new baseline on which to undertake future appropriate studies on transmission, epidemiology and control.

1. Introduction

Freshwater lymnaeid snails transmit fascioliasis, a highly pathogenic liver parasitosis caused by trematode species of the genus *Fasciola* which affects humans and livestock species almost everywhere (Mas-Coma et al., 2009a). Distribution, both in space (latitudinal, longitudinal and altitudinal) and time (seasonal, yearly), of fascioliasis depends on the presence and population dynamics of the specific intermediate host species in its turn linked to the presence of the appropriate water bodies and on adequate climate characteristics enabling fluke development. In the last two decades, this disease is emerging in many countries of Latin America, Europe, Africa and Asia. This emergence phenomenon has partly been related to climate change, given the high dependence of both fasciolid larval stages and their freshwater lymnaeid snail hosts on climatic and environmental characteristics (Mas-Coma et al., 2008, 2009b). The increasing importance of human fascioliasis also relies on recent results showing a great morbidity impact on children in long-term infection (Valero et al., 2003, 2006, 2008).

Within the several human fascioliasis hotspot regions known, South America stands out due to the human hyperendemic areas caused by *Fasciola hepatica* in many Andean countries, such as Bolivia (Esteban et al., 1997, 1999) and Peru (Esteban et al., 2002; Gonzalez et al., 2011). In Argentina the human fascioliasis situation seems to be underestimated (Mera y Sierra et al., 2011), in Colombia appropriate studies are still pending (Bargues et al., 2011a), and in Venezuela a potential underestimation of the situation has recently been highlighted (Bargues et al., in press-a).

In Chile, human fascioliasis reports have been numerous, including individual case descriptions (e.g., Venturelli et al., 2003; Lopez et al., 2004; Rosas et al., 2008; Morales et al., 2009), epidemic situations and familiar outbreaks (Subercaseaux et al., 1985; Borie et al., 1990), and even studies on human fascioliasis endemic areas (Apt et al., 1992, 1993). A large random survey in the provinces of Curico, Talca and Linares showed prevalences of 0.6%, 0.75% and 0.71%, respectively, with an estimation of 2000 people infected in the area analysed (Apt et al., 1993). Only in the capital Santiago,

Abbreviations: rDNA, nuclear ribosomal DNA; mtDNA, mitochondrial DNA; ITS-1, first internal transcribed spacer; ITS-2, second internal transcribed spacer; COX1, cytochrome c oxidase subunit I nucleotide sequence (*cox*1) and amino acid sequence.

* Corresponding author. Tel.: +34 96 3544905; fax: +34 96 3544769.
E-mail address: S.Mas.Coma@uv.es (S. Mas-Coma).

0001-706X/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.actatropica.2011.09.002

between 20 and 30 liver fluke infected patients were diagnosed yearly and this only concerned symptomatic subjects (Apt, 1987; Alcaino and Apt, 1989).

Fascioliasis is also a great veterinary problem in Chile (Alcaino and Apt, 1989; Morales and Luengo, 1995; Alcaino and Gorman, 1999). Official data from slaughterhouses indicate that the disease affects livestock in all regions, with countrywide high yearly prevalences in cattle (28.5–31.8%, mean 30.1%), sheep (1.8–2.75, mean 1.9%), goats (11.2–18.3%, mean 14.0%), pigs (1.0–2.1%, mean 1.4%), equines (9.1–14.1%, mean) and camelids (0.03–8.37%, mean 0.99%) according to data from the 1989 to 1995 period (Morales et al., 2000).

Despite the importance of fascioliasis in Chile, lymnaeid snails have been only the focus of a very few studies in that country. A total of seven lymnaeid species have been described or noted to be present in Chile (Hubendick, 1951; Paraense, 1982, 1984; Sielfeld, 2001; Valdovinos, 2006):

- *Lymnaea diaphana* King and Broderip, 1832: originally described from Cape Gregory, Strait of Magallanes (King and Broderip, 1832) and later also cited from Argentina and Peru (Bargues et al., in press-b); the genus *Pectinidens* proposed by Pilsbry (1911) for *diaphana* as type species has recently been molecularly demonstrated to be valid (Bargues et al., in press-b);
- *L. viator* D'Orbigny, 1835 (=*L. viatrix sensu* Paraense, 1976): originally described under var. A ventricosa from Rio Negro in Argentina as well as surrounding Santiago and Casablanca in Chile, and under var. B elongata from Rimac river canals around Callao and Lima in Peru (D'Orbigny, 1835, 1837; Paraense, 1976; Bargues et al., 2007a);
- *Lymnaea chilensis* Beck, 1838: originally noted from Chile without further geographical detail (Beck, 1838);
- *Lymnaea lebruni* Mabille, 1884: originally described from Punta Arenas, Patagonia (Región XII) (Mabille, 1884);
- *Lymnaea pictonica* Rochebrune and Mabille, 1885: originally described from Isla Picton (Rochebrune and Mabille, 1885);
- *Lymnaea cousini* Jousseaume, 1887: cited once in Valdivia (Hubendick, 1951), but originally described from Ecuador and later also from Colombia (Bargues et al., 2011a);
- *L. patagonica* Strebel, 1907: originally described from Agua Fresca, Strait of Magallanes (Strebel, 1907).

However, *L. chilensis* may be deleted from that list because neither a description nor a locality were provided by Beck (1838: species name noted as "*L. Chilensis* B. – Chili" in page 112 but without description in the Appendix where the descriptions of all the new species were included), so that it is taxonomically considered a *nomen nudum*, as already stated by Hubendick (1951). Moreover, different synonymies have been proposed between these species, authors sometimes not in agreement one another. For instance, *L. lebruni* is considered a synonym of *L. diaphana* by Pilsbry (1911) because Mabille (1884) overlooked the article of King and Broderip (1832). *Lymnaea patagonica* is considered a synonym of *L. pictonica* by Hubendick (1951), whereas *L. patagonica* is a valid species according to Malek (1985).

The species noted to be involved in fascioliasis transmission in Chile is *L. viator*, specimens of which collected from Macul and Bucalemu proved to be susceptible to experimental infection already long ago (Tagle, 1944).

The classification of individual lymnaeids poses serious difficulties when only applying malacological methods, due to anatomic similarities and large intraspecific variation of shell shape and size (Bargues et al., 2001; Bargues and Mas-Coma, 2005). Although shell shape may help in particular species and populations (Samadi et al., 2000), there are groups, as the "fossarine" or *Galba/Fossaria* group, in which specimen classification may be very difficult when based only on phenotype, as is the case of the aforementioned *L. viator* and *L. cousini* (Bargues et al., 2007a, 2011a,b, in press-a, in press-b). The implications of lymnaeids for fascioliasis transmission, epidemiology and control urged to develop new tools to facilitate specimen classification, genetic characterisation of natural populations and laboratory strains, and to elucidate the systematics and taxonomy of the Lymnaeidae. This is the purpose of the worldwide lymnaeid molecular characterisation initiative (Mas-Coma et al., 2009a). Nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) markers proved to be useful for this endeavour in invertebrates in general, although disadvantages and limitations depending on each marker should be taken into account (Mas-Coma and Bargues, 2009). Their application also showed their usefulness in lymnaeid snails for specific, generic and suprageneric taxon levels (Bargues and Mas-Coma, 2005).

The internal transcribed spacers of the rDNA, mainly ITS-2 and secondarily ITS-1, are the most useful sequences for lymnaeid species classification (Bargues and Mas-Coma, 2005). A fragment of the cytochrome c oxidase subunit I gene (*cox*1) of mtDNA also proved to be useful in lymnaeids (Bargues et al., 2007a), although mtDNA markers should be used with great caution in lymnaeids (Bargues et al., 2011b), due to (i) problems of nucleotide saturation making it unappropriate for comparison of genera and even well separated species within the same genus and (ii) biased information furnished by only a gene fragment (Mas-Coma and Bargues, 2009).

The aim of the present article is to expose the results of ITS-2, ITS-1 and *cox*1 sequencing of lymnaeid species present in Chile and to ascertain their systematic status. The final analysis has the purpose to offer a new baseline on which to design and launch further lymnaeid studies and appropriate assessments on human and animal fascioliasis in Chile from now on. The implications of the new intermediate host scenario on the disease in that country are finally discussed.

2. Materials and methods

2.1. Lymnaeid snail materials

The snail specimens studied were collected in the field, from lymnaeid populations present in geographical areas with human infection and/or animal fascioliasis endemicity. In order to be systematically conclusive, sequenced specimens of the species *L. diaphana*, *L. viator*, *L. lebruni*, and *L. patagonica* were from their respective type localities. Unfortunately, the present impossibility to visit Picton island due to the distribution of anti-personnel mines throughout the island did not allow for the collection of *L. pictonica*. An effort to find *L. cousini* was made in Valdivia and its surroundings. Localities and their coordinates and altitudes furnishing the lymnaeid specimens sequenced are noted in Table 1 and Fig. 1. The number of specimens analysed for each species is noted in Table 1.

2.2. Molecular techniques

2.2.1. DNA extraction

DNA was extracted from more than one specimen of a given population when this was deemed necessary for sequence verification. DNA was only isolated from the foot of each snail (Bargues et al., 1997, 2007a). Snail feet fixed in 70% ethanol were used for DNA extraction procedures. After dissection under a microscope, half of the foot was suspended in 400 μl of lysis buffer (10 mM Tris–HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% sodium dodecyl sulfate SDS) containing 500 μg/ml Proteinase K (Promega, Madison, WI, USA) and digested for 2 h at 55 °C with alternate shaking each 15 min. Total DNA was isolated according to the

Table 1
Nuclear ribosomal and mitochondrial DNA haplotype code identification for lymnaeid species and populations studied from Chile.

Preliminary classification (*n* = collected/sequenced)	Populations				rDNA ITS-2		rDNA ITS-1		mtDNA *cox*1		Definitive classification	
	Locality	Coordinates	Altitude	Region	H	Acc. No.	H	Acc. No.	H[b]	Acc. No.	Species	Combined H nomenclature
L. viator (*n* = 15/4)	Casa Blanca	S 33° 19′ 12.9″w 71° 24′ 47.8″	270 m	V Valparaiso	2[a]	JN051366	B[a]	JN051368	b[a]	JN051373	*L. viator*	L.via-2B,cox1b
L. viator (*n* = 8/2)	Casa Blanca	S 33° 19′ 12.9″w 71° 24′ 47.8″	270 m	V Valparaiso	2[a]	JN051366	B[a]	JN051368	c[a]	JN051374	*L. viator*	L.via-2B,cox1c
L. viator (*n* = 52/6)	Rosario (Sector Rengo)	S 34° 22′ 35.0″w 70° 51′ 31.3″	320 m	VI O'Higgins	3	AJ272051	C	AJ272052	c[a]	JN051372	*G. truncatula*	G.tru-3C,*cox*1c
L. viator (*n* = 47/4)	Chillán (Sector Alazán)	S 36° 32′ 42.6″w 71° 46′ 24.2″	250 m	VIII Bío-Bío	3	AJ272051	C	AJ272052	c[a]	JN051372	*G. truncatula*	G.tru-3C,*cox*1c
L. viator (*n* = 4/2)	Isla Teja, Granja UACH Valdivia	S 39° 48′ 10.4″w 73° 15′ 05.2″	10 m	XIV Los Ríos	3	AJ272051	C	AJ272052	c[a]	JN051372	*G. truncatula*	G.tru-3C,*cox*1c
L. diaphana (*n* = 27/5)	Cape San Gregory (vegas bajas)	S 52° 37′ 52.3″w 70° 15′ 18.0″	2–5 m	XII Magallanes	1	JF909498	A	JF909499	a	JF909501	*P. diaphana*	L.dia-1A,*cox*1a
L. diaphana (*n* = 23/4)	Cape San Gregory (vegas bajas)	S 52° 37′ 52.3″w 70° 15′ 18.0″	2-5 m	XII Magallanes	1	JF909498	A	JF909499	b[a]	JN051375	*P. diaphana*	L.dia-1A,*cox*1b
L. diaphana (*n* = 18/4)	Cape San Gregory (vegas bajas)	S 52° 37′ 52.3″w 70° 15′ 18.0″	2–5 m	XII Magallanes	1	JF909498	A	JF909499	d[a]	JN051377	*P. diaphana*	L.dia-1A,*cox*1d
L. diaphana (*n* = 11/3)	Cape San Gregory (vegas altas)	S 52° 34′ 02.4″w 70° 12′ 05.9″	21 m	XII Magallanes	1	JF909498	B[a]	JN051369	c[a]	JN051376	*P. diaphana*	L.dia-1B,*cox*1c
L. patagonica (*n* = 31/5)	Agua Fresca	S 53° 23′ 58,8″w 71° 07′ 08,6″	157 m	XII Magallanes	1	JF909498	C[a]	JN051370	e[a]	JN051378	*P. diaphana*	L.dia-1C*cox*1e
L. patagonica (*n* = 25/3)	Agua Fresca	S 53° 23′ 58,8″w 71° 07′ 08,6″	157 m	XII Magallanes	1	JF909498	C[a]	JN051370	f[a]	JN051379	*P. diaphana*	L.dia-1C,*cox*1f
L. patagonica (*n* = 19/2)	Agua Fresca	S 53° 23′ 58,8″w 71° 07′ 08,6″	157 m	XII Magallanes	1	JF909498	C[a]	JN051370	g[a]	JN051380	*P. diaphana*	L.dia-1C,*cox*1g
L. lebruni (*n* = 7/2)	Punta Arenas	S 53° 24′ 25.1″w 71° 15′ 45,7″	296 m	XII Magallanes	2[a]	JN051367	D[a]	JN051371	h[b]	JN051381	*P. diaphana*	L.dia-2D,*cox*1h
L. cousini (*n* = 5/3)	Isla Teja, Río Cau-Cau Valdivia	S 39° 48′ 05.3″w 73° 15′ 09.4″	7 m	XIV Los Ríos	–	–	–	–	a[a]	JN051382	*Chilina* sp.	C.sp.-*cox*1a

n, number of specimens collected and malacologically studied for classification/number of specimens sequenced; H, haplotype.

[a] New haplotypes for the corresponding lymnaeid species.

[b] Only preliminary haplotypes due to incomplete gene sequence.

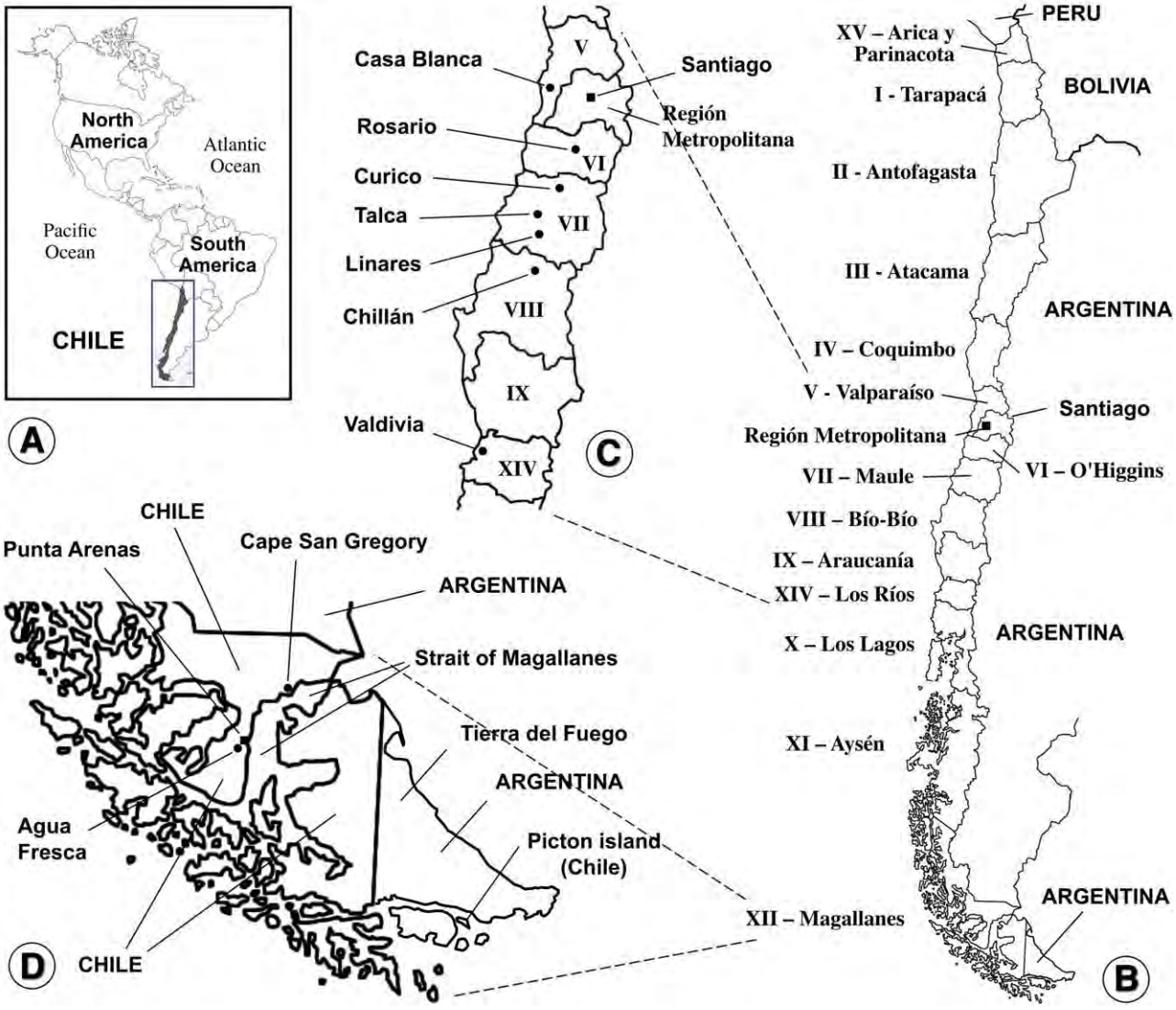


Fig. 1. Map of Chile showing localities where lymnaeids were collected: (A) location of Chile in South America; (B) division of country in 15 political regions; (C) central regions showing lymnaeid sampling localities (Casa Blanca, Rosario, Chillán and Valdivia) and localities where high human prevalences were described (Curico, Talca and Linares); (D) Región XII of Magallanes showing lymnaeid sampling localities (Cape San Gregory, Punta Arenas and Agua Fresca); note location of Picton island.

phenol–chloroform extraction and ethanol precipitation method. The procedure steps were performed according to methods outlined previously (Bargues and Mas-Coma, 1997; Bargues et al., 2001, 2007a). The pellet was dried and resuspended in 30 μl sterile TE buffer (pH 8.0). This suspension was stored at −20 °C until use.

2.2.2. DNA sequence amplification

Each one of the three DNA markers were PCR amplified independently for each lymnaeid specimen and each PCR product was sequenced for a bona-fide haplotype characterisation. The rDNA spacers ITS-2 and ITS-1 were amplified using primers previously described (Bargues et al., 2001, 2006a, 2007a; Mas-Coma et al., 2001). A mitochondrial DNA *cox*1 gene fragment was amplified using universal primers (Folmer et al., 1994). Amplifications were generated in a Mastercycle ep*gradient* (Eppendorf, Hamburg, Germany) using 4–6 μl of genomic DNA for each 50 μl PCR reaction. PCR conditions were 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C, preceded by 30 s at 94 °C and followed by 7 min at 72 °C for ITS-2 and ITS-1, and by 40 cycles of 30 s at 90 °C, 1 min at 48 °C and 1 min at 72 °C, preceded by 2.5 min at 94 °C and followed by 10 min at 72 °C for *cox*1. Ten μl of each PCR product was checked by staining with ethidium bromide on 1% Nusieve® GTG agarose (FMC) gel electrophoresis, using the Molecular Weight Marker VI (Boehringer Mannheim) at 0.1 μg DNA/μl as control.

2.2.3. Purification and quantification of PCR products

Primers and nucleotides were removed from PCR products by purification on Wizard™ PCR Preps DNA Purification System (Promega, Madison, WI, USA) according to the manufacturer's protocol and resuspended in 50 μl of 10 mM TE buffer (pH 7.6). The final DNA concentration was determined by measuring the absorbance at 260 and 280 nm.

2.2.4. DNA sequencing

The sequencing of the complete rDNA ITS-2 and ITS-1 and the fragment of the mtDNA *cox*1 gene was performed on both strands by the dideoxy chain-termination method (Sanger et al., 1977). It was carried out with the Taq dye-terminator chemistry kit for ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), using PCR primers.

2.2.5. Sequence alignments

Sequences were aligned using CLUSTAL-W version 1.8 (Thompson et al., 1994) and MEGA 4.0 (Tamura et al., 2007). Subsequently, minor corrections were manually introduced for a better fit of nucleotide correspondences in microsatellite sequence regions. Homologies were performed using the BLASTN programme from the National Center for Biotechnology information web site (http://www.ncbi.nlm.nih.gov/BLAST).

2.2.6. DNA haplotype nomenclature

The codes for the sequences obtained follow the standard nomenclature proposed for lymnaeid snails previously (Bargues and Mas-Coma, 2005; Bargues et al., 2006a; Mas-Coma et al., 2009a). It shall be noted that haplotype codes are only definitive in the case of complete sequences. When dealing with fragments or incomplete sequences, haplotype codes are provisional, as is the case here of the mtDNA *cox*1 fragment obtained.

2.2.7. Sequence comparisons

The following sequences from GenBank-EMBL have been used for comparison and/or phylogenetic analyses (species names as noted in respective articles):

- rDNA ITS-2: *Galba truncatula* H1 (AJ296271), H2 (AJ243017) and H3 (=*Lymnaea viatrix sensu* Ueno et al., 1975; =*L. cubensis sensu* Ueno et al., 1975) (AJ272051) (Bargues et al., 2001; Mas-Coma et al., 2001; Bargues et al., 2007a); *L. cubensis* H1 (AM412223), H2 (FN182200), H3 (FN182201) and H4 (JF514088) (Bargues et al., 2007a, 2011b, in press-a), *L. viatrix* H1 (type locality Rio Negro, Argentina) (AM412224) (Bargues et al., 2007a), *L. neotropica* H1 (AM412225) and H2 (JF514089) (Bargues et al., 2007a, in press-a), *L. humilis* H1 (FN182191) and H2 (FN182192) (Bargues et al., 2011b), *L. diaphana* H1 (JF909498) (Bargues et al., in press-b); and *Pseudosuccinea columella* H1 (FN598155) and H2 (FN598156) (Bargues et al., 2011a).
- rDNA ITS-1: *G. truncatula* HA (AJ243018), HB (AJ296270), HC (=*L. viatrix sensu* Ueno *et al.*, 1975; =*L. cubensis sensu* Ueno et al., 1975) (AJ272052) and HD (JF514090) (Mas-Coma et al., 2001; Bargues et al., 2001, 2006a, 2007a, in press-a); *L. cubensis* HA (AM412226), HB (FN182202) and HC (FN182203) (Bargues et al., 2007a, 2011b), *L. viatrix* HA (type locality Rio Negro, Argentina) (AM412227) (Bargues et al., 2007a), *L. neotropica* (AM412228) (Bargues et al., 2007a), *L. humilis* HA (FN182193) and HB (FN18219) (Bargues et al., 2011b), *L. diaphana* HA (JF909499) (Bargues et al., in press-b); and *P. columella* HA (FN598160) (Bargues et al., 2011a).
- mtDNA *cox*1 gene: *G. truncatula cox*1a (AM494011), *cox*1b (JF461487) (Bargues et al., 2007a, in press-a and *G. truncatula* (EU818799) (Albrecht et al., 2008); *L. cubensis cox*1a (AM494009) and *cox*1b (FN182205), *L. viatrix* cox1a (type locality Rio Negro, Argentina) (AM494010) (Bargues et al., 2007a), *L. neotropica cox*1a (AM494008), *cox*1b (FN356741), *cox*1c (JF461485) and *cox*1d (JF461486) (Bargues et al., 2007a, 2011b, in press-a; Mera y Sierra et al., 2009), *L. humilis cox*1a, *cox*1b and *cox*1c (FN182197-9) (Bargues et al., 2011b), *L. diaphana cox*1a (JF909501) (Bargues et al., in press-b); *P. columella* (FN598165) (Bargues et al., 2011a) and *P. columella* (AY227366) (Remigio and Hebert, 2003).

2.3. Phylogenetic inference

Phylogenetic analysis of ITS-2 and ITS-1 combined haplotypes was reconstructed using the Maximum Likelihood (ML) method implemented in PAUP version 4.0b10 and PhyML program version 3.0 aLRT. ML parameters and the evolutionary model best fitting our dataset were determined using Akaike and Bayesian information criteria (AIC and BIC) (Akaike, 1974; Posada and Buckley, 2004), implemented in jModeltest vesion 0.1.1 (Posada, 2008). Starting branch lengths were obtained using the least-squares method with ML distances.

To provide an assessment of the reliability of the nodes in the ML tree, three methods were used. First, a distance-based phylogeny using the neighbor-joining (NJ) algorithm (Saitou and Nei, 1987) with the ML pairwise distances was obtained and statistical support for the nodes was evaluated with 1000 bootstrap replicates, with and without removal of gapped positions, in PAUP. Second, a bootstrap analysis using 1000 replicates was made by using branch-swapping algorithm (tree-bisection-reconnection TBR) with full heuristic search in PAUP. Third, a Bayesian phylogeny reconstruction procedure was applied to obtain posterior probabilities (BPP) for the nodes in the ML tree, by using the same evolutionary model as above, implemented in MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). Four Markov Chain Monte Carlo (MCMC) chains were run for 10,000 generations, sampling every 100 generations, with the first 100 sampled trees discarded as "burn-in". Finally, a 50% majority rule consensus tree was constructed. Reliability for internal branches in the PhyML tree was also assessed using the aLRT test (SH-Like) for comparative purposes.

Phylogenetic analyses were performed after adding the following reference sequences of rDNA ITS-2 and ITS-1 of lymnaeids stored in the GenBank database: *L.* (*Stagnicola*) *palustris palustris* (AJ319620, AJ626849); *L.* (*S.*) *fuscus* (AJ319621, AJ626856) (Bargues et al., 2001); *Catascopia catascopium* (AF013143, AF013143); *Hinkleyia caperata* (AF013139, AF013139) (Remigio and Blair, 1997); *Radix auricularia* (AJ319628, JF922878); *R. balthica (=R. peregra)* (AJ319633, JF922879) (Bargues et al., 2001, in press-a). The intergenic region sequence (AY030361) including both ITSs of the planorbid species *Biomphalaria pfeifferi* (De Jong et al., 2001) was used as outgroup.

3. Results

Nuclear rDNA ITS-2 and ITS-1 and mtDNA *cox*1 nucleotide sequence data reported in this article are available in the GenBank™, EMBL and DDBJ databases under the accession numbers noted in Table 1.

3.1. Lymnaea viator

Snail specimens collected from Valle Casa Blanca, Valparaiso, preliminarily classified as *L. viatrix*, proved to have been well identified after rDNA and mtDNA marker sequencing (Table 1).

- **rDNA ITS-2**. All the specimens analysed presented the same ITS-2 sequence, of 444 bp and 53.9% GC content. When compared to the ITS-2 haplotype of *L. viatrix* from Rio Negro, Argentina, available in GenBank (L.via-H1: AM412224), it proved to be different in one mutation (transition C/T in H1/H2) in position 414 and in 8 polymorphic sites corresponding to 8 indels related to a different number of repeats in the microsatellites GCTT and GCTC, being $(GCTT)_4$ and $(GCTC)_1$ in Casa Blanca whereas $(GCTT)_5$ and $(GCTC)_2$ in Rio Negro (Table 2). The code H2 has been ascribed to this new haplotype (Table 1).
- **rDNA ITS-1**. All the lymnaeid individuals showed identical ITS-1 sequence, of 574 bp long and 54.0% GC content. When compared to the ITS-1 haplotype of *L. viatrix* from Rio Negro (L.via-HA: AM412227), it proved to be different in 10 polymorphic sites corresponding to two mutations, one tetranucleotide repeat and four insertion/deletions (Table 2). The code HB has been ascribed to this new haplotype (Table 1).
- **mtDNA cox1**. Two different nucleotide sequences were found, both of 672 bp but with different biased AT content of 69.4% and 69.5%. These haplotypes differ in only one mutation in position

Table 2
Nucleotide differences and microsatellite repeats found in the ITS-2 and ITS-1 rDNA haplotypes of *Lymnaea viator* from Casa Blanca, Chile, and Rio Negro, Argentina.

ITS-2 rDNA	positions	
	microsatellite repeats	
	53-68	69-76
L. viator H1** (Rio Negro, Argentina)	$(GCTT)_5$	$(GCTC)_2$
L. viator H2 (Casa Blanca, Chile)	$(GCTT)_4$	$(GCTC)_1$

ITS-1 rDNA	positions	
		microsatellite repeats
	111122224 3000027779 9345627897	103-106
L. viator HA* (Rio Negro, Argentina)	G----C---A	$(ATTG)_1$
L. viator HB (Casa Blanca, Chile)	AATTGTTGA-	$(ATTG)_2$

Positions refer to variable positions obtained in the alignment made with MEGA 4.0 and CLUSTALW 1.8.
* AM412227.
** AM412224.

488 of their alignment, which represent one aminoacid change (Table 3). When both haplotypes are compared with *cox*1a haplotype of *L. viatrix* from Rio Negro (AM494010), a total of 13 variable positions appear, all of them singleton sites (Table 3). The codes *cox*1b and *cox*1c have been ascribed to these new haplotypes (Table 1). Interestingly, nucleotide differences give rise to three amino acid differences between the Chilean and Argentinian haplotypes of *L. viator* (Table 3).

3.2. Galba truncatula

Specimens from the localities of Rosario, Chillán and Isla Teja, Valdivia, preliminarily classified as *L. viatrix*, proved to be *G. truncatula* by rDNA and mtDNA sequences (Table 1).

- **rDNA ITS-2**. Specimens studied showed the same ITS-2 sequence, of 401 bp and 58.6% GC content. When compared with the three ITS-2 haplotypes of *G. truncatula* (H1, H2, H3), the sequence from Chile proved to be identical to the haplotype H3 (AJ272051).
- **rDNA ITS-1**. All specimens analysed presented the same ITS-1 sequence, of 504 bp and 57.5% GC content. When compared with the four ITS-1 haplotypes of *G. truncatula* available (HA, HB, HC and HD), it proved to be identical to the haplotype HC (AJ272052).
- **mtDNA cox1**. Only one haplotype was detected, of 672 bp and 68.6% AT content. This haplotype proved to be different from the previously described haplotypes *cox*1a and *cox*1b for this species. Hence, the provisional code G.tru-*cox*1c has been assigned (Table 1). In a 672 bp-long alignment, including *cox*1a–c haplotypes of *G. truncaltula* and *cox*1 sequence of *G. truncatula* from Germany (EU818799), a total of 30 variable positions appeared, including 1 parsimony informative position and 29 singleton sites (Table 4). The *cox*1c haplotype of *G. truncatula* of Chile differs by only one amino acid change in position 16 when compared with *cox*1a and *cox*1b amino acid haplotypes (Table 4).

3.3. Pectinidens diaphana

Specimens from Cape San Gregory, Magallanes, preliminarily classified as *L. diaphana*, proved to be correctly classified by rDNA and mtDNA sequences (Table 1).

- **rDNA ITS-2**. All specimens studied presented the same ITS-2 sequence of 495 bp and 57.0% GC content. This sequence was compared with the ITS-2 haplotype of *L. diaphana* available in GenBank (L.dia-H1) and proved to be identical.
- **rDNA ITS-1**. The specimens analysed presented two different ITS-1 sequences, one of 520 bp and 54.0% GC content found close to the Magallanes Strait ("vegas bajas") and the other of 524 bp and 54.0% GC content found somewhat more northward ("vegas altas"). The first is identical to the haplotype L.dia-HA of topotypic specimens of *L. diaphana* (JF909499). Consequently, the new code HB has been assigned to the second one (Table 1). Nucleotide differences between both HA and HB haplotypes concern a different number of repeats of the microsatellites CAGT, CTAG and TCAG, being $(CAGT)_5$, $(CTAG)_3$ and $(TCAG)_2$ in HA, and $(CAGT)_6$, $(CTAG)_4$ and $(TCAG)_1$ in HB (Table 5).
- **mtDNA cox1**. Four different haplotypes were detected in the specimens studied, with a length of 672 bp and an AT content of

Table 3
Nucleotide (Nt) and amino acid (Aa) differences found in the mtDNA *cox*1 gene sequence of *L. viator* populations studied from Chile and Argentina.

	Nt	Aa
	22344445556	11
	1913926884882	769
	3946608081341	935
*L. viator cox*1a (Rio Negro, Argentina)*	CCCGTTCTCTCTT	STL
*L. viator cox*1b (Casa Blanca, Chile)	TTTACCTC.CTGC	K.C
*L. viator cox*1c (Casa Blanca, Chile)	TTTACCTCTCTGC	KIC

Position, numbers (to be read in vertical) refer to variable positions obtained in the alignment made with MEGA 4.0;. = identical; haplotype codes only provisional due to incomplete sequences of the gene.
* AM494010.

Table 4
Nucleotide (Nt) and amino acid (Aa) differences found in the mtDNA *cox*1 gene sequence of *Galba truncatula* populations studied from Chile and other countries.

	Nt	Aa
	1111122223333333344455666666	
	4781138811331224567906817022 35	1
	6871750969178145795068030047 07	6
*G. truncatula cox*1a*	GGAACAAAATTAGAGCCCCCCTCGATACGT	V
*G. truncatula cox*1b**	.AGGTGGGGCCGAGATTTT.TCTAG.GTCC	.
G. truncatula Germany***	........G.................. A-	.
*G. truncatula cox*1c (Chile)	A..................T.....C....	I

Position = numbers (to be read in vertical) refer to variable positions obtained in the alignment made with MEGA 4.0;. = identical; - = indel; haplotype codes only provisional due to incomplete sequences of the gene.
* AM494011.
** JF461487.
*** EU818799.

69.4%, 69.6%, 69.8% and 69.3%. The first is identical to the haplotype *cox*1a of topotypic specimens of *L. diaphana* (JF909501). The codes *cox*1b, *cox*1c and *cox*1d have been ascribed to the three new ones (Table 1). Nucleotide and amino acid differences between the four detected *cox*1 haplotypes are listed in Table 6. In the amino acidic sequence alignment (224 aa long), *cox*1a, *cox*1b and *cox*1d haplotypes appear to be identical. Amino acidic differences detected between the four *L. diaphana cox*1 haplotypes only concern one amino acid change, generated by the haplotype *cox*1c in position 133 (Table 6).

3.4. *Lymnaea patagonica*

Specimens from Agua Fresca, Magallanes, originally ascribed to *L. patagonica* and corresponding to the type locality of that species, proved in fact to be *L. diaphana* by rDNA and mtDNA (Table 1).

- **rDNA ITS-2**. One haplotype was found in the specimens studied. Their length and GC content was 495 bp and 57.0%, respectively. This sequence fits exactly with the topotypic haplotype L.dia-H1 of the species *L. diaphana* (JF909498).
- **rDNA ITS-1**. The specimens from this type locality presented one ITS-1 sequence of 516 bp and 54.0% GC content. When comparing with haplotypes HA and HB of *L. diaphana*, nucleotide differences only concerned a different number of repeats of the microsatellites CAGT, CTAG and TCAG. Consequently, the sequence of *L. patagonica* was assigned a new haplotype for *L. diaphana* and deposited under the code L.dia-HC (Table 1). Differences between microsatellite repeats and their position in the alignment of *L. diaphana* (=*L. patagonica*) haplotypes are shown in Table 5.
- **mtDNA cox1**. Three different haplotypes were detected in the specimens from Agua Fresca, with a length of 672 bp and an AT content of 69.5%, 69.6% and 69.2%. When comparing these *L. patagonica cox*1 haplotypes with those known in *L. diaphana*, the three proved to be new and hence the codes *cox*1e, *cox*1f and *cox*1g were respectively assigned (Table 1). Only a very few nucleotide differences appear when comparing *L. patagonica* and *L. diaphana* haplotypes. The three sequences of *L. patagonica* give rise to only one COX1 amino acid sequence, which appears to be identical to the one corresponding to *cox*1a of *L. diaphana* topotypic specimens (Table 6).

3.5. *Lymnaea lebruni*

Specimens from Punta Arenas, Magallanes, originally ascribed to *L. lebruni* and corresponding to the type locality of that species, proved in fact to be *L. diaphana* by rDNA and mtDNA (Table 1).

- **rDNA ITS-2**. One haplotypes of a length of 498 bp and a GC content of 56.8% was found in the specimens studied. Nucleotide differences between this haplotype and L.dia-H1 (JF909498) were very few, including only one transversion (C/A in *patagonica*/*lebruni* haplotypes) in position 63 of their respective alignment and a different number of repeats in the microsatellite CGT, being $(CGT)_2$ in the first and $(CGT)_3$ in the second (Table 5). The code L.dia-H2 has been assigned to the latter (Table 1).

Table 5
Nucleotide differerences and microsatellite repeats found in the ITS-2 and ITS-1 rDNA haplotypes of *Lymnaea diaphana* and *L. patagonica* from Chile.

ITS-2 rDNA	Position	
	Tv	Microsatellite repeats
	63	86-94
L. diaphana H1 (=*L. patagonica*)	C	$(CGT)_2$
L. diaphana H2 (=*L. lebruni*)	A	$(CGT)_3$

ITS-1 rDNA	Position		
	Microsatellite repeats		
	42–65	222–241	
L. diaphana HA	$(CAGT)_5$	$(CTAG)_3$	$(TCAG)_2$
L. diaphana HB	$(CAGT)_6$	$(CTAG)_4$	$(TCAG)_1$
L. diaphana HC (=*L. patagonica*)	$(CAGT)_4$	$(CTAG)_3$	$(TCAG)_2$
L. diaphana HD (=*L. lebruni*)	$(CAGT)_6$	$(CTAG)_3$	$(TCAG)_1$

Positions refer to variable positions obtained in the alignment made with MEGA 4.0 and CLUSTALW 1.8; Tv, Transversion.

Table 6
Nucleotide (Nt) and amino acid (Aa) differences found in the mtDNA *cox*1 gene sequence of *Lymnaea diaphana* and *L. patagonica* populations studied from Chile.

	Nt	Aa
	23334445666	1
	5634691685236	3
	7718074808160	3
*L. diaphana cox*1a*	ACTGTAGCTTTGG	I
*L. diaphana cox*1b	C..A. .....A.	.
*L. diaphana cox*1c	CT.A.GA. ..A.	V
*L. diaphana cox*1d	C..A. ...CCA.	.
*L. diaphana cox*1e (= *L. patagonica*)	C.CA...T.C.A.	.
*L. diaphana cox*1f (= *L. patagonica*)	C..A...T.C.A.	.
*L. diaphana cox*1g (= *L. patagonica*)	C..AC...CC.A.	.
*L. diaphana cox*1h (= *L. lebruni*)	C.CA...T.C.AA	.

Position = numbers (to be read in vertical) refer to variable positions obtained in the alignment made with MEGA 4.0;. = identical; - = indel; haplotype codes only provisional due to incomplete sequences of the gene.
* JF909501.

- **rDNA ITS-1**. The specimens from Punta Arenas type locality showed an ITS-1 sequence of 520 bp and 54.0% GC content. Nucleotide differences with regard to haplotype L.dia-HC of *L. diaphana* (=*L. patagonica*) also concerned a different number of repeats of the microsatellites CAGT and TCAG, being $(CAGT)_4$ and $(TCAG)_2$ in the first, and $(CAGT)_6$ and $(TCAG)_1$ in the second. When comparing with haplotypes HA and HB of *L. diaphana*, nucleotide differences only concerned a different number of the same microsatellite repeats. Hence, the sequence of *L. lebruni* was assigned new haplotype for *L. diaphana* and deposited under the code L.dia-HD (Table 1). Differences between microsatellite repeats and their position in the alignment of *L. diaphana* (=*L. lebruni*) haplotypes are shown in Table 5.
- **mtDNA cox1**. One haplotype was found in the specimens from Punta Arenas, with a length of 672 bp and an AT content of 69.6%. When comparing this *L. lebruni cox*1 haplotype with those known in *L. diaphana*, it proved to be new and therefore the code *cox*1h was assigned (Table 1). Nucleotide differences between *L. lebruni*, *L. patagonica* and *L. diaphana* haplotypes were only a very few, including 13 variable positions in the 672 bp-long alignment, of which 3 parsimony informative positions and 10 singleton sites. In the amino acid sequence alignment (224 aa long), *L. lebruni* shows no difference regarding *L. patagonica* and is thus also identical to the one corresponding to L.dia-*cox*1a (Table 6).

3.6. *Chilina sp.*

The small specimens from Isla Teja, Río Cau-Cau, in Valdivia, preliminarily identified as *L. cousini* according to shell shape, proved to belong to the family Chilinidae after the BLASTAn comparison performed by using the mtDNA *cox*1 sequence obtained (no ITS sequences could be obtained).

3.7. *Phylogenetic analysis*

The ML model best fitting the ITS-1 and ITS-2 combined haplotype dataset was found to be HKY85+G+I, using a ts/tv ratio of 1.44 (kappa = 2.8353224), base frequencies for A, C, G and T of 0.224205, 0.263705, 0.230633 and 0.281457, respectively, gamma (continuous) with shape parameter alpha of 0.75, and a proportion of invariable sites equal to 0.036 (-ln *L*9206.34542).

The combination of the two internal transcribed spacers in a single dataset generated a robust tree, indicating phylogenetic accordance between the two spacers in the ML tree obtained (Fig. 2).

The monophyly of the ITSs haplotypes of the five lymnaeid species of the *Galba/Fossaria* group, which comprises the *F. hepatica* main intermediate host species, was strongly supported (100/100/100 in NJ/ML/BBP). In this clade, the haplotypes of *L. viator* and *G. truncatula* detected in Chile appear clustering together with other previously described haplotypes of the same species, respectively. Concerning the four combined haplotypes of *P. diaphana*, all of them cluster together in a monophyletic branch included in the same clade comprising the North American stagnicolines, with a very high bootstrap support of 96%, 97% and 100% with NJ/ML/BPP algorithms, respectively. The only difference detected in the topologies obtained with PAUP, PhyML and MrBayes, concerns *P. columella*, appearing as a basal branch of stagnicoles and *Galba/Fossaria* groups in ML trees obtained in PAUP and PHyML (73/56 of support in NJ/ML), whereas appearing as a sister group within the *L. diaphana*-American stagnicolines clade in the ML tree obtained with MrBayes (100% support). Moreover, the ingroup branches including *Pseudosuccinea*, *Galba/Fossaria* and stagnicolines appears well separated from the *Radix* branch, with high supports (86/65–88/100 in NJ/ML/BBP).

4. Discussion

4.1. *Lymnaeid species analyses*

Lymnaea viator specimens collected in the second type locality Casa Blanca, Valparaiso, have proved to be unexpectedly different from topotypic specimens of the same species from Rio Negro, Argentina, at the level of the three markers analysed. Although nucleotide differences in the species level markers ITS-2 and ITS-1 do not justify two different species (most differences related to different microsatellite repeat numbers), according to interspecific genetic distances known in invertebrates (Mas-Coma and Bargues, 2009) and also in Lymnaeidae (Bargues et al., 2001), these few ITS differences together with the nucleotide and amino acid differences in *cox*1 suggest a relatively old divergence of these *L. viator* populations from one and another side of the Andean chain. Additionally, it should be remembered that the masculine spelling *viator* used by D'Orbigny (1835) when originally describing the species within the genus *Lymnaeus* Lamarck, 1799, was later modified to the feminine spelling *viatrix* to use it with the feminine *Lymnaea* by Paraense (1976). However, this does not fit articles 31.2.1 and 34.2.1 of the International Code of Zoological Nomenclature. Hence, the correct species name for the specimens from Casa Blanca is *Lymnaea viator* D'Orbigny, 1835.

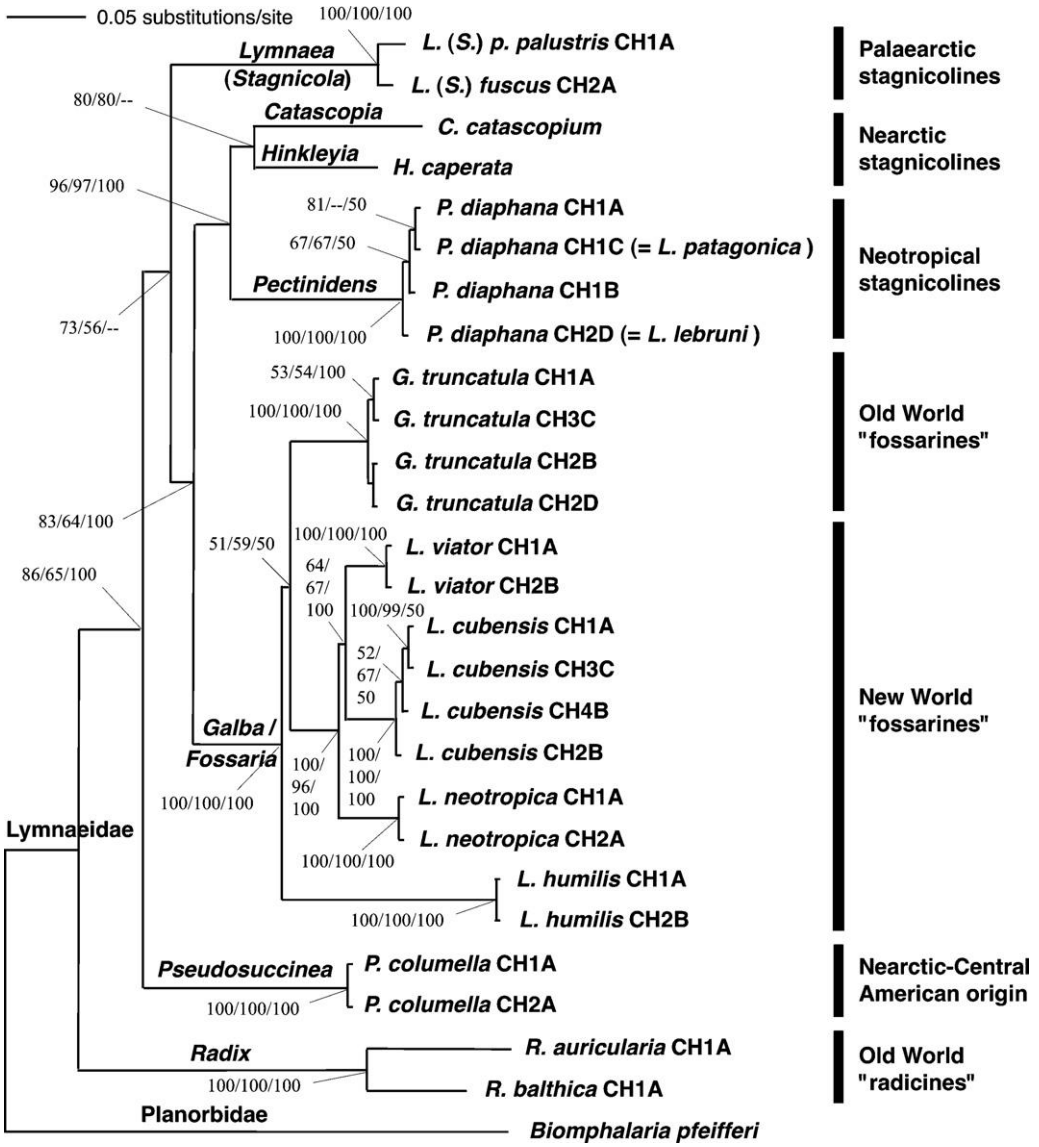


Fig. 2. Phylogenetic tree based on maximum-likelihood (ML) estimates, including lymnaeid species studied in Chile together with selected species representing the different lymnaeid groups of interest for comparison purposes, obtained using the planorbid *B. pfeifferi* as outgroup. Scale bar indicates the number of substitutions per sequence position. Support for nodes a/b/c: (a): bootstrap with NJ reconstruction using PAUP with ML distances and 1000 replicates; (b): bootstrap with ML reconstruction using PAUP with 1000 full-heuristic replicates; (c): Bayesian posterior probability with ML model using MrBayes.

Galba truncatula is here molecularly confirmed to be present in the three Región VI (O'Higgins), Región VIII (Bio-Bío) and Región XIV (Los Ríos). Thus, Chile becomes another country to add to the already wide geographical spread of this lymnaeid in South America, whose presence has already been shown by DNA sequencing methods in Bolivia (Bargues and Mas-Coma, 1997; Bargues et al., 1997; Mas-Coma et al., 2001), Peru (Esteban et al., 2002), Argentina (Bargues et al., 2006b, 2007b) and recently also Venezuela (Bargues et al., in press-a). The *G. truncatula* haplotypes H3 of ITS-2 and HC of ITS-1 found in Chile are worth mentioning, as they indicate that Chilean populations of *G. truncatula* are identical to its populations in Bolivia and Argentina at the level of rDNA (Bargues et al., 2007a).

Pectinidens diaphana specimens from Cape San Gregory, Magallanes, were phenotypically re-described by Paraense (1984). Its DNA sequencing showed a large intraspecific variability, with one new haplotype in ITS-1 and three new ones in *cox*1 when compared to topotypic specimens (Bargues et al., in press-b). This variation is remarkable within a particular area and suggests that the type locality is most probably included within the area of origin of that species where it has evolved during a long period. In its turn, this suggests that *P. diaphana* may be a species preferentially adapted to inhabit cold regions.

L. patagonica specimens from the type locality of the species, Agua Fresca in Magallanes, proved that it is a synonym of *P. diaphana*, as already proposed by Hubendick (1951). Moreover, the very few nucleotide differences at ITS-2 and ITS-1 levels, most of them related to different microsatellite repeat numbers, together with the silent few mutations in the *cox*1 mtDNA gene, do not even support the possibility to keep *patagonica* as a subspecies of *P. diaphana*. This result does therefore show that *P. diaphana* is (i) more widespread southward, (ii) it has a pronounced intraspecific genetic variability which concerns both nuclear rDNA and mtDNA, and (iii) this supports the aforementioned hypothesis of dealing with a lymnaeid species linked to a cold climate.

L. lebruni specimens from the type locality of the species, Punta Arenas in Magallanes, showed morphological features of the shell in agreement with the original description of Mabille (1884) and slightly different from the *L. patagonica* topotypic specimens found. Nucleotide differences between both at the level of all markers may be related to that phenotypic difference. However, these differences regarding *L. patagonica* as well as *P. diaphana* are too few to consider it a different taxon, whether at species or at subspecies level. Hence, it additionally supports the aforementioned conclusions on *P. diaphana*.

4.2. Characterisation of the lymnaeid fauna from Chile

According to the DNA sequencing results and the taxonomic synonymies, the lymnaeid fauna of Chile originally including seven species should be reduced to the following only three valid species: *L. viator*, *G. truncatula* and *P. diaphana* (=*L. patagonica*; =*L. lebruni*).

The quotation of *L. cousini* in Valdivia, Chile by Hubendick (1951) represents the southernmost report of this species and poses acceptance problems already highlighted (Paraense, 1995). This species is hitherto only known in Ecuador and Colombia (Bargues et al., 2011a) and its presence in such a southern locality as Valdivia is surprising. The brief description of the shell and the male copulatory organ made by Hubendick (1951) is insufficient to draw clear conclusions on the correct classification of the Chilean specimen. Although the climate of the lowland of Valdivia is of temperate type and thus not so different from the environmental characteristics of the high altitude in the Andean areas of Ecuador and Colombia, such a southern isolated location becomes bewildering and should be verified (Bargues et al., 2011a). We were unfortunately unable to find it.

The molecular and phylogenetic data prove that *L. patagonica* is not a valid species, as argued by Malek (1985), but a synonym of *P. diaphana*. Similarly, the probable synonymy of *L. lebruni*, from Punta Arenas as type locality, with *P. diaphana* proposed by Pilsbry (1911) may be considered definitive according to the DNA sequencing results obtained. And finally, *L. chilensis* is taxonomically considered a *nomen nudum* (Hubendick, 1951) and may therefore be excluded.

Thus, the lymnaeid fauna of mainland Chile appears composed by two authochthonous species, *L. viator* and *P. diaphana*, and a third introduced species of Palaearctic origin *G. truncatula*. It sould be highlighted that *L. viator* and *G. truncatula* belong both to the *Galba/Fossaria* group of lymnaeid intermediate hosts, a group within which specimen classification is usually very difficult due to the pronounced morphological similarity between species. In the case of *P. diaphana*, the phylogenetic analysis shows that it is not a *Galba/Fossaria* member but rather a stagnicoline (Fig. 2), as already emphasized very recently (Bargues et al., in press-b). However, this does not mean that this species may be very easily confused with both *L. viator* and *G. truncatula* when only basing on shell characteristics (see Paraense, 1984), mainly when dealing with small-medium-sized specimens. The great lymnaeid similarity problem is undoubtedly the responsible for the overlook of the presence of *G. truncatula* in Chile up to the present. Hence the usefulness of the DNA markers here furnished to help in both field and laboratory studies on disease transmission, epidemiology and control of fascioliasis in Chile from now on.

The presence of *G. truncatula* should consequently be added to the list of seven exotic freshwater snail species already described in Chile (Letelier et al., 2007). The introduction success of these snail invaders in Chile appears to be related to increasing commercial activities and trade in the south-eastern Pacific, and their subsequent mainland spread to given global environmental and climate changes or the rises in temperature produced in the Chilean coastal areas by the oceanographic El Niño events. Several of these exotic species registered in Chile are unfortunately well known due to their capacity to transmit helminthic diseases, such as *Melanoides tuberculata* and *Pomacea* spp. (Letelier et al., 2007).

4.3. Implications for human and animal fascioliasis epidemiology

Although there are only three species of lymnaeid snails in mainland Chile, from the public health point of view the problem lies in the fascioliasis transmission capacity of all of them. Whereas in Chile the transmission of the liver fluke has only been demonstrated in the case of *L. viator* so far (Tagle, 1944), this lymnaeid species has repeatedly proved to be involved in similar latitudes in Argentina (Venturini, 1978; Rossanigo et al., 1983; Kleiman et al., 2004). *Pectinidens diaphana* has also been reported to participate in the transmission, at least in Peru (Cordova et al., 1961; Santa Cruz, 1979; Larrea et al., 1994), and its phylogenetic relationship with stagnicolines (Fig. 2) suggests an intermediate host capacity of secondary level (Bargues et al., in press-b). Finally, *G. truncatula* is known as both the original and main intermediate host of *F. hepatica* wherever this lymnaeid species is present, including a very large body of literature from throughout (see reviews in Bargues et al., 2001; Mas-Coma et al., 2009a) and has also proved to be the responsible for fascioliasis transmission in South America (Mas-Coma et al., 2001).

The combined ITS-2–ITS-1 haplotype G.tru-H3C found in Chile merits additional comments, as it is the same as the one responsible for the disease transmission in the endemic area of fascioliasis with the highest prevalences and intensities known in humans: the Northern Bolivian Altiplano. In this area, prevalences detected in some communities were of up to 72% and 100% in coprological and serological surveys, respectively, and intensities reached up to more than 8000 eggs per gram (epg) in children (Esteban et al., 1997, 1999; Mas-Coma et al., 2009a). Although this human fascioliasis hyperendemic area is located at 3800-4100 m altitude and hence environmental conditions and climatic variables evidently differ from the pronouncedly lower altitudes where *G. truncatula* has been collected in Chile, temperatures may be similar because of the most southern latitude (Fuentes et al., 1999). Indeed, studies on the influences of temperature and rainfall on *F. hepatica* egg development have shown how the liver fluke is able to be transmitted in given seasons even in southern areas as Valdivia and Temuco (Valenzuela, 1979), under environmental characteristics which are similar to those where *G. truncatula* plays its intermediate host role in Europe.

Moreover, two other aspects shall be considered. First, the wide ecological features of *G. truncatula* do allow it to come close to human settings. This freshwater snail species may be even found in polluted waters such as those present in villages, as detected in the Northern Bolivian Altiplano (Mas-Coma et al., 1999). This anthropophylic ecology enables this lymnaeid to be in the background of many human infections. Second, *G. truncatula* is markedly linked to areas where livestock is present. This allows it to be passively transported by domestic animals as sheep or cattle from one place to another and explains the great spreading power of this lymnaeid (Mas-Coma et al., 2009a). This suggests a high liver fluke infection risk for both humans and animals in those areas of Chile where this intermediate host may be present.

In Chile, prevalence data differ according to domestic animal host species and regions. The higher prevalences in cattle are found in Región VII (85.7%), followed by Región VIII (66.8%), Región VI (50.5%) and Región IV (44.7%). In sheep, the highest prevalences are in Región VIII (17.9%), followed by Region IV (15.2%), Region III (15.1%) and Region IX (13.9%). In goats, the highest prevalences are in Región IV (20.9%), followed by Región II (19.9%), Región VII (14.6%) and Región VI (10.6%). In pigs, the highest prevalences are in Región VII (16.4%), followed by Región VIII (8.0%), Región II (9.6%) and Región IV (5.9%). In equines, the highest prevalences are in

Región VII (37.3%), followed by Región III (24.1%), Región IV (15.7%) and Región II (13.3%) (Morales et al., 2000). The high prevalence data from regions IV, VII and VIII should be highlighted, as in their slaughterhouses mainly animals from their respective regions are slaugthered (Alcaino and Apt, 1989). The Región VII has been the objective of particular studies due to its high prevalences (Gorman et al., 1979; Alcaino and Mozo, 1983; Alcaino et al., 1993).

An annual risk map was created for fascioliasis in the central regions of Chile by using both a climatic index and NDVI (normalized difference vegetation index) values as valid forecasting tools to assess disease transmission risk based on satellite remote sensing data (Fuentes and Malone, 1999). Despite the usefulness of these indexes previously verified in the human endemic area of Bolivia (Fuentes et al., 1999, 2001), final results obtained suggested transmission risk values which did not correlate cattle prevalence data for high endemic regions as Región VI and Región VII. Factors other than natural climate were highlighted to explain the lack of fit, such as irrigation practices and the effect of irregular topography on availability of springs, seeps and water collection sites that keep habitats wet through part of the dry season, thus extending the suitable transmission period (Fuentes and Malone, 1999). DNA sequencing results suggest that the absence of correlation between remote sensing data and bovine prevalences could also, or perhaps mainly, be due to differences of geographical distribution, ecological preferences, population dynamics and liver fluke infection suitability of each lymnaeid intermediate host species, *L. viator* and *G. truncatula*. Indeed, the endemic area in Bolivia where such methods proved to be useful is caused by only one lymnaeid species, *G. truncatula* (Mas-Coma et al., 2001).

A patchy distribution of infection is typical within fascioliasis endemic areas given its water-borne transmission linked to freshwater lymnaeid intermediate hosts (Mas-Coma et al., 1999). Within this patchy epidemiological frame, and considering that the infectivity of the metacercarial stage from different livestock species isolates has shown to be similar (Valero and Mas-Coma, 2000; Valero et al., 2001), domestic animal prevalence data alone do not explain why human infection data appears so high in the provinces of Curico, Talca and Linares in Región VII (Apt et al., 1992, 1993). Indeed, geographical distribution, prevalences and intensities of both human and animal infection are known to pronouncedly depend on the lymnaeid species involved in the local transmission and on characteristics such as their population dynamics, anthropophylic characteristics, type of water bodies, etc. (Bargues and Mas-Coma, 2005).

All in all, results of DNA sequencing of lymnaeid snails suggest an hypothesis for Chile of three lymnaeid intermediate hosts presenting different geograpical distributions and transmission capacities: (i) *G. truncatula*, as introduced species, would be the responsible for most of the human infection cases throughout a more or less large area restricted to central regions of the country, (ii) *L. viator* would be the main intermediate host involved in animal infection, mainly in areas which *G. truncatula* has not yet have the time to colonise, and (iii) *P. diaphana* may play a secondary intermediate host role in southern latitudes if overlapped with *L. viator* and/or *G. truncatula* or be the only transmitter in the most extreme southern areas of the country where it is the only lymnaeid present. The DNA sequencing results here exposed furnish a new baseline on which to undertake future appropriate studies on transmission, epidemiology and control of both human and animal fascioliasis in Chile. Elucidating the detailed geographical distribution of each one of the aforementioned lymnaeid intermediate hosts becomes crucial. Only after obtaining sufficient knowledge on intermediate host species distribution by using DNA markers could useful tools for disease assessment and control, as mathematical modelling based on climatic data and remote sensing and geographical information systems, be developed for Chile.

Acknowledgements

Studies funded by Projects No. SAF2006-09278 and SAF No. 2010-20805 of the Ministry of Education and Ministry of Science and Innovation, Madrid; and by the Red de Investigación de Centros de Enfermedades Tropicales – RICET (Projects No. C03/04, No. ISCIII2005-PI050574 and No. ISCIII-RETIC RD06/0021/0017 of the Programa de Redes Temáticas de Investigación Cooperativa RETICS/FEDER), FIS, Ministry of Health, Madrid, Spain.

Part of this work was made thanks to a personal fellowship funded to P. Artigas from Chile by the Agencia Española de Cooperación Internacional (AECI), Ministry of Foreign Affairs, Madrid, Spain. Thanks are given to Dr. Gerhard Falkner (Stuttgart, Germany) for taxonomic information funrnished concerning the old original descriptions of *L. viator* and *L. diaphana*. The donation of freshwater snail material from Isla Teja, Valdivia, by Lic. Pablo Cuervo (Mendoza, Argentina) is greatly acknowledged.

Field work activities performed within the worldwide initiative of WHO (Headquarters Geneva, Switzerland) against human fascioliasis. Technical support provided by the Servicio Central de Secuenciación para la Investigación Experimental (SCSIE) of the Universidad de Valencia (Dr. A. Martínez).

References

Akaike, H., 1974. A new look at the statistical model identification. IEEE Trans. Automat. Constr. 19, 716–723.

Albrecht, C., Wolf, C., Glöer, P., Wilke, T., 2008. Concurrent evolution of ancient sister lakes and sister species: the freshwater gastropod genus *Radix* in lakes Ohrid and Prespa. Hydrobiologia 615, 157–167.

Alcaino, H., Apt, W., 1989. Algunos antecedentes sobre la fascioliasis animal y humana. Monogr. Med. Vet. 11, 14–29.

Alcaino, H., Gorman, T., 1999. Parásitos de los animales domésticos en Chile. Parasitol. Día 23, 33–41.

Alcaino, H., Mozo, P., 1983. Antecedentes sobre el parasitismo en bovinos de la provincia de Linares (VII Región de Chile). Parasitol. Día 7, 108–112.

Alcaino, H., Vega, F., Gorman, T., 1993. Epidemiología de la fascioliasis hepatica en la VII Región, Chile. Parasitol. Día 17, 99–106.

Apt, W., 1987. Parasitic disease in Chile. Trop. Dis. Bull. 84, 2–11.

Apt, W., Aguilera, X., Vega, F., Zulantay, I., Retamal, C., Apt, P., Sandoval, J., 1992. Fasciliasis en la población rural de las provincias de Curico, Talca y Linares. Rev. Med. Chile 120, 621–626.

Apt, W., Aguilera, X., Vega, F., Alcaino, H., Zulantay, I., Apt, P., Gonzalez, V., Retamal, C., Rodriguez, J., Sandoval, J., 1993. Prevalencia de fascioliasis en humanos, caballos, cerdos y conejos silvestres en tres provincias de Chile. Bol. Of. Sanit. Panam. 115, 405–414.

Bargues, M.D., Mas-Coma, S., 1997. Phylogenetic analysis of lymnaeid snails based on 18S rDNA sequences. Mol. Biol. Evol. 14, 569–577.

Bargues, M.D., Mas-Coma, S., 2005. Reviewing lymnaeid vectors of fascioliasis by ribosomal DNA sequence analyses. J. Helminthol. 79, 257–267.

Bargues, M.D., Mangold, A.J., Muñoz-Antoli, C., Pointier, J.P., Mas-Coma, S., 1997. SSU rDNA characterization of lymnaeid snails transmitting human fascioliasis in South and Central America. J. Parasitol. 83, 1086–1092.

Bargues, M.D., Vigo, M., Horak, P., Dvorak, J., Patzner, R.A., Pointier, J.P., Jackiewicz, M., Meier-Brook, C., Mas-Coma, S., 2001. European Lymnaeidae (Mollusca: Gastropoda), intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS-2 sequences. Inf. Gen. Evol. 1, 85–107.

Bargues, M.D., Artigas, P., Jackiewicz, M., Pointier, J.P., Mas-Coma, S., 2006a. Ribosomal DNA ITS-1 sequence analysis of European stagnicoline Lymnaeidae (Gastropoda). Heldia. München 6, 29–40.

Bargues, M.D., Mera-y-Sierra, R., Gomez, H.G., Artigas, P., Mas-Coma, S., 2006b. Ribosomal DNA ITS-1 sequencing of *Galba truncatula* (Gastropoda: Lymnaeidae) and its potential impact on fascioliasis transmission in Mendoza, Argentina. Anim. Biodivers. Conserv. 29, 191–194.

Bargues, M.D., Artigas, P., Mera-y-Sierra, R.L., Pointier, J.P., Mas-Coma, S., 2007a. Characterisation of *Lymnaea cubensis*, *L. viatrix* and *L. neotropica* n. sp., the main vectors of *Fasciola hepatica* in Latin America, by analysis of their ribosomal and mitochondrial DNA. Ann. Trop. Med. Parasitol. 101, 621–641.

Bargues, M.D., Mera y Sierra, R., Gomez, H.G., Artigas, P., Mas-Coma, S., 2007b. Caracterización molecular de *Galba truncatula*, vector principal de la Fasciolíasis, en Argentina. Implicaciones en salud pública. Enf. Emerg., Barcelona 9, 77–82.

Bargues, M.D., Artigas, P., Khoubbane, M., Mas-Coma, S., 2011a. DNA sequence characterization and phylogeography of *Lymnaea cousini* and related species, vectors of fascioliasis in northern Andean countries, with description of *Lymnaea meridensis* n. sp. (Gastropoda: Lymnaeidae). Parasite Vector 4, 132.

Bargues, M.D., Artigas, P., Khoubbane, M., Flores, R., Glöer, P., Rojas, Garcia, R., Ashrafi, K., Falkner, G., Mas-Coma, S., 2011b. Lymnaea schirazensis, an overlooked snail

distorting fascioliasis data: genotype, phenotype, ecology, worldwide spread, susceptibility, applicability. PLoS ONE, doi:10.1371/journal.pone.0024567.
Bargues, M.D., Gonzalez, L.C., Artigas, P., Mas-Coma, S., in press-a. A new baseline for fascioliasis in Venezuela: lymnaeid vectors ascertained by DNA sequencing and analysis of their relationships with human and animal infection. Parasite Vector.
Bargues, M.D., Mera y Sierra, R.L., Artigas, P., Mas-Coma, S., in press-b. DNA multigene sequencing of topotypic specimens of the fascioliasis vector Lymnaea diaphana and phylogenetic analysis of the genus Pectinidens (Gastropoda). Mem. Inst. Osw. Cruz. 106.
Beck, H., 1838. Index molluscorum praesentis aevi musei Principis Augustissimi Christiani Frederici. Appendix: Specierum novarum in indice molluscorum praesentis aevi musei Principis Christiani Frederici ab autore propositarum characteres breves. Hafniae 1, 1–124 + 1–8.
Borie, C., Corona, S., Garin, A., Olea, P., Salcedo, M., Perez, C., Apt, W., 1990. Brote familiar de fascioliasis hepática aguda. Rev. Méd. Chile 118, 67–72.
Cordova, E., Naquira, F., Naquira, C., 1961. *Lymnaea diaphana* King como huésped intermediario de *Fasciola hepatica* en Arequipa (Perú). Arch. Peru Pathol. Clin. 15, 165–172.
D'Orbigny, A., 1835. Synopsis terrestrium et fluviatilium molluscorum, in suo per Americam Meridionalem itinere collectorum. Mag. Zool. 5, Classe 5 (61–62), 1–44.
D'Orbigny, A., 1837. Voyage dans l'Amérique Méridionale, Vol. 5, 3ème Partie: Mollusques. P. Bertrand, Paris.
De Jong, R.J., Morgan, J.A.T., Paraense, W.L., Pointier, J.P., Amarista, M., Ayeh-Kumi, P.F.K., Babiker, A., Barbosa, C.S., Bremond, Ph., Canese, A.P., Pereira de Souza, C., Dominguez, C., File, S., Gutierrez, A., Incani, R.N., Kawano, T., Kazibwe, F., Kpikpi, J., Lwambo, N.J.S., Mimpfoundri, R., Njiokou, F., Poda, J.N., Sene, M., Velasquez, L.E., Yong, M., Adema, C.M., Hofkin, B.V., Mkoji, G.M., Loker, E.S., 2001. Evolutionary relationships and biogeography of *Biomphalaria* (Gastropoda: Planorbidae) with implications regarding its role as host of the human bloodfluke, *Schistosoma mansoni*. Mol. Biol. Evol. 18, 2225–2239.
Esteban, J.G., Flores, A., Angles, R., Strauss, W., Aguirre, C., Mas-Coma, S., 1997. A population-based coprological study of human fascioliasis in a hyperendemic area of the Bolivian Altiplano. Trop. Med. Int. Health 2, 695–699.
Esteban, J.G., Flores, A., Angles, R., Mas-Coma, S., 1999. High endemicity of human fascioliasis between Lake Titicaca and La Paz valley, Bolivia. Trans. R. Soc. Trop. Med. Hyg. 93, 151–156.
Esteban, J.G., Gonzalez, C., Bargues, M.D., Angles, R., Sanchez, C., Naquira, C., Mas-Coma, S., 2002. High fascioliasis infection in children linked to a man-made irrigation zone in Peru. Trop. Med. Int. Health 7, 339–348.
Folmer, O., Black, M., Hoch, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3, 294–299.
Fuentes, M.V., Malone, J.B., 1999. Development of a forecast system for fascioliasis in central Chile using remote sensing and climatic data in a Geographic Information System. Res. Rev. Parasitol. 59, 129–134.
Fuentes, M.V., Valero, M.A., Bargues, M.D., Esteban, J.G., Angles, R., Mas-Coma, S., 1999. Analysis of climatic data and forecast indices for human fascioliasis at very high altitude. Ann. Trop. Med. Parasitol. 93, 835–850.
Fuentes, M.V., Malone, J.B., Mas-Coma, S., 2001. Validation of a mapping and predicting model for human fasciolosis transmission in Andean very high altitude endemic areas using remote sensing data. Acta Trop. 79, 87–95.
Gonzalez, L.C., Esteban, J.G., Bargues, M.D., Valero, M.A., Ortiz, P., Naquira, C., Mas-Coma, S., 2011. Hyperendemic human fascioliasis in Andean valleys: an altitudinal transect analysis in children of Cajamarca province, Peru. Acta Trop. 120, 119–129.
Gorman, T., Alcaino, H., Vidal, E., Berardi, L., 1979. Prevalencias y tendencias de la distomatosis y triquinosis en la VII Región. Arch. Med. Vet. (Suppl. 1), 123–128.
Hubendick, B., 1951. Recent Lymnaeidae. Their variation, morphology, taxonomy, nomenclature, and distribution. Kungliga Svenska Vetenskapsakademiens Handlingar, Fjärde Serien, 3, 1–223 + 5 pl.
King, P.P., Broderip, W.J., 1832. Description of the Cisrrhipeda Conchifera and Mollusca, in a collection formed by the Officers of H.M.S. Adventure and Beagle employed between the years 1826 and 1830 in surveying the Southern Coasts of South America, including the Straits of Magalhaens and the Coast of Tierra del Fuego. Zool. J. 5 (19), 332–349, No. 47.
Kleiman, F., Pietrovsky, S., Paraense, W.L., Wisnivesky-Colli, C., 2004. Southernmost finding of *Lymnaea viatrix* Orbigny 1835 (Pulmonata: Lymnaeidae), intermediate host of *Fasciola hepatica* (Linnaeus, 1758) (Trematoda: Digenea), in urban and rural areas of Patagonia, Argentina. Mem. Inst. Osw. Cruz 99, 23–24.
Larrea, H., Oviedo, M.L., Huaman, P., 1994. Estudio anatómico de Lymnaea diaphana King, 1830, agente transmisor de la fascioliasis en la zona Sur del Perú. Bol. Lima 16 (91–96), 95–100.
Letelier, S., Ramos, A.M., Huaquin, L.G., 2007. Moluscos dulceaquícolas exóticos en Chile. Rev. Mex. Biodiv. 78 (Suppl.), 9–13.
Lopez, A., Silva, C., Busel, D., 2004. Fascioliasis hepática: reporte de un caso y revisión de la literatura. Rev. Chile Radiol. 10, 118–123.
Mabille, M.J., 1884. Notices malacologiques. Bull. Soc. Philomath. Paris 7ème Série 8, 39–49.
Malek, E.A., 1985. Snail hosts of schistosomiasis and other snail-transmitted diseases in tropical America: a manual. Pan American Health Organization, Washington, Scientific Publication No. 478, 325 pp.
Mas-Coma, S., Bargues, M.D., 2009. Populations, hybrids and the systematic concepts of species and subspecies in Chagas disease triatomine vectors inferred from nuclear ribosomal and mitochondrial DNA. Acta Trop. 110, 112–136.
Mas-Coma, S., Angles, R., Esteban, J.G., Bargues, M.D., Buchon, P., Franken, M., Strauss, W., 1999. The Northern Bolivian Altiplano: a region highly endemic for human fascioliasis. Trop. Med. Int. Health 4, 454–467.
Mas-Coma, S., Funatsu, I.R., Bargues, M.D., 2001. *Fasciola hepatica* and lymnaeid snails occurring at very high altitude in South America. Parasitology 123, S115–S127.
Mas-Coma, S., Valero, M.A., Bargues, M.D., 2008. Effects of climate change on animal and zoonotic helminthiases. Rev. Sci. Technol. OIE 27 (2), 443–457.
Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009a. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv. Parasitol. 69, 41–146.
Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009b. Climate change effects on trematodiases, with emphasis on zoonotic fascioliasis and schistosomiasis. Vet. Parasitol. 163, 264–280.
Mera y Sierra, R., Artigas, P., Cuervo, P., Deis, E., Sidoti, L., Mas-Coma, S., Bargues, M.D., 2009. Fascioliasis transmission by *Lymnaea neotropica* confirmed by nuclear rDNA and mtDNA sequencing in Argentina. Vet. Parasitol. 166, 73–79.
Mera y Sierra, R., Agramunt, V.H., Cuervo, P., Mas-Coma, S., 2011. Human fascioliasis in Argentina: retrospective overview, critical analysis and baseline for future research. Parasite Vector 4, 104.
Morales, J.L., Arriagada, R., Salas, L., Morales, C., Fuentes, F., Santana, R., 2009. Hematoma subcapsular por fasciolasis. Rev. Chile Cirugía 61, 73–77.
Morales, M.A., Luengo, J., 1995. Beneficio y causales de decomiso de ovinos, porcinos, equinos, caprinos y camélidos en Chile. Avances Cienc. Vet. 11, 136–146.
Morales, M.A., Luengo, J., Vasquez, O., 2000. Distribución y tendencia de la fasciolasis en el ganado de abasto en Chile, 1989–1995. Parasitol. Día 24, 115–118.
Paraense, W.L., 1976. *Lymnaea viatrix*: a study of topotypic specimens (Mollusca: Lymnaeidae). Rev. Bras. Biol. 36, 419–428.
Paraense, W.L., 1982. *Lymnaea viatrix* and *Lymnaea columella* in the Neotropical region: a distributional outline. Mem. Inst. Osw. Cruz 77, 181–188.
Paraense, W.L., 1984. *Lymnaea diaphana:* a study of topotypic specimens (Pulmonata: Lymnaeidae). Mem. Inst. Osw. Cruz 79, 75–81.
Paraense, W.L., 1995. Lymnaea cousini Jousseaume, 1887 from Ecuador (Gastropoda: Lymnaeidae). Mem. Inst. Osw. Cruz 90, 605–609.
Pilsbry, H.A., 1911. Non-marine mollusca of Patagonia. Reports of Princeton University Expeditions to Patagonia, 1896–1899, 3 (5), 513–633.
Posada, D., 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256.
Posada, D., Buckley, T.R., 2004. Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. Syst. Biol. 53, 793–808.
Remigio, E.A., Blair, D., 1997. Relationships among problematic North American stagnicoline snails (Pulmonata: Lymnaeidae) reinvestigated using nuclear ribosomal DNA internal transcribed spacer sequences. Can. J. Zool. 75, 1540–1545.
Remigio, E.A., Hebert, P.D., 2003. Testing the utility of partial COI sequences for phylogenetic estimates of gastropod relationships. Mol. Phylogenet. Evol. 29, 641–647.
Rochebrune, A.-T., de Mabille, J., 1885. Diagnoses de Mollusques nouveaux, recueillis par les membres de la mission du Cap Horn et M Lebrun, Préparateur au Muséum, chargé d'une mission à Santa-Cruz de Patagonie. Bull. Soc. Philomath. Paris 7ème Série 9, 100–111.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
Rosas, H., Fiestas, C., Torres, R., Aita, G., Lozada, D., 2008. Hematoma subcapsular hepático por Fasciolasis. Rev. Chile Radiol. 14, 20–24.
Rossanigo, C.E., Avila, J.D., Vasquez, R., Sager, R.L., 1983. Incidencia, distribución e identificación del huésped intermediario de la distomatosis bovina en la provincia de San Luis. Gaceta Vet., Buenos Aires 45 (382), 739–749.
Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
Samadi, S., Roumegoux, A., Bargues, M.D., Mas-Coma, S., Yong, M., Pointier, J.P., 2000. Morphological studies of lymnaeid snails from the human fascioliasis endemic zone of Bolivia. J. Mollus. Stud. 66, 31–44.
Sanger, F., Nicklen, S., Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. Proc. Nat. Acad. Sci. USA 74, 5463–5467.
Santa Cruz, A.C.M., 1979. Comportamiento de *Limnaea diaphana* King en condiciones de laboratorio. Veterinaria 3, 29–40.
Sielfeld, W., 2001. Phylum Mollusca. Guías de Identificación y Biodiversidad. Fauna Chilena. Apuntes de Zoología. Universidad Arturo Prat, Iquique, Chile, 15 pp.
Strebel, H., 1907. Beiträge zur Kenntnis der Molluskenfauna der Magalhaen-Provinz. No. 5. Mit Tafel 1-8 und 6 Abbildungen im Text. Zoologische Jahrbücher, Abteilung für Systematik, Geographie und Biologie der Tiere, Verlag Von Gustav Fischer, Jena, 25 (1, 29 August 1907), 79–196+8 figure plates.
Subercaseaux, B., Tapia, S., Guglielmetti, A., Stanley, W., Muñoz, N., 1985. Brote epidémico de fascioliasis hepática humana en Valparaíso. Parasitol. Día 9, 10–14.
Tagle, I., 1944. Observaciones sobre la evolución de la Fasciola hepatica Linneo 1758. Comprobación del huésped intermediario en Chile. Rev. Chile Hist. Nat. 46/47, 232–241.
Tamura, K., Dudley, J., Nei, M.B., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetic Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity and progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Valdovinos, C., 2006. Estado de conocimiento de los gastropodos dulceaquícolas de Chile. Gayana 70, 88–95.

Valenzuela, G., 1979. Estudio epidemiológico acerca del desarrollo de huevos de *Fasciola* hepatica en el medio ambiente en Valdivia. Chile Bol. Chil. Parasitol. 34, 31–35.

Valero, M.A., Mas-Coma, S., 2000. Comparative infectivity of *Fasciola hepatica* metacercariae from isolates of the main and secondary reservoir animal host species in the Bolivian Altiplano high human endemic region. Folia Parasitol. 47, 17–22.

Valero, M.A., Darce, N.A., Panova, M., Mas-Coma, S., 2001. Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the Northern Bolivian Altiplano hyperendemic region. Vet. Parasitol. 102, 85–100.

Valero, M.A., Santana, M., Morales, M., Hernandez, J.L., Mas-Coma, S., 2003. Risk of gallstone disease in advanced chronic phase of fascioliasis: an experimental study in a rat model. J. Infect. Dis. 188, 787–793.

Valero, M.A., Navarro, M., Garcia-Bodelon, M.A., Marcilla, A., Morales, M., Garcia, J.E., Hernandez, J.L., Mas-Coma, S., 2006. High risk of bacterobilia in advanced experimental chronic fasciolosis. Acta Trop. 100, 17–23.

Valero, M.A., Girones, N., Garcia-Bodelon, M.A., Periago, M.V., Chico-Calero, I., Khoubbane, M., Fresno, M., Mas-Coma, S., 2008. Anaemia in advanced chronic fasciolosis. Acta Trop. 108, 35–43.

Venturelli, A., Monje, M., Assef, V., Venturelli, F., 2003. Fascioliasis hepática. Cuad. Cir. 17, 43–46.

Venturini, L., 1978. Revisión del ciclo biológico de *Fasciola hepatica*. Analecta Vet. 10, 13–20.

Journal of Zoo and Wildlife Medicine 42(2): 354–356, 2011

NATURAL *FASCIOLA HEPATICA* INFECTION IN NUTRIA (*MYOCASTOR COYPUS*) IN URUGUAY

Valeria Gayo, M.V., M.Sc., Pablo Cuervo, Vet., Diego Rosadilla, Br., Soledad Birriel, Br., Luc´ıa Dell'Oca, Br., Alfredo Trelles, Tec. Agrop., Ulises Cuore, M.V., and Roberto Mera y Sierra, M.V., M.Sc.

Abstract: Fascioliasis, the zoonotic disease caused by the trematode *Fasciola hepatica*, is expanding worldwide, with a 17 million people at risk. Rodents, often recognized as a major source of zoonotic diseases, are affected by *F. hepatica*, with some species playing important roles in the disease epidemiology. The case reported here in a nutria or kiyá (*Myocastor coypus*) is the first documented case of *F. hepatica* in this species in Uruguay. Parasitic burden and total egg production detected are markedly higher than reported previously for this species, confirming its potential role as an effective reservoir and disseminator of liver flukes. Although further research is needed, nutria should be considered when designing effective control programs for fascioliasis.

Key words: *Fasciola hepatica*, kiyá, *Myocastor coypus*, nutria, Uruguay.

BRIEF COMMUNICATION

Fascioliasis, an important zoonotic parasitic disease caused by the trematode *Fasciola hepatica*, has the potential to be transmitted globally, placing an estimated 17 million people at risk of infection in 51 countries.[14] This disease primarily affects domestic ruminants, but wild herbivorous mammals are also susceptible.[14] The epidemiologic role of these wild species is currently being evaluated as well as its potential conservation threat.[6] In Uruguay, the disease occurs throughout the country, with mean bovine prevalences of 57%, a prevalence consistently higher than the prevalence found in ovines.[13] Between 1909 and 1993, approximately 91 human cases were reported in Uruguay, but it is possible that this number may be underestimated because it is not a disease of mandatory incidence reporting.[13]

Several rodent species have been described as effective reservoirs of *F. hepatica*. *Rattus norvegicus*, *Mus musculus*, and *Cavia porcellus* are commonly used as experimental hosts and are sporadically found naturally infected.[5,7,18,19,20] *Rattus rattus* and introduced *Myocastor coypus* have been described as fascioliasis reservoirs of specific biotopes, such as in Corsica, Italy, and in Loire-Atlantique, France, respectively.[15,19] Natural infections in South American wild rodents have been described in *Cavia aperea* (Peru), *Hydrochaeris hydrochaeris* (Argentina, Brazil, and Uruguay), *M. coypus* (Argentina and Brazil), and *Lagidium viscacia* (Argentina).[2,8,9,12,17,21,22]

Myocastor coypus is a large, semiaquatic rodent known in Uruguay as nutria after the name given by the Spanish conquerors, or as kiyá after the aboriginal Guarani name.[11] Exclusive of South America, the species is present in eastern Bolivia, central and southern Chile, southern Brazil, and is widespread in Argentina, Paraguay, and Uruguay.[3] Nutria have been introduced into North America, Europe, and Asia via escapes and releases from fur farms.[4] Nutria live in marshes and on the banks of lakes and rivers, especially in areas with abundant floating and emergent vegetation.[3] Nutria often defecate in the water, and feces are easily recognized because of their tendency to float and by their size and elongated shape.[3]

Worldwide, rodents are recognized as one of the most important sources of zoonoses.[16] *Myocastor coypus* is not an exception; several studies have highlighted the importance of this species as a reservoir of zoonotic diseases.[9,10]

The sampling site was located in Minas locality (34824.0649S, 55821.2139W), Lavalleja region, Uruguay, in a farm with a recognized bovine and ovine fascioliasis problem. In March 2009, *M. coypus*' feces were surveyed and collected from a small pond where several individuals were known to dwell. The samples were identified by its typical characteristics, manually collected, and transported, fresh, to the DILAVE laboratory facilities for analysis. Five feces were processed with a rapid sedimentation technique, consisting of 3 min

From Divisio´n de Laboratorios Veterinarios (DILAVE), Ministerio de Ganaderı´a, Agricultura y Pesca (MGAP), Ruta 8 Km 17.500, 12100 Montevideo, Uruguay (Gayo, Rosadilla, Birriel, Dell'Oca, Trelles, Cuore); and Parasitologı´a y Enfermedades Parasitarias, Facultad de Ciencias Veterinarias y Ambientales, Universidad J.A. Maza, Av. Acceso Este–Lateral Sur 2245, 5519, Guaymalle´n, Mendoza, Argentina (Cuervo, Mera y Sierra). Correspondence should be directed to Dr. Cuervo (pablofcuervo@gmail.com).

consecutive sedimentations, with the elimination of supernatant. A couple of months later, an adult *M. coypus* from the same farm was sent to the DILAVE for a necropsy. Liver and gall blader were thoroughly examined, and gall blader contents were collected for additional examination. Three milliliters of bile (total volume) were diluted up to a total volume of 50 ml. An aliquot of 100]l was evaluated, and fluke eggs were enumerated. *Fasciola hepatica* and eggs were recovered and measured. Egg size and shape were estimated by multiplying length 3 width and dividing length/width (length/width ¼ 1 in round eggs and length/width . 1 in elliptical eggs), respectively.[1] Finally, parasitic burdens and total egg production were compared with those of two naturally infected sheep (*Ovis aries*).

Fasciola hepatica eggs were detected in every fecal sample examined, with 7.86 6 7.11 (mean 6 SE; range, 1.5–15.6) eggs per gram (epg). Twenty-four adult specimens of *F. hepatica* were recovered from the liver; whereas the bile analysis for fluke eggs revealed 1.4 million eggs in total. Thirty-four and 59 liver flukes were recovered from the two sheep livers, with 140,500 and 586,500 eggs in total, respectively. Flukes collected from *M. coypus* measured 2–2.5 cm in length and 1 cm in width. One hundred eggs were measured, with a length of 130 6 9.6]m (mean 6 SE; range, 105–150]m) and a width of 74 6 5]m (60–90]m). Estimated size was 9,698.62 6 1,086.3 (mean 6 SE; range 7,200–12,375]m), and eggs elliptical (mean shape, 1.77 6 0.2; range, 1.4–2.25).

Despite the low numbers of feces analyzed, all were found positive for *F. hepatica* eggs. The epg count is low compared with data reported previously (mean 6 SE, 26.9 6 9.4),[15] and it is possible that all the feces may have come from the same individual. Furthermore, when considering parasitic burdens, the number of *F. hepatica* recovered from a single liver (from the individual recovered from the affected farm) is markedly higher than that reported by the same study.[15] Eggs length and width values obtained are similar to those from previous reports, as were liver fluke dimensions.[1,9,15] *Fasciola hepatica* eggs from *M. coypus* origin are elliptical and medium-sized and range in dimensions similarly to those reported for cattle and lagomorphs.[1] The egg shape observed in this study is more similar to that reported from rabbits than rodents.[1] When taking into account the morphologic characteristics mentioned, it is clear that further research on viability and hatchability needs to be conducted.

Finally, when comparing parasitic burdens and total egg production, the total load of *F. hepatica* eggs in *M. coypus* is surprisingly higher than that reported in naturally infected sheep, despite the lower quantity of adult liver flukes. Although *M. coypus* has been described as naturally infected with *F. hepatica* in Argentina, Brazil, and France, to our knowledge, this is the first report of this species infected by *F. hepatica* in Uruguay.[9,15,21]

It seems that the nutria or kiyá are capable of dispersing *F. hepatica* by contaminating natural water sources with their feces, thereby increasing exposure of lymnaeid snails to *F. hepatica*'s miracidium. Although further studies are required, nutria should be considered as an effective wildlife reservoir and integrated into the design of effective control programs for animal and human fascioliasis in Uruguay.

LITERATURE CITED

1. Abrous, M., A. M. Comes, N. Gasnier, D. Rondelaud, G. Dreyfuss, A. Chauvin, A. Ménard, A. Agoulon, and J. Cabaret. 1998. Morphological variability in *Fasciola hepatica* eggs in ruminants, rodents and lagomorphs. J. Helminthol. 72: 313–317.

2. Alvarez, J. D., R. A. Moriena, M. I. Ortiz, and O. Racciopi. 2009. Hallazgo de *Fasciola hepatica* (Trematoda: Digenea) en un carpincho (*Hydrochaeris hydrochaeris*) de la provincia de Corrientes, Argentina. Rev. Vet. 20: 132–134.

3. Canevari, M., and O. Vaccaro. 2007. Gui´a de Mamíferos del sur de América del Sur. L.O.L.A. (Literature of Latin America), Buenos Aires, Argentina. Pp. 252–254.

4. Carter, J., and B. P. Leonard. 2002. A review of the literature on the worldwide distribution, spread of, and efforts to eradicate the coypu (*Myocastor coypus*). Wildl. Soc. B 30: 162–175.

5. Coles, G. C. 1975. Activity of commercial fasciolicides in small laboratory mammals. J. Parasitol. 61: 54–58.

6. Cuervo, P., R. Mera y Sierra, E. Deis, and A. Zarco. 2008. La introducción de *Fasciola hepatica* en América: ¿un posible riesgo para la conservación de especies en peligro de extinción de Argentina? III Congreso Nacional de Conservación de la Biodiversidad, Buenos Aires, Argentina.

7. De Paula, R. C., G. D. Cassali, D. Negrão-Corrêa, and M. P. Guimarães. 2010. Development and pathology of *Fasciola hepatica* in CCL3-deficient mice. Vet. Parasitol. 173: 147–151.

8. Dittmar, K. 2002. Arthropod and helminth parasites of the wild guinea pig, *Cavia aperea*, from the Andes and the Cordillera in Perú, South America. J. Parasitol. 88: 409–411.

9. El-Kouba, M. M., S. M. Marques, C. Pilati, and W. Hamman. 2009. Presence of *Fasciola hepatica* in

feral nutria (*Myocastor coypus*) living in a public park in Brazil. J. Zoo Wildl. Med. 40: 103–106.

10. Howerth, E. W., A. J. Reeves, M. R. McElveen, and F. W. Austin. 1994. Survey for selected diseases in nutria (*Myocastor coypus*) from Louisiana. J. Wildl. Dis. 30: 450–453.

11. Latourrette Sapriza, O. 1955. El *Myocastor coypus*, su clasificación. An. Fac. Vet. Urug. 6: 17–26.

12. Led, J. E., F. G. Yannarella, D. A. Scasso, and G. M. Denegri. 1979. *Lagidium viscaccia boxi* nuevo reservorio silvestre de *Fasciola hepatica* (Linnaeus, 1758) en la República Argentina. Veterinaria 2: 31–39.

13. López Lemes, M. H., S. Hernández, A. M. Acuña, and A. Nari. 1996. Fascioliasis en la República Oriental del Uruguay. Rev. Med. Urug. 12: 37–43.

14. Mas-Coma, S., M. A. Valero, and M. D. Bargues. 2009. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv. Parasitol. 69: 41–146.

15. Ménard, A., A. Agoulon, M. L'Hostis, D. Rondelaud, S. Collard, and A. Chauvin. 2001. *Myocastor coypus* as a reservoir host of *Fasciola hepatica* in France. Vet. Res. 32: 499–508.

16. Perkins, S. E., I. Cattadori, and P. J. Hudson. 2005. The role of mammals in emerging zoonoses. Mamm. Stud. 30: S67–S71.

17. Santarem, V. A., R. A. Tostes, H. Alberti, and O. Carvalho Sanches. 2006. *Fasciola hepatica* in capybara. Acta Trop. 98: 311–313.

18. Tewari, H. C. 1968. Studies on experimental *Fasciola hepatica* infections in the guinea-pig. Ann. Trop. Med. Parasitol. 62: 495–501.

19. Valero, M. A., M. D. Marcos, R. Fons, and S. Mas-Coma. 1998. *Fasciola hepatica* development in the experimentally infected black rat *Rattus rattus*. Parasitol. Res. 84: 188–194.

20. Valero, M. A., M. Panova, A. M. Comes, R. Fons, and S. Mas-Coma. 2002. Patterns in size and shedding of *Fasciola hepatica* eggs by naturally and experimentally infected murid rodents. J. Parasitol. 88: 308–313.

21. Wolffhügel, K. 1909. Sobre trematodes (*Fasciola hepatica* L. y *Prosthogonimus cuneatus* (Rud.) parásitos de los animales domésticos de la República Argentina. Centro de Estudiantes de Agronomía y Veterinaria 7: 3–8.

22. Wolffhügel, K. 1916. El carpincho (*Hydrochoerus capybara*) huésped de *Fasciola hepatica*. Primary Congreso Médico Nacional, Montevideo, Uruguay. (Abstr.)

Received for publication 1 December 2010

DNA multigene sequencing of topotypic specimens of the fascioliasis vector *Lymnaea diaphana* and phylogenetic analysis of the genus *Pectinidens* (Gastropoda)

Maria Dolores Bargues[1]/+, Roberto Luis Mera y Sierra[2], Patricio Artigas[1], Santiago Mas-Coma[1]

[1]Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia, Av. Vicente Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain [2]Cátedra de Parasitología y Enfermedades Parasitarias, Facultad de Ciencias Veterinarias, Universidad Juan Agustín Maza, Guaymallén, Mendoza, Argentina

Freshwater lymnaeid snails are crucial in defining transmission and epidemiology of fascioliasis. In South America, human endemic areas are related to high altitudes in Andean regions. The species Lymnaea diaphana *has, however, been involved in low altitude areas of Chile, Argentina and Peru where human infection also occurs. Complete nuclear ribosomal DNA 18S, internal transcribed spacer (ITS)-2 and ITS-1 and fragments of mitochondrial DNA 16S and* cytochrome c oxidase *(*cox*)1 genes of* L. diaphana *specimens from its type locality offered 1,848, 495, 520, 424 and 672 bp long sequences. Comparisons with New and Old World* Galba/Fossaria, *Palaearctic stagnicolines, Nearctic stagnicolines, Old World* Radix *and* Pseudosuccinea *allowed to conclude that (i)* L. diaphana *shows sequences very different from all other lymnaeids, (ii) each marker allows its differentiation, except* cox*1 amino acid sequence, and (iii)* L. diaphana *is not a fossarine lymnaeid, but rather an archaic relict form derived from the oldest North American stagnicoline ancestors. Phylogeny and large genetic distances support the genus* Pectinidens *as the first stagnicoline representative in the southern hemisphere, including colonization of extreme world regions, as most southern Patagonia, long time ago. The phylogenetic link of* L. diaphana *with the stagnicoline group may give light to the aforementioned peculiar low altitude epidemiological scenario of fascioliasis.*

Key words: *Lymnaea diaphana* - Lymnaeidae - nuclear rDNA - mtDNA - phylogeny - fascioliasis vectors

Freshwater snails of the family Lymnaeidae are of great importance in public health due to their capacity to transmit fascioliasis, a parasitic disease caused by the two liver fluke species *Fasciola hepatica* and *Fasciola gigantica* (Mas-Coma et al. 2009a). Whereas the consequences of fascioliasis are the cause of concern in livestock husbandry since long ago (Spithill et al. 1999, Torgerson & Claxton 1999), its impact on human communities has shown to progressively increase from an amount of 2,500 human cases in 1990 (Chen & Mott 1990) to a global estimation of 17 million people affected which may be even worse if the lack of knowledge in many regions of Africa and Asia are considered (Mas-Coma et al. 2009a). This recent emergence appears to be at least in part related to climate change (Mas-Coma et al. 2008, 2009b). General concern about fascioliasis has moreover risen due to the large long-term pathogenicity of fasciolid flukes (Valero et al. 2003, 2006, 2008) and their immunosuppression effect (Gironés et al. 2007) recently demonstrated in the advanced chronic stage of the disease, which appears to be the usual situation of infected subjects in the human endemic areas.

Within the several human fascioliasis hotspot regions known, South America is characterized by the so-called Andean transmission pattern, including the Altiplano subpattern and Valley subpattern. Both subpatterns are characterized by high altitude endemic areas, including high prevalences and intensities in humans caused by *F. hepatica*, such as in Bolivia (Hillyer et al. 1992, Esteban et al. 1997a, b, 1999) and Peru (Esteban et al. 2002, Gonzalez et al. 2011). In Argentina the human fascioliasis situation, although underestimated, also shows a link to altitude areas (Mera y Sierra et al. 2011). However, human infection in South America has also been described to be relatively frequent in given low altitude areas, such as in Arequipa region, Peru and southern Chile, where the species *Lymnaea diaphana* King, 1830 has been noted to be directly involved in the transmission (Cordova et al. 1961, Tantalean et al. 1974, Larrea et al. 1994) or known to be present in the transmission area (Sielfeld 2001, Valdovinos 2006), respectively. Additionally, *L. diaphana* is known to inhabit the southernmost areas of South America (Hubendick 1951) where animal fascioliasis has been described, in both Chile (Alcaino & Apt 1989, Morales et al. 2000) and Argentina (Olaechea 1994).

In spite of their applied interest, our knowledge on lymnaeid snails is far from sufficient regarding both their genetics and their vector role. This situation is well illustrated by the systematic-taxonomic controversy in which this molluscan family is immersed (see review in

Financial support: SAF2006-09278, SAF2010-20805 (MICINN, Spain), PI030545, RD06/0021/0017 (RICET, FIS, Spain), RLA5049 (AIEA, Austria), Secretaría de Ciencia, Técnica y Posgrado, UNCU, Area de Investigación, UMAzA, Argentina
The aim of this study is included within the worldwide initiative of WHO (Headquarters Geneva, Switzerland) against human fascioliasis.
+ Corresponding author: m.d.bargues@uv.es
Received 12 May 2011
Accepted 19 July 2011

Bargues et al. 2001). At lymnaeid species level, the problems are mainly due to the interspecific morphological and anatomic uniformity numerous species show, usually presenting serious difficulties in specimen classification, sometimes even impeding it. Moreover, intraspecific variation of shell shape is particularly well marked within lymnaeids depending on environmental conditions, although a genetic component in shell shape has been shown at least in some lymnaeid populations (Samadi et al. 2000). In the Americas, there are many specimen classification problems, mainly concerning fascioliasis vector species of the so-called "fossarine" or *Galba*/*Fossaria* group (Bargues et al. 2007), which is characterized by a shell shape and size range within which *L. diaphana* fits (Paraense 1984).

Recent studies have shown that nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) sequences furnish appropriate markers to clarify the systematics of this snail group as well as lymnaeid specimen classification even in particularly confusing lymnaeid groups (Bargues & Mas-Coma 1997, 2005, Bargues et al. 1997, 2001, 2003, 2006, 2007, Remigio & Blair 1997a, b, Remigio 2002, Remigio & Hebert 2003). Additionally, analyses have recently shown that the only way to perform a correct, definitive species ascription to a DNA sequence is by comparing with the sequence of the same marker obtained in specimens collected in the type locality of the species (Bargues et al. 2011a), as already followed concerning shell and morphoanatomical characteristics by Paraense (1976, 1984).

The current trend in molecular population genetics is to use increasing numbers of genes in the analysis. Here we describe a multigenic sequence analyses of *L. diaphana* thanks to the attainment of complete sequences of the 18S gene and the first and second internal transcribed spacers (ITS), ITS-1 and ITS-2, of the rDNA and partial sequences of the 16S gene and cytochrome c oxidase subunit I (*cox*1) coding gene. To avoid any possible doubt, the molecular characterization is based only on specimens collected in the type locality of this species. These sequences are analyzed in full detail, by means of pairwise comparisons and phylogenetic methods, with those of the same markers in (i) other American lymnaeid species of the "fossarine" or *Galba*/*Fossaria* group, most of them represented by specimens from the respective type localities of the species, (ii) the main fascioliasis vector species throughout the world *Galba truncatula*, which also belongs to the *Galba*/*Fossaria* group and (iii) morphologically close Nearctic and Palaearctic species of the "stagnicoline" group. Finally, the study is also used for the analysis of the lymnaeid genus *Pectinidens*, which was proposed with *L. diaphana* as type species long ago (Pilsbry 1911). *Pectinidens* has also been used at subgenus level to even include other *Galba*/*Fossaria* vector species such as *Lymnaea viatrix* (Alcaino & Apt 1989).

MATERIALS AND METHODS

Lymnaeid snail materials - The snail specimens studied were collected in the field, at the type locality included in the original description of the species *L. diaphana*: neighbourhood of Cape Gregory, which is on the continental side of the eastern end of the Strait of Magalhaens, province of Magallanes, Chile (King & Broderip 1832, Pilsbry 1911, Paraense 1984). For a complete description of the shell and anatomy of topotypic specimens of this species, including detailed drawings, see Paraense (1984). A typic specimen of the lymnaeid snails collected is illustrated in Fig. 1. The terra typica of this lymnaeid (S52º37'52.3" W70º15'18.0", altitude 2-5 m) is constituted by different neighbouring water collections resulting from subsoil effluences in a wide area of sheep farming (see kind of biotopes in Fig. 2A, B). Specimens were usually found inside cold water, mostly on stony waterbottom (Fig. 2C) and only very rarely outside water as on floating leafs (Fig. 2D). No other lymnaeid species was found in the water collections studied.

Molecular techniques - DNA was only isolated from the foot of each alcohol-fixed snail (Bargues et al. 1997, 2007). Total DNA was isolated according to the phenol-chloroform extraction and ethanol precipitation method. The procedure steps were performed according to methods outlined previously (Bargues & Mas-Coma 1997, Bargues et al. 2001, 2007). The pellet was dried and resuspended in 30 µL sterile tris-ethylenediamine tetraacetic acid (TE) buffer (pH 8.0). This suspension was stored at -20ºC until use.

A combined set of nuclear rDNA and mtDNA markers were polymerase chain reaction (PCR) amplified independently for each lymnaeid specimen and each PCR product was sequenced for a bona-fide haplotype characterization. The complete 18S rRNA gene was amplified using specific primers (Bargues et al. 1997, 2011a). The rDNA spacers ITS-2 and ITS-1 were amplified using primers designed in conserved positions of 5.8S and 28S rRNA genes and 18S and 5.8S rRNA genes, respectively (Bargues et al. 2001, 2006, 2007). The target 16S gene region was amplified using a set of universal primers (Simon et al. 1991). Amplification procedures and thermal cycler conditions were carried out as previously described for lymnaeids (Remigio & Blair 1997a). A *cox*1 gene fragment was amplified using other universal primers (Folmer et al. 1994). Amplifications were generated in a Mastercycle ep gradient (Eppendorf, Hamburg, Germany) using specific PCR conditions for each marker, as previously described (Bargues et al. 2011a). Ten microlitres of each PCR product were checked by

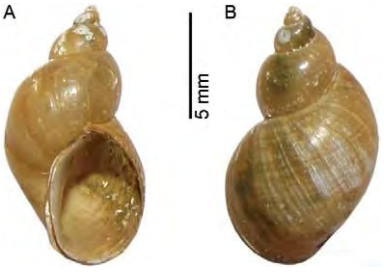


Fig. 1: specimen of *Lymnaea diaphana* collected at its type locality of Cape Gregory, Strait of Magellan, province of Magallanes. Chile. A: ventral view; B: dorsal view.

staining with ethidium bromide on 1% Nusieve® Genetic Technology Grade agarose (FMC Bioproducts) gel electrophoresis using the Molecular Weight Marker VI (Boehringer Mannheim) at 0.1 µg DNA/µL as control.

Primers and nucleotides were removed from PCR products by purification on Wizard™ PCR Preps DNA Purification System (Promega, Madison, WI, USA), according to the manufacturer's protocol, and suspended in 50 µL of 10 mM TE buffer (pH 7.6). The final DNA concentration was determined by measuring the absorbance at 260 and 280 nm.

DNA sequencing was performed on both strands by the dideoxy chain-termination method (Sanger et al. 1977). It was carried out with the Taq dye-terminator chemistry kit for ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), using PCR primers. Sequences were aligned using CLUSTALW version 1.8 (Thompson et al. 1994) and MEGA 5.0 (Tamura et al. 2011). Minor corrections were manually introduced for a better fit of nucleotide correspondences in microsatellite sequence regions.

For 18S rRNA secondary structure representation, the previously published secondary structure prediction for *Limicolaria kambeul* 18S rRNA (Winnepennickx et al. 1992) based on the general eukaryote 18S rRNA secondary structure (De Rijk et al. 1992) was used and extended to encompass lymnaeid sequences.

DNA haplotype nomenclature - The codes for the sequences obtained follow the standard nomenclature proposed for lymnaeid snails previously (Bargues & Mas-Coma 2005, Bargues et al. 2006, Mas-Coma et al. 2009a). It shall be noted that haplotype codes are only definitive in the case of complete sequences. When dealing with fragments or incomplete sequences, haplotype codes are provisional.

Sequence comparisons - The following sequences from GenBank and European Molecular Biology Laboratory (EMBL) have been used for comparison and/or phylogenetic analyses: (i) 18S rRNA gene: complete sequences of *Lymnaea (Lymnaea) stagnalis* (GenBank accession z73984), *Lymnaea (Stagnicola) palustris* (z73983), *Omphiscola glabra* (z73982) and *G. truncatula* (z73985) (Bargues & Mas-Coma 1997), *Lymnaea cubensis* (z83831) (Bargues et al. 1997, 2007), *L. viatrix* and *Lymnaea neotropica* (both species with the same sequence AM412222) (Bargues et al. 2007), *Lymnaea humilis* (FN182190) (Bargues et al. 2011a) and *Pseudosuccinea columella* (FN598152) (Bargues et al. 2011b), *Radix auricularia* (z73980) and *Radix balthica* (z73981) (Bargues & Mas-Coma 1997, Bargues et al. 1997). Other lymnaeid incomplete sequences available in the GenBank have not been used to avoid problems in comparative sequence analyses. The planorbids *Biomphalaria glabrata*

Fig. 2: type locality of *Lymnaea diaphana* at San Gregorio, Strait of Magellan, province of Magallanes. Chile: A: water collection inhabited by the species in a view to the Strait (see sea on the background); B: another water collection inhabited by the species in a view to the inland; C: specimens rarely found out of water, on a floating leaf; D: stony water ground on which specimens were usually found.

(Hanelt et al. 1997) and *Bulinus truncatus* (Jorgensen et al. 2011) were included for comparison purposes; (ii) rDNA ITS-2: *L.* (*S.*) *palustris palustris* (AJ319620), *Lymnaea* (*Stagnicola*) *fuscus* (AJ319621) and *Catascopia occulta* (AJ319642) (Bargues et al. 2001, 2003), *Catascopia catascopium* (AF013143), *Catascopia emarginata* (AF013141, AF013142), *Catascopia elodes* (AF013138) and *Hinkleyia caperata* (AF013139) (Remigio & Blair 1997b), *G. truncatula* H1 (AJ296271) (Bargues et al. 2001, Mas-Coma et al. 2001), *L. cubensis* H1 (AM412223) and H2 (FN182200) (Bargues et al. 2007, 2011a), *L. viatrix* H1 (AM412224) and *L. neotropica* H1 (AM412225) (Bargues et al. 2007), *L. humilis* H1 (FN182191) (Bargues et al. 2011a), *P. columella* H1 (FN598155) (Bargues et al. 2011b), *R. auricularia* (AJ319628) and *R. balthica* (AJ319633) (Bargues et al. 2001); (iii) rDNA ITS-1: *L.* (*S.*) *palustris palustris* (AJ626849), *L.* (*S.*) *fuscus* (AJ626856) and *C. occulta* (AJ626858) (Bargues et al. 2006), *C. catascopium* (AF013143), *C. emarginata* (AF013142), *C. elodes* (AF013138) and *H. caperata* (AF013139) (Remigio & Blair 1997b), *G. truncatula* HA (AJ243018) (Mas-Coma et al. 2001), *L. cubensis* HA (AM412226) and HB (FN182202) (Bargues et al. 2007, 2011a), *L. viatrix* HA (AM412227) and *L. neotropica* HA (AM412228) (Bargues et al. 2007), *L. humilis* HA (FN182193) (Bargues et al. 2011a), *P. columella* HA (FN598160) (Bargues et al. 2011b); *R. auricularia* (JF922878) and *R. balthica* (JF922879) (Bargues et al. 2011b); (iv) 16S rRNA gene of the mtDNA: *Fossaria bulimoides* (AF485657), *Fossaria obrussa* (AF485658) and *Stagnicola bonnevillensis* (AF485655) (Remigio 2002), *F. bulimoides* (EU038315) and *Stagnicola elodes* isolate 44106 (EU038305) (Wethington & Lydeard 2007), *L. humilis* (FN182195) and *L. cubensis* (FN182204) (Bargues et al. 2011a), *P. columella* (PCU82073) (Remigio & Blair 1997a); (v) mtDNA *cox*1 gene: *S. elodes* (AY227368), *F. bulimoides* (AY227367) and *Austropeplea tomentosa* (AY227365) (Remigio & Hebert 2003), *G. truncatula* from Spain (AM494011) (Bargues et al. 2007) and Germany (EU818799) (Albrecht et al. 2008), *L. cubensis cox*1a (AM494009) and *cox*1b (FN182205), *L. neotropica cox*1a (AM494008) and *cox*1b (FN356741), *L. humilis cox*1a, *cox*1b and *cox*1c (FN182197-9) (Bargues et al. 2007, 2011a, Mera y Sierra et al. 2009), *P. columella* (FN598165) (Bargues et al. 2011b) and *P. columella* (AY227366) (Remigio & Hebert 2003), *Radix rubiginosa* (GU451737) (Liu et al. 2010).

Phylogenetic inference - Phylogenetic analysis of ITS-1 and ITS-2 combined haplotypes was firstly performed with a maximum likelihood (ML) approach using Phylogenetic Analysis Using Parsimony (PAUP) version 4.0b10 (Swofford 2002) and the PhyML program version 3.0 aLRT. ML parameters and the evolutionary model best fitting our dataset were determined using Akaike and Bayesian information criteria (Akaike 1974, Posada & Buckley 2004), implemented in jModeltest version 0.1.1 (Posada 2008). Starting branch lengths were obtained using the least-squares method with ML distances. The intergenic region sequence (AY030361) (De Jong et al. 2001) including both ITSs of a planorbid species, *Biomphalaria pfeifferi*, was used as outgroup.

To provide an assessment of the reliability of the nodes in the ML tree, four methods were used. First, a distance-based phylogeny using the neighbour-joining (NJ) algorithm (Saitou & Nei 1987) with the ML pairwise distances was obtained and statistical support for the nodes was evaluated with 1,000 bootstrap replicates, with and without removal of gapped positions, in PAUP. Second, a bootstrap analysis using 1,000 replicates was made by using branch-swapping algorithm (tree-bisection-reconnection) with full heuristic search in PAUP. Third, a Bayesian phylogeny reconstruction procedure was applied to obtain posterior probabilities (BPP) for the nodes in the ML tree, by using the same evolutionary model as above, implemented in MrBayes 3.1 (Ronquist & Huelsenbeck 2003) with four chains during 1,000,000 generations and trees being sampled every 100 generations. The first 1,000 trees sampled were discarded ("burn-in") and clade posterior probabilities were computed from the remaining trees. Fourth, reliability for internal branch was assessed with a fast method using the aLRT test (SH-like) implemented in PhyML for final comparison purposes.

RESULTS

DNA sequences - Nuclear rDNA 18S, ITS-2 and ITS-1 and mtDNA 16S and *cox*1 nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DNA Data Bank of Japan databases under the accessions noted in Table I.

18S rRNA gene - The 18S rDNA sequences obtained in the *L. diaphana* specimens analyzed from the type locality are identical base to base, with a length of 1,848 bp and a guanine-cytosine (GC) content of 51.68% (Table I).

A multiple sequence alignment of 11 different 18S sequences, including several *Galba*/*Fossaria* vector species such as *L. cubensis*, *L. viatrix* (with 18S sequence identical to that of *L. neotropica*), *G. truncatula* and *L. humilis*, the peculiar species *P. columella*, three representatives of stagnicolines such as *L. (L.) stagnalis*, *L. (S.) palustris* and *O. glabra* and two species of the *Radix* group such as *R. auricularia* and *R. balthica*, was 1,867 bp long, show-

TABLE I

Nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) barcode haplotype identification and respective GenBank accessions for the lymnaeid species *Lymnaea diaphana* from its type locality

DNA marker	Haplotype code	Accession
18S rRNA	L.dia 18S-H1	JF909497
rDNA ITS-2	L.dia ITS2-H1	JF909498
rDNA ITS-1	L.dia ITS2-HA	JF909499
mtDNA 16S	L.dia 16S-HA[a]	JF909500
mtDNA *cox*1	L.dia *cox*1-Ha[a]	JF909501

a: mtDNA haplotype codes only preliminary due to incomplete gene sequence. H: haplotypes.

ing a total of 62 variable positions (3.32% nucleotide divergence). Thirty of these 62 polymorphic sites appear grouped in the short sequence between positions 233 and 266, which corresponds to the helix E10-1 of the variable area V2 of the secondary structure (Supplementary data). Pairwise nucleotide differences at the level of the 18S between *L. diaphana* and the other 10 aforementioned lymnaeid species, as well as with representatives of Planorbidae to comparatively assess distances at family level, are shown in Table II.

rDNA ITS-2 - Specimens of *L. diaphana* from the type locality present a 495 bp-long ITS-2, with a 56.97% GC content, which has been ascribed to *L. diaphana* haplotype 1 (L.dia ITS2-H1) (Table I).

Worth mentioning is the very high number of nucleotide differences detected in the pairwise comparisons of this ITS-2 sequence of *L. diaphana* with the ITS-2 of other European and American *Galba*/*Fossaria*, stagnicolines and Pseudosuccinea species available in GenBank. The ITS-2 dataset distance matrix obtained with PAUP shows that the number of total and mean character differences between *L. diaphana* and the other species considered are considerably high in all cases (Table III). These total differences ranged between 68-116 (average 88.6), when comparing with *Galba*/*Fossaria* species, and between 105-108 (average 106.5) and 66-80 (71.5), when *L. diaphana* is compared with European and American stagnicolines, respectively. Regarding *P. columella*, the differences are 81 (Table IV). According to these ITS-2 pairwise distance results, the group of species appearing to be more close to *L. diaphana* is the one of the American stagnicolines. Genetic distances between *L. diaphana* and its molecularly closest lymnaeid species according to PAUP (*L. neotropica*, *C. catascopium*, *C. elodes* and *H. caperata*) are shown in Table V.

rDNA ITS-1 - *L. diaphana* specimens collected in the type locality present an ITS-1 with a length of 520 bp and a GC content of 54.05%. This ITS-1 sequence has been ascribed to *L. diaphana* haplotype A (L.dia ITS1-HA) (Table I).

Similarly as in the case of ITS-2, a very high number of nucleotide differences appear in the pairwise comparisons of this ITS-1 sequence of *L. diaphana* with the ITS-1 of species of *Galba*/*Fossaria* and stagnicolines, as well as to *P. columella* available in GenBank. The ITS-1 dataset distance matrix obtained with PAUP shows that the number of total and mean character differences between *L. diaphana* and the other species considered are considerably high in all cases (Table VI). These total differences ranged between 73-95 (average 85.6) when comparing with *Galba*/*Fossaria* species and between 100-101 (100.5) and 76-93 (86.7) when *L. diaphana* is compared with European and American stagnicolines, respectively. Regarding *P. columella*, a total of 105 differences appear (Table IV). According to these ITS-1 pairwise distance results and similarly as detected in the ITS-2 analysis, the group of lymnaeids appearing to be more close to *L. diaphana* is the one of the American stagnicolines. Genetic distances between *L. diaphana* and its molecularly closest lymnaeid species according to PAUP (*G. truncatula* and *H. caperata*) are shown in Table V.

16S mtDNA - Snail specimens from the type locality furnished a 16S fragment sequence of a length of 424 bp characterized by a considerable adenine-thymine (AT) biased average nucleotide composition of 70.75%. The provisional code L.dia 16S-HA has been assigned for this fragment (Table I).

Estimates of evolutionary divergence and base composition bias differences in the 16S sequence alignment including *L. diaphana* and other species available in GenBank demonstrate that *L. diaphana* is different from any other species of *Galba*/*Fossaria* and stagnicolines, as well as from *P. columella*, at the level of this mtDNA gene (Table VII, Supplementary data). In pairwise comparisons, minimum differences were 31 mutations when *L. diaphana* is compared with *S. bonnevillensis* and a maximum of 46 mutations appeared with *F. bulimoides*. Nucleotide differences were numerous and very similar when comparing *L. diaphana* with *Galba*/*Fossaria* (41-46, average 42.0), with stagnicolines (31-41, 36.0) and with *P. columella* (42) (Table VII). The eight species 445-bp-long alignment shows a total of 108 polymorphic sites, including 89 variable positions (20%), of which 43 were parsimony informative (p-info), 46 were singleton sites and 19 gapped or ambiguous sites (Supplementary data). These variable positions do not appear regularly distributed throughout the 16S fragment and show evident concentrations in given hot spot regions (Supplementary data).

mtDNA cox1 - The code L.dia *cox*1-Ha has been ascribed for the provisional haplotype obtained for this fragment. The sequence in question is 672-bp long and shows a high AT-biased composition of 69.40% (Table I).

In a multiple 672-bp-long sequence alignment restricted to 17 sequences similar in nucleotide length, a total of 473 positions were conserved and 199 variable, comprising 166 p-info and 33 singleton sites (alignment not shown). When comparing the *L. diaphana* *cox*1 sequence with these other proximal lymnaeid species, including species of the *Galba*/*Fossaria* group, stagnicolines such as *S. elodes*, *P. columella* and also species of the *Radix* group such as *A. tomentosa* and *R. rubiginosa*, available in GenBank, the high number of nucleotide differences appears evident in a pairwise *cox*1 distance matrix (Table VIII).

The code L.dia COX1-HI has been assigned to the provisional haplotype represented by the 224-aa-long protein sequence of that *cox*1 gene fragment (Table I). In the protein alignment comprising *L. diaphana* and the aforementioned species, a total of 213 positions appeared to be conserved and 11 were variable, including five p-info and six singleton sites. A pairwise comparison showed a 100% identity between this *L. diaphana* haplotype *cox*1-a and *L. neotropica* *cox*1-b form Argentina and only one amino acid change (S/G) when compared with *F. bulimoides, G. truncatula, L. cubensis* and *L. neotropica* haplotype *cox*1-a (Table IX).

Phylogenetic analysis - The combination of the two ITS in a single data-set generated a robust tree, indicating phylogenetic accordance between the two spacers. The ML model best fitting this data-set was HKY85+G+I, using a ts/tv ratio of 1.32 (kappa = 2.5975285), base fre-

TABLE II

Pairwise distances between ribosomal DNA 18S nucleotide sequences according to Phylogenetic Analysis Using Parsimony including the lymnaeid species studied, together with other proximal lymnaeid species available in GenBank

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 *Lymnaea (Lymnaea) stagnalis*	-	0.00108	0.00325	0.00813	0.00705	0.00761	0.00704	0.00923	0.00758	0.00597	0.00923	0.03168	0.03444
2 *Lymnaea (Stagnicola) palustris*	2	-	0.00325	0.00813	0.00650	0.00760	0.00811	0.01030	0.00865	0.00651	0.01030	0.03273	0.03559
3 *Omphiscola glabra*	6	6	-	0.00814	0.00813	0.00870	0.00813	0.01032	0.00867	0.00706	0.01032	0.03169	0.03387
4 *Radix auricularia*	15	15	15	-	0.00216	0.00489	0.00812	0.00869	0.00866	0.00651	0.00706	0.02837	0.03084
5 *Radix balthica*	13	12	15	4	-	0.00380	0.00812	0.00869	0.00866	0.00651	0.00706	0.02946	0.03203
6 *Galba truncatula*	14	14	16	9	7	-	0.00597	0.00706	0.00597	0.00706	0.00544	0.02787	0.02973
7 *Lymnaea viatrix*	13	15	15	15	15	11	-	0.00271	0.00215	0.00433	0.00595	0.03050	0.03254
8 *Lymnaea humilis*	17	19	19	16	16	13	5	-	0.00271	0.00541	0.00596	0.02999	0.03201
9 *Lymnaea cubensis*	14	16	16	16	16	11	4	5	-	0.00433	0.00595	0.03106	0.03316
10 *Lymnaea diaphana*	11	12	13	12	12	13	8	10	8	-	0.00434	0.02944	0.03142
11 *Pseudosuccinea columella*	17	19	19	13	13	10	11	11	11	8	-	0.02840	0.03147
12 *Biomphalaria glabrata*	58	60	58	52	54	51	56	55	57	54	52	-	0.01480
13 *Bulinus truncatus*	58	60	57	52	54	50	55	54	56	53	53	25	-

the planorbids *B. glabrata* and *B. truncatus* included for comparison purposes of family distances. Sequence correspondences detailed in Materials and Methods section. Below diagonal: total character differences; above diagonal: mean character differences (adjusted for missing data).

TABLE III

Pairwise distances between ribosomal DNA internal transcribed spacer nucleotide-2 sequences according to Phylogenetic Analysis Using Parsimony including the lymnaeid species studied, together with other proximal lymnaeid species available in GenBank

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 *Lymnaea (Stagnicola) palustris*-H1	-	0.02998	0.24737	0.29398	0.28362	0.25693	0.23200	0.23174	0.22922	0.21782	0.22879	0.25899	0.20460
2 *Lymnaea (Stagnicola)*fuscus-H2	14	-	0.25397	0.28467	0.28889	0.26209	0.23720	0.22251	0.21995	0.21197	0.22798	0.25547	0.20308
3 *Galba truncatula*-H1	94	96	-	0.14767	0.09819	0.07752	0.07937	0.16992	0.16992	0.17080	0.18732	0.20968	0.20000
4 *Lymnaea humilis*-H1	122	117	57	-	0.20920	0.18313	0.16203	0.23940	0.24190	0.25776	0.26799	0.27751	0.27632
5 *Lymnaea cubensis*-H2	116	117	38	91	-	0.04556	0.04380	0.21120	0.21628	0.20603	0.21558	0.24010	0.25000
6 *Lymnaea viatrix*-H1	102	103	30	76	20	-	0.02651	0.17016	0.17016	0.17708	0.17935	0.21429	0.22841
7 *Lymnaea neotropica*-H1	87	88	30	64	18	11	-	0.15342	0.15342	0.16120	0.17280	0.18329	0.20178
8 *Catascopia catascopium*	92	87	61	96	83	65	56	-	0.00676	0.05301	0.10579	0.15603	0.17297
9 *Stagnicola elodes*	91	86	61	97	85	65	56	3	-	0.05301	0.10327	0.15603	0.17838
10 *Catascopia occulta*-H1	88	85	62	108	82	68	59	22	22	-	0.13382	0.18476	0.18684
11 *Hinkleyia caperata*	89	88	65	108	83	66	61	42	41	55	-	0.17831	0.17204
12 *Lymnaea diaphana*-H1	108	105	78	116	97	84	68	66	66	80	74	-	0.20876
13 *Pseudosuccinea columella*-H1	80	79	67	105	93	82	68	64	66	71	64	81	-

sequence correspondences detailed in Materials and Methods section. H: haplotype; below diagonal: total character differences; above diagonal: mean character differences (adjusted for missing data).

quencies for A, C, G and T of 0.22830, 0.26280, 0.22550 and 0.28340, respectively, gamma (continuous) with shape parameter alpha of 1.07 and a proportion of invariable sites equal to 0.121 (-ln L 9275.68103).

In the ML tree obtained (Fig. 3), the species of the *Radix* group appear clearly independent from all other lymnaeids, this independence always showing the highest support values. Worth emphasizing is also the presence of *P. columella* located basal to the branch, including species of both the *Galba*/*Fossaria* group and the stagnicolines, although such a location is not supported in the Heuristic analysis in which *P. columella* appears in a paraphyly regarding the fossarines and all stagnicolines nor in the Bayesian phylogeny reconstruction in which it appears basal to the *Galba*/*Fossaria* group (trees not shown).

In all trees obtained, European stagnicolines appear clustering together with the *Galba*/*Fossaria* group, whereas American stagnicolines (*Hinkleyia* and *Catascopia*, the latter also including the Palaearctic species *C. occulta*) appear separately, grouped within the same branch. *L. diaphana* appears basal to that clade comprising the American stagnicolines, with a bootstrap support of 74%, 76% and 88% with NJ, Heuristic and BPP algorithms, respectively. However, in the ML tree obtained with PhyML (tree not shown), the reliability for this *L. diaphana* - American stagnicolines cluster appear with a lower support of only 45% when applying the aLRT test.

DISCUSSION

Molecular characterization of L. diaphana - Phenotypically, this species was well described at the levels of both shell features and anatomical characteristics from specimens collected in the same type locality time ago (Paraense 1984).

Recently, broad analyses on the usefulness of the molecular markers offered by DNA in different organism groups have shown that nuclear rDNA correlates with the phenotype (shape, size, anatomy), while mtDNA does not (Mas-Coma & Bargues 2009, Mas-Coma et al. 2009a), and that mtDNA poses many problems causing erroneous results when used to compare genetically distant taxa as distant species within the same genus or different genera (Lin & Danforth 2004, Ballard & Rand 2005, Mas-Coma & Bargues 2009). This has obvious implications on the usefulness of these markers, as in fact traditional systematics and taxonomy, as those always applied to snails in malacology have fundamentally relied on morphology (Bargues et al. 2011a). Therefore, in Lymnaeidae it has been more recently concluded that (i) rDNA markers are the appropriate targets when dealing

TABLE IV

Total character differences (extreme and average values of nucleotide differences) at ribosomal DNA internal transcribed spacer (ITS)-2 and ITS-1 sequences according to Phylogenetic Analysis Using Parsimony in the pairwise distance comparisons between *Lymnaea diaphana* and the different lymnaeid groups studied

DNA marker	*Galba*/*Fossaria* group	European stagnicolines	American stagnicolines	*Pseudosuccinea columella*
ITS-2	68-116 (88.6)	105-108 (106.5)	66-80 (71.5)	81 (81)
ITS-1	73-95 (85.6)	100-101 (100.5)	76-93 (86.7)	105 (105)

data set contains 736 and 765 characters for ITS-2 and ITS-1, respectively.

TABLE V

Genetic distances in internal transcribed spacer (ITS)-2 and ITS-1 detected in pairwise comparisons of *Lymnaea diaphana* with its molecularly closest lymnaeid species

Pairwise sequence comparisons	Alignment length (bp long)	Conserved positions (n)	Nucleotide differences n (%)	Mutations (transitions + transversions) n (%)	Insertions + deletions (indels) n (%)
ITS-2					
L. diaphana vs. *Lymnaea neotropica*	512	323	189 (36.91)	77 (15.04)	112 (21.87)
L. diaphana vs. *Catascopia catascopium*	511	361	150 (29.35)	67 (13.11)	83 (16.24)
L. diaphana vs. *Catascopia elodes*	499	340	159 (31.86)	104 (20.84)	55 (11.02)
L. diaphana vs. *Hinkleyia caperata*	502	331	171 (34.06)	96 (19.12)	75 (14.94)
ITS-1					
L. diaphana vs. *Galba truncatula*	536	399	137 (25.56)	89 (16.60)	48 (8.95)
L. diaphana vs. *Hinkleyia caperata*	591	427	164 (27.75)	89 (15.01)	75 (12.69)

TABLE VI

Pairwise distances between ribosomal DNA internal transcribed spacer (ITS)-1 nucleotide sequences according to Phylogenetic Analysis Using Parsimony including the lymnaeid species studied, together with other proximal lymnaeid species available in GenBank

	1	2	3	4	5	6	7	8	9	10	11	12	13
1 *Lymnaea (Stagnicola) palustris*-HA	-	0.01512	0.21258	0.21849	0.20211	0.21268	0.21118	0.23409	0.24280	0.23984	0.23320	0.20739	0.25319
2 *Lymnaea (Stagnicola)fuscus*-HA	8	-	0.20950	0.21757	0.19874	0.21341	0.21237	0.23770	0.24486	0.24646	0.23123	0.20534	0.25319
3 *Galba truncatula*-HA	98	97	-	0.15208	0.12000	0.14286	0.14639	0.22009	0.22698	0.21097	0.18298	0.16115	0.24890
4 *Lymnaea humilis*-HA	104	104	73	-	0.14868	0.15551	0.15569	0.25859	0.26316	0.26000	0.23846	0.20085	0.25652
5 *Lymnaea cubensis*-HA	96	95	57	73	-	0.04864	0.05952	0.23061	0.23950	0.21946	0.18672	0.17570	0.25217
6 *Lymnaea viatrix*-HA	104	105	71	79	25	-	0.05642	0.23374	0.24033	0.22088	0.20160	0.18737	0.25934
7 *Lymnaea neotropica*-HA	102	103	71	78	30	29	-	0.24746	0.25000	0.23695	0.19960	0.19108	0.24034
8 *Catascopia catascopium*	114	116	103	128	110	115	122	-	0.00741	0.07129	0.22543	0.18699	0.25684
9 *Stagnicola elodes*	118	119	106	130	114	118	123	4	-	0.07910	0.22929	0.18941	0.25367
10 *Catascopia occulta*-HA	118	122	100	130	106	110	118	38	42	-	0.20992	0.17304	0.25156
11 Hinkleyia caperata	118	117	86	124	90	101	99	117	119	110	-	0.14729	0.23701
12 *Lymnaea diaphana*-HA	101	100	73	95	81	89	90	92	93	86	76	-	0.22532
13 *Pseudosuccinea columella*-HA	119	119	113	118	116	125	112	122	121	121	114	105	-

sequence correspondences detailed in Materials and Methods section. H: haplotype; below diagonal: total character differences; above diagonal: mean character differences (ad- justed for missing data).

with systematic-taxonomic and phylogenetic aspects, as well as for molecular characterization of species by haplotyping, (ii) mtDNA markers are more convenient for population and intraspecific variability studies and (iii) both rDNA and mtDNA markers may be used for the classification of specimens (Bargues et al. 2011a).

The 18S sequence of *L. diaphana* (1848 bp) is slightly longer than that of *G. truncatula* (1,843 bp) (Bargues et al. 1997), equally long than that of *L. humilis* (1,848 bp) (Bargues et al. 2011a), similar to that of the European stagnicolines *L. stagnalis*, *O. glabra* and *L.* (*S.*) *palustris*, the radicines *R. auricularia* and *R. balthica,* as well as to *P. columella* (ranging between 1,849-1,852 bp) (Bargues et al. 1997, 2011b), but pronouncedly shorter than that of *L. cubensis*, *L. viatrix* and *L. neotropica* (all three 1,860-bp long) (Bargues et al. 2007). This suggests that *L. diaphana* may be considered an old species within the family Lymnaeidae, according to their phylogeny in which the oldest lymnaeid fossil known is *Galba* from the Jurassic (zilch 1959-1960, Inaba 1969), a shorter sequence would be the plesiomorphic condition and an increase in sequence length would have occurred during lymnaeid evolution (Bargues et al. 2001).

Pairwise nucleotide comparisons in a slowly evolving gene as the rRNA small subunit (Table II) show *L. diaphana* to be at a genetic distance from Old World radicines similar to that from Palaearctic stagnicolines, as well as from *Galba*. Only New World fossarines and Pseudosuccinea appear somewhat closer, suggesting a biogeographic background for such a relationship. Unfortunately, the 18S sequence is not known for any Nearctic stagnicoline so far, in the way to verify an additional support for such an assumption.

With regard to the ITS-2, *L. diaphana* presents a sequence whose 495-bp length fits within the group of lymnaeids having the longest ITS-2 sequences (Bargues et al. 2001). This might be interpreted as a species having long time derived from the old form suggested by the 18S. In this context, *L. diaphana* shows evolutionary characteristics similar to the Nearctic species *L. humilis* (Bargues et al. 2011a). Additionally, the very high number of nucleotide differences it shows when compared to all other lymnaeid species (Table III) and groups (Table IV) is surprising. Moreover, contrary to what was expected, the distances regarding Nearctic stagnicolines appear to be lower than those regarding Holarctic *Galba*/*Fossaria* species (Table IV). This indicates that *L. diaphana* should not be included in the *Galba*/*Fossaria* group as its shell and anatomic characteristics suggest (Paraense 1984).

Such a relationship with American stagnicolines does, however, not appear so clear at ITS-1 level, a marker in which the lower *L. diaphana* differences appear with Old World originary *G. truncatula* (73) and North American *H. caperata* (76) (Table VI). Interestingly, the length of ITS-1 in *L. diaphana* is the shortest hitherto known in Lymnaeidae (520 bp), only surpassed by *G. truncatula* (504 bp).

Particular aspects of the results obtained in mtDNA 16S sequences should be highlighted: (i) AT composition appears to be pronouncedly biased, which should be taken into account when analyzing the significance of the

information this marker offers, (ii) variable positions do not appear regularly distributed throughout the sequence, but concentrated in hot spot regions (Supplementary data), which indicates that the information furnished by the fragment may not appropriately reflect whole gene evolution, as already seen in other organisms (Mas-Coma & Bargues 2009), and (iii) nucleotide differences appear to be less in number than those logically expected from mtDNA (Table VII), which suggests a low mutation rate indeed only apparent, as a consequence of an evolutionary parallelism of its rRNA gene function inside the mitochondrial genome with fast evolving mtDNA coding genes giving rise to position saturation, as already seen in lymnaeids and other freshwater molluscs (Bargues et al. 2011a). Thus, the somewhat closer 16S sequence of *L. diaphana* to that of American stagnicolines, represented by *S. bonnevillensis*, may be considered with great caution.

Nucleotide saturation and biased composition may also pose a significance question mark on the potential relationships of *L. diaphana* with the different lymnaeid groups, as suggested by the high number of nucleotide differences in the mtDNA *cox*1 gene (Table VIII). This mtDNA saturation problem has already been highlighted in lymnaeids very recently (Bargues et al. 2011a). Moreover, the very few amino acid differences in the protein sequence (Table IX) indicate that most of the nucleotide differences are silent. Additionally, there is unfortunately only one stagnicoline from which the *cox*1 fragment in question is available (*S. elodes*), so that no conclusions may be obtained from that comparison.

Summing up, the following conclusions may be obtained from the sequence analyses of nuclear rDNA and mtDNA markers: (i) *L. diaphana* shows sequences very different from all hitherto lymnaeid sequences available at the level of both nuclear rDNA and mtDNA markers, (ii) each one of the five markers analyzed, including rDNA 18S, ITS-2 and ITS-1, as well as mtDNA 16S and *cox*1, allow the differentiation of *L. diaphana* specimens from all other lymnaeids; only the COX1 amino acid sequence does not, (iii) nuclear rDNA suggest that *L. diaphana* is not a fossarine lymnaeid, but rather a relict form related to ancestral stagnicolines and (iv) mtDNA markers do not furnish genetic distance information useful for the analysis of the relationships of *L. diaphana* with the different lymnaeid groups.

Phylogenetic relationships and Pectinidens genus assessment - *L. diaphana* was selected as type species of the new section *Pectinidens* within the genus *Lymnaea* Lamarck by Pilsbry (1911). The erection of *Pectinidens* was justified on the characteristics of the radular teeth of *L. diaphana*, noted to have peculiarities different from all other lymnaeids known at that time. *Pectinidens* was considered at genus level until its synonymization with *Lymnaea* by Hubendick (1951). From that moment, it disappeared from the lymnaeid literature, although a few authors still sporadically referred to it, whether at genus level (Inaba 1969) or at subgenus level (Alcaino & Apt 1989).

In the phylogenetic reconstructions performed, *L. diaphana* does not cluster together with other morphologically similar fossarine lymnaeids as the New World *L. cubensis*, *L. viatrix* and *L. neotropica*, the Nearctic *L. humilis*, or the Old World *G. truncatula*. Contrary to what would be phenotypically expected, *L. diaphana* appears basal to the Nearctic stagnicolines (Fig. 3). Values supporting such a phylogenetic relationship were higher than 70% in most of the node reliability assessment methods. Although it may be argued that higher node supports would be better as to conclude that this result is definitive, the phylogenetic tree agrees with the very numerous nucleotide differences and very large genetic distances shown by DNA markers with verified usefulness at specific and supraspecific levels as both ITSs. Thus, in ITS-2 and ITS-1 the nucleotide differences separating *L. diaphana* from all other lymnaeids appear to be of supraspecific level in both spacers (see Table

TABLE VII

Pairwise distances between mitochondrial DNA 16S ribosomal DNA gene data set nucleotide sequences according to Phylogenetic Analysis Using Parsimony including the lymnaeid species studied together with other proximal lymnaeid species available in GenBank

	1	2	3	4	5	6	7	8	9
1 *Lymnaea diaphana*	-	0.09762	0.09739	0.10072	0.09785	0.09927	0.09716	0.11005	0.07329
2 *Lymnaea humilis*	41	-	0.04276	0.10526	0.00000	0.00241	0.04976	0.08115	0.08789
3 *Lymnaea cubensis*	41	18	-	0.10048	0.04265	0.04589	0.01174	0.07619	0.08706
4 *Pseudosuccinea columella*	42	44	42	-	0.10526	0.10706	0.10263	0.10287	0.10476
5 *Stagnicola elodes*	41	0	18	44	-	0.00242	0.04965	0.07889	0.08768
6 *Fossaria obrussa*	41	1	19	44	1	-	0.05301	0.08495	0.09179
7 *Fossaria bulimoides*[a]	41	21	5	43	21	22	-	0.07601	0.08216
8 *Fossaria bulimoides*[b]	46	34	32	43	34	35	32	-	0.10214
9 *Stagnicola bonnevillensis*	31	37	37	44	37	38	35	43	-

a: AF485657; *b*: EU038315. Sequence correspondences detailed in Materials and Methods section. H: haplotype; below diagonal: total character differences; above diagonal: mean character differences (adjusted for missing data).

TABLE VIII

Pairwise distances between mitochondrial DNA cytochrome c oxidase (*cox*)1 nucleotide sequences according to Phylogenetic Analysis Using Parsimony including the lymnaeid species studied together with other proximal lymnaeid species available in GenBank

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 *Lymnaea diaphana cox*1-a	-	0.11737	0.13889	0.13426	0.13542	0.13889	0.13839	0.14333	0.11905	0.12054	0.11161	0.11012	0.11756	0.13542	0.13690	0.13839	0.15696
2 *Fossaria bulimoides*	75	-	0.14867	0.13302	0.14867	0.14867	0.10798	0.10829	0.03443	0.03286	0.02191	0.02347	0.05008	0.10955	0.10955	0.11111	0.14950
3 *Stagnicola elodes*	90	95	-	0.13889	0.14815	0.14815	0.15432	0.16000	0.14660	0.14815	0.14815	0.14969	0.14043	0.13735	0.13735	0.13889	0.16694
4 *Austropeplea tomentosa*	87	85	90	-	0.14352	0.14352	0.13117	0.13833	0.13426	0.13580	0.12500	0.12654	0.13117	0.13735	0.13889	0.14043	0.14894
5 *Pseudosuccinea columella*[a]	91	95	96	93	-	0.00000	0.14286	0.15000	0.14583	0.14435	0.14435	0.14583	0.14881	0.13542	0.13542	0.13690	0.14725
6 *Pseudosuccinea columella*[b]	90	95	96	93	0	-	0.14815	0.15000	0.15123	0.14969	0.14969	0.15123	0.15278	0.14043	0.14043	0.14198	0.14894
7 *Galba truncatula*[c]	93	69	100	85	96	96	-	0.00333	0.11161	0.11012	0.09970	0.10119	0.10119	0.08929	0.08780	0.08929	0.15372
8 *Galba truncatula*[d]	86	64	96	83	90	90	2	-	0.12000	0.11833	0.10667	0.10833	0.10500	0.09667	0.09500	0.09667	0.15878
9 *Lymnaea cubensis cox*1-a	80	22	95	87	98	98	75	72	-	0.00149	0.02083	0.02232	0.05655	0.10714	0.10714	0.10565	0.15372
10 *Lymnaea cubensis cox*1-b	81	21	96	88	97	97	74	71	1	-	0.01935	0.02083	0.05804	0.10565	0.10565	0.10417	0.15210
11 *Lymnaea neotropica cox*1-a	75	14	96	81	97	97	67	64	14	13	-	0.00149	0.04315	0.10119	0.09821	0.09970	0.14725
12 *Lymnaea neotropica cox*1-b	74	15	97	82	98	98	68	65	15	14	1	-	0.04464	0.10268	0.09970	0.10119	0.14887
13 *Lymnaea viatrix cox*1-a	79	32	91	85	100	99	68	63	38	39	29	30	-	0.10714	0.11012	0.11161	0.15372
14 *Lymnaea humilis cox*1-a	91	70	89	89	91	91	60	58	72	71	68	69	72	-	0.00744	0.00595	0.14401
15 *Lymnaea humilis cox*1-b	92	70	89	90	91	91	59	57	72	71	66	67	74	5	-	0.00149	0.14563
16 *Lymnaea humilis cox*1-c	93	71	90	91	92	92	60	58	71	70	67	68	75	4	1	-	0.14401
17 *Radix rubiginosa*	97	90	102	91	91	91	95	94	95	94	91	92	95	89	90	89	-

a: FN598165; *b*: AY227366; *c*: AM494011; *d*: EU818799. Haplotype codes only provisional due to incomplete sequences of the gene. Sequence correspondences detailed in Materials and Methods section. Below diagonal: total character differences; above diagonal: mean character differences (adjusted for missing data).

IV for p-info positions). A deep analysis of genetic distances between *L. diaphana* and its molecularly closest lymnaeid species belonging to the Nearctic stagnicoline group and the *Galba*/*Fossaria* group according to PAUP (Tables III, VI) shows very high values (ITS-2: 29.35-36.91%; ITS-1: 25.56-27.75%) of unquestionable genus level (Table V) when compared with known genetic distances between lymnaeid genera (Bargues et al. 2001, 2006). Consequently, taxonomic validity should be restored to the genus *Pectinidens*.

It should be added that, in the literature, *L. diaphana* has usually been ascribed to King, 1830. In the original article including the description of this species, both in the second page of the contents index and in page 332 at the beginning of the article 47, it is noted "By Captain Phillip P. King, R.N., F.R.S., & c. assisted by W.J. Broderip, Esq. F.R.S., & c". Moreover, although the issue 19 of The zoological Journal corresponds, as clearly stated in the issue front cover, to the period of July 1830-September 1831, at the bottom of this front cover it is also noted "London: printed by… and Published by… 1832". Finally, different misunderstandings have appeared in the literature with regard to the original reference; the correct one should include Year 1832, Volume 5 (from 1832-1834), Issue 19 (July 1830-September 1831), article 47, species described within article list under 43 in page 344. Therefore, according to present rules, the correct type species taxon of *Pectinidens* would thus become *Pectinidens diaphana* (King & Broderip, 1832) Pilsbry, 1911.

Stagnicolines are generally characterized by its elongate and pointed shell form, relatively long size, very numerous in species number, hitherto known to be restricted to the northern hemisphere and mostly lymnaeids adapted to live in cold waters (Bargues et al. 2003). The smaller size of only up to 14.6 mm long and 8.8 mm wide of five-whorl *L. diaphana* specimens (Paraense 1984) may be interpreted as not being sufficient as to manifest the typical stagnicoline elongate trend, similarly as has been recently shown in *L. humilis* (Bargues et al. 2011a). This would explain the usual including of *L. diaphana* within the *Galba*/*Fossaria* group. Inaba (1969) even considered *Pectinidens* as a form presumably derived at the beginning of the Pleistocene from *Fossaria* species characterized by harbouring 18 chromosomes. Accepting the ascription of *L. diaphana* to the stagnicoline group would, thus, represents not only its first representative in the southern hemisphere, but also the colonization of extreme world regions, as it is the case of the most southern Patagonia, by stagnicolines long time ago.

Whether such an old southward spreading phenomenon only concerned *L. diaphana* or additionally other lymnaeid species still remains an open question. In fact, the present molecular study furnishes the baseline on which to clarify the systematic/taxonomic validity of numerous lymnaeid species described in the southernmost mainland areas and islands of South America, in Chile as well as in Argentina. All of these species are very similar to *L. diaphana* and several of them have already been proposed to be synonyms of *L. diaphana*, although the opinion about the validity of particular species differ according to authors: *Lymnaea lebruni* Mabille 1883, *Lymnaea falklandiana* Smith, 1884, *Lymnaea pictonica* Rochebrune and

Mabille, 1889, *Lymnaea patagonica* Strebel, 1907, *Lymnaea brunneoflavida* Preston, 1910, *Lymnaea andeana* Pilsbry, 1911, *Lymnaea inelegans* Pilsbry 1911, *Lymnaea riochicoensis* Pilsbry, 1911 and *Lymnaea plicata* Hylton Scott, 1954 (Rochebrune & Mabille 1885, Pilsbry 1911, Hubendick 1951, Hylton Scott 1954, Malek 1985). Indeed, many of these lymnaeid species were already included within *Pectinidens* by Pilsbry (1911).

L. diaphana and fascioliasis transmission - With regard to epidemiological characteristics and transmission patterns in human fascioliasis endemic areas, its zoonotic aspect seems to have only a relative influence due mainly to the scarce differences related to different livestock species playing a role of reservoir, given the similar infectivity of the metacercarial stage from different livestock species isolates (Valero & Mas-Coma 2000, Valero et al. 2001). On the contrary, its vector-borne aspect has proved to have a pronounced influence on human infection. The different fascioliasis patterns appear related to the different lymnaeid species, their ecology, type of water bodies they inhabit, their anthropophilic preferences, population dynamics, climatic links and transmission capacity (Bargues & Mas-Coma 2005, Mas-Coma et al. 2009a).

Fascioliasis endemic areas inhabited by *L. diaphana* are low altitude areas (Cordova et al. 1961, Tantalean et al. 1974, Larrea et al. 1994, Sielfeld 2001, Valdovinos 2006), which do not appear to fit the hitherto two main disease transmission scenarios where human infection epidemiology has been characterized: the Altiplano subpattern and Valley subpattern, both in high altitude endemic zones (Mas-Coma 2005, Mas-Coma et al. 2009a).

The phylogenetic link of *L. diaphana* with the stagnicoline group may give light to the aforementioned peculiar low altitude epidemiological scenario. Indeed, stagnicolines are only considered secondary vectors of fascioliasis when compared to the main vector species of *F. hepatica* included in the *Galba*/*Fossaria* group (Bargues et al. 2001) and to play an important transmission role only rarely in particular places where no other lymnaeid vector is present or under special natural conditions (Czapski 1962, 1997, Bouix-Busson & Rondelaud 1985, 1986, Dreyfuss et al. 1994, 2000). The existence of lymnaeid species of high transmission capacity, belonging to the *Galba*/*Fossaria* group, has already been molecularly confirmed in low altitude endemic areas of Peru (Bargues et al. 2007). The participation of *L. diaphana* in disease transmission in the southernmost fascioliasis endemic areas of South America, in both Chile (Alcaino & Apt 1989, Morales et

TABLE IX

Cytochrome c oxidase (*cox*)1 amino acid sequence differences detected in pairwise comparisons between haplotypes of *Lymnaea diaphana* and other proximal lymnaeid species available in GenBank

Nucleotidic haplotype *cox*1	GenBank accession	Country	Variable position									
			1	1	1	1	1	1	1	1	1	2
			1	3	9	9	9	0	2	6	7	0
			8	1	2	6	8	9	0	5	0	6	4
L.dia-*cox*1-a	JF909501	Chile	I	I	T	I	L	L	C	S	P	V	S
F. bulimoides	AY227367	Canada	.	.	.	.	.	.	.	G	.	.	.
S. elodes	AY227368	Canada	.	V	L	V	.	.	.	G	.	.	.
A. tomentosa	AY227365	Australia	.	V	M	.	.	.	?	S	I	T	
P. columella	FN598165	Venezuela	V	V	M	.	.	.	.	G	.	.	T
P. columella	AY227366	Australia	V	V	M	.	.	.	.	G	.	.	T
G.tru-*cox*1-a	AM494011	Spain	.	.	.	.	.	.	.	G	.	.	.
G. truncatula	EU818799	Germany	-	-	.	.	.	.	.	G	.	.	.
L.cub-*cox*1-a	AM494009	Cuba	.	.	.	.	.	.	.	G	.	.	.
L.cub-*cox*1-b	FN182205	USA	.	.	.	.	.	.	.	G	.	.	.
L.neo-*cox*1-a	AM494008	Peru	.	.	.	.	.	.	.	G	.	.	.
L.neo-*cox*1-b	FN356741	Argentina	.	.	.	.	.	.	.	.	.	.	.
L.via-*cox*1-a	AM494010	Argentina	.	V	.	.	.	.	.	G	.	.	.
L.hum-*cox*1-a	FN182197	USA	.	V	.	.	.	.	.	G	.	.	.
L.hum-*cox*1-b	FN182198	USA	.	V	.	.	.	.	.	G	.	.	.
L.hum-*cox*1-c	FN182199	USA	.	V	.	.	.	.	.	G	.	.	.
R. rubiginosa	GU451737	Thailand	.	V	S	.	F	F	V	G	.	.	.

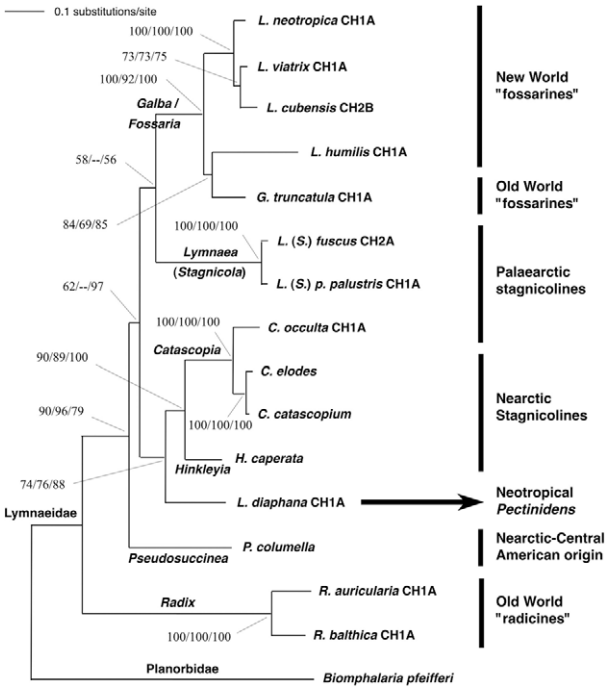


Fig. 3: phylogenetic tree of lymnaeid species studied obtained using the planorbid *Biomphalaria pfeifferi* as outgroup, based on maximum-likelihood (ML) estimates. CH: internal transcribed spacers composite haplotype. Scale bar indicates the number of substitutions per sequence position. Support for nodes a/b/c: *a*: bootstrap with neighbour-joining reconstruction using Phylogenetic Analysis Using Parsimony (PAUP) with ML distance and 1,000 replicates; *b*: bootstrap with ML reconstruction using PAUP with 1,000 heuristic replicates; *c*: Bayesian posterior probability with ML model using MrBayes.

al. 2000) and Argentina (Olaechea 1994), whether as only vector or coexisting with other lymnaeid vector species (e.g., *L. viatrix*) (Kaczorkiewick 1983, Rubel et al. 2005, Kleiman et al. 2007) should be assessed.

Molecular markers established in the present study furnish the needed baseline on which to (i) address the validity or synonymy of each one of the several aforementioned lymnaeid species cited in southern Patagonia, (ii) clarify the geographical distribution and intraspecific genetic variability of *L. diaphana* and finally (iii) assess its population dynamics correlation with fascioliasis transmission. The cold weather typical of such extreme latitudes suggest, in the southern low altitude Patagonian plains, a marked transmission seasonality restriction due to the minimum temperature 9-10ºC threshold of *F. hepatica* (Fuentes et al. 1999, 2001). Such a seasonality has already been verified somewhat more northward in Andean valleys (Kleiman et al. 2007).

ACKNOWLEDGEMENTS

To Mr. Ricardo Vladilo Bartulovich, owner of the land where snail collections were made, for his valuable help, and to the Servicio Agricola Ganadero of Chile, through its office of Punta Arenas, for the useful information they provide.

REFERENCES

Akaike H 1974. A new look at the statistical model identification. *Ieee Trans Automat Control 19*: 716-723.

Albrecht C, Wolf C, Glöer P, Wilke T 2008. Concurrent evolution of ancient sister lakes and sister species: the freshwater gastropod genus *Radix* in lakes Ohrid and Prespa. *Hydrobiologia 615*:157-167.

Alcaino H, Apt W 1989. Algunos antecedentes sobre la fascioliasis animal y humana. *Monogr Med Vet 11*: 14-29.

Ballard JWO, Rand DM 2005. The population biology of mitochondrial DNA and its phylogenetic implications. *Ann Rev Ecol Evol Syst 36*: 621-642.

Bargues MD, Artigas P, Khoubbane M, Flores R, Glöer P, Rojas-Garcia R, Ashrafi K, Falkner G, Mas-Coma S 2011a. *Lymnaea schirazensis*, an overlooked snail distorting fascioliasis data: genotype, phenotype, ecology, worldwide spread, susceptibility, applicability. *PLoS ONE 6*: e24567.

Bargues MD, Artigas P, Jackiewicz M, Pointier JP, Mas-Coma S 2006. Ribosomal DNA ITS-1 sequence analysis of European stagnicoline Lymnaeidae (Gastropoda). *Heldia München 6*: 29-40.

Bargues MD, Artigas P, Mera y Sierra RL, Pointier JP, Mas-Coma S 2007. Characterisation of *Lymnaea cubensis*, *L. viatrix* and *L. neotropica* n. sp., the main vectors of *Fasciola hepatica* in Latin America, by analysis of their ribosomal and mitochondrial DNA. *Ann Trop Med Parasitol 101*: 621-641.

Bargues MD, Gonzalez LC, Artigas P, Mas-Coma S 2011b. A new baseline for fascioliasis in Venezuela: lymnaeid vectors ascertained by DNA sequencing and analysis of their relationships with human and animal infection. *Parasit Vectors 4*: 200.

Bargues MD, Horak P, Patzner RA, Pointier JP, Jackiewicz M, Meier-Brook C, Mas-Coma S 2003. Insights into the relationships of Palaearctic and Nearctic lymnaeids (Mollusca: Gastropoda) by rDNA ITS-2 sequencing and phylogeny of stagnicoline intermediate host species of *Fasciola hepatica*. *Parasite 10*: 243-255.

Bargues MD, Mangold AJ, Muñoz-Antolí C, Pointier JP, Mas-Coma S 1997. SSU rDNA characterization of lymnaeid snails transmitting human fascioliasis in South and Central America. *J Parasitol 83*: 1086-1092.

Bargues MD, Mas-Coma S 1997. Phylogenetic analysis of lymnaeid snails based on 18S rDNA sequences. *Mol Biol Evol 14*: 569-577.

Bargues MD, Mas-Coma S 2005. Reviewing lymnaeid vectors of fascioliasis by ribosomal DNA sequence analyses. *J Helminthol 79*: 257-267.

Bargues MD, Vigo M, Horak P, Dvorak, Patzner RA, Pointier JP, Jackiewicz M, Meier-Brook C, Mas-Coma S 2001. European Lymnaeidae (Mollusca: Gastropoda), intermediate hosts of trematodiases, based on nuclear ribosomal DNA ITS-2 sequences. *Infect Genet Evol 1*: 85-107.

Bouix-Busson D, Rondelaud D 1985. Etude de l'aptitude à l'infestation fasciolienne chez *Lymnaea glabra* Müller et chez *L. truncatula* dans des peuplements mono et bispécifiques. *Bull Soc Fran Parasitol 33*: 95-98.

Bouix-Busson D, Rondelaud D 1986. L'infestation de *Lymnaea glabra* Müller par *Fasciola hepatica* L. Etude expérimentale sur le terrain. *Ann Parasitol Hum Comp 61*: 215-225.

Cordova E, Naquira F, Naquira C 1961. *Lymnaea diaphana* King como huésped intermediario de *Fasciola hepatica* en Arequipa (Perú). *Arch Per Patol Clin 15*: 165-172.

Czapski z 1962. The snail *Galba occulta* Jackiewicz, 1959, another intermediate host of *Fasciola hepatica* L. *Z Tropenmed Parasitol 13*: 332-337.

Czapski z 1997. Biologiczne aspekty epidemiologii fasciolozy. *Monog Podreczniki Skrpty AWF w Poznaniu 95*: 1-143.

Chen MG, Mott KE 1990. Progress in assessment of morbidity due to *Fasciola hepatica* infection: a review of recent literature. *Trop Dis Bull 87*: R1-R38.

De Rijk P, Neefs JM, Van de Peer Y, De Wachter R 1992. Compilation of small ribosomal subunit RNA sequences. *Nucl Acids Res 20*: 2075-2089.

De Jong RJ, Morgan JAT, Paraense WL, Pointier JP, Amarista M, Ayeh-Kumi PFK, Babiker A, Barbosa CS, Bremond PH, Canese AP, Pereira da Souza C, Dominguez C, File S, Gutierrez A, Incani, RN, Kawano T, Kazibwe F, Kpikpi J, Lwambo NJS, Mimpfoundi R, Njiokou F, Poda JN, Sene M, Velasquez LE, Yong M, Adema CM, Hofkin BV, Mkoji GM, Loker ES 2001. Evolutionary relationships and biogeography of *Biomphalaria* (Gastropoda: Planorbidae) with implications regarding its role as host of the human bloodfluke, *Schistosoma mansoni*. *Mol Biol Evol 18*: 2225-2239.

Dreyfuss G, Abrous M, Rondelaud D 2000. The susceptibility of *Lymnaea fuscus* to experimental infection with *Fasciola hepatica*. *J Parasitol 86*: 158-160.

Dreyfuss G, Moukrim A, Rondelaud D, Vareille-Morel C 1994. Field observations concerning infection of *Lymnaea palustris* by *Fasciola hepatica*. *J Helminthol 68*: 115-118.

Esteban JG, Flores A, Aguirre C, Strauss W, Angles R, Mas-Coma S 1997a. Presence of very high prevalence and intensity of infection with *Fasciola hepatica* among Aymara children from the northern Bolivian Altiplano. *Acta Trop 66*: 1-14.

Esteban JG, Flores A, Aguirre C, Strauss W, Angles R, Mas-Coma S 1997b. A population-based coprological study of human fascioliasis in a hyperendemic area of the Bolivian Altiplano. *Trop Med Int Health 2*: 695-699.

Esteban JG, Flores A, Angles R, Mas-Coma S 1999. High endemicity of human fascioliasis between Lake Titicaca and La Paz valley, Bolivia. *Trans Roy Soc Trop Med Hyg 93*: 151-156.

Esteban JG, Gonzalez C, Bargues MD, Angles R, Sanchez C, Naquira C, Mas-Coma S 2002. High fascioliasis infection in children linked to a man-made irrigation zone in Peru. *Trop Med Int Health 7*: 339-348.

Folmer O, Black M, Lutz R, Vrijenhoek R 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol 3*: 294-299.

Fuentes MV, Malone JB, Mas-Coma S 2001.Validation of a mapping and predicting model for human fasciolosis transmission in Andean very high altitude endemic areas using remote sensing data. *Acta Trop 79*: 87-95.

Fuentes MV, Valero MA, Bargues MD, Esteban JG, Angles R, Mas-Coma S 1999. Analysis of climatic data and forecast indices for human fascioliasis at very high altitude. *Ann Trop Med Parasi- tol 93*: 835-850.

Gironés N, Valero MA, García-Bodelón MA, Chico-Calero MI, Punzón C, Fresno M, Mas-Coma S 2007. Immune suppression in advanced chronic fascioliasis: an experimental study in a rat model. *J infect Dis 195*: 1504-1512.

Gonzalez LC, Esteban JG, Bargues MD, Valero MA, Ortiz P, Naquira C, Mas-Coma S 2011. Hyperendemic human fascioliasis in Andean valleys: an altitudinal transect analysis in children of Cajamarca province, Peru. *Acta Trop 120*: 119-129.

Hanelt B, Adema CM, Mansour MH, Loker ES 1997. Detection of *Schistosoma mansoni* in *Biomphalaria* using nested PCR. *J Parasitol 83*: 387-394.

Hillyer GV, Soler de Galanes M, Rodriguez-Perez J, Bjorland J, Silva de Lagrava M, Guzman SR, Bryan RT 1992. Use of the Falcon™ assay screening test-enzyme-linked immunosorbent assay (FAST-ELISA) and the enzyme-linked immunoelectrotransfer blot (EITB) to determine the prevalence of human fascioliasis in the Bolivian altiplano. *Am J Trop Med Hyg 46*: 603-609.

Hubendick B 1951. Recent Lymnaeidae. Their variation, morphology, taxonomy, nomenclature and distribution. *Kungl Svenska Vetenskapsakad Handl Fjärde Ser 3*: 1-223 + 5 pl.

Hylton Scott MI 1954. Sobre Limnaeidae argentinos (Mol. Pulm.). *Physis Buenos Aires 20*: 401-408.

Inaba A 1969. Cytotaxonomic studies of lymnaeid snails. *Malacologia 7*: 143-168.

Jorgensen A, Madsen H, Nalugwa A, Nyakaana S, Rollinson D, Stothard JR, Kristensen TK 2011. A molecular phylogenetic analysis of *Bulinus* (Gastropoda: Planorbidae) with conserved nuclear genes. *Zool Scr 40*: 126-136.

Kaczorkiewick AJ 1983. Distomatosis en la provincia de Neuquén. *Rev Med Vet Buenos Aires 64*: 354-356.

King PP, Broderip WJ 1832. Description of the Cirrhipeda, Conchifera and Mollusca, in a collection formed by the officers of H.M.S. Adventure and Beagle employed between the years 1826 and 1830 in surveying the southern coasts of South America, including the Straits of Magalhaens and the coast of Tierra del Fuego. *Zoological J 5*: 332-349.

Kleiman F, Pietrokovsky S, Prepelitchi L, Carbajo AE, Wisnivesky-Colli C 2007. Dynamics of *Fasciola hepatica* transmission in the Andean Patagonian valleys, Argentina. *Vet Parasitol 145*: 274-286.

Larrea H, Oviedo ML, Huaman P 1994. Estudio anatómico de *Lymnaea diaphana* King, 1830, agente transmisor de la fascioliasis en la zona sur del Perú. *Bol Lima 16*: 95-100.

Lin CP, Danforth BN 2004. How do insect nuclear and mitochondrial gene substitution patterns differ? Insights from Bayesian analyses of combined datasets. *Mol Phylogenet Evol 30*: 686-702.

Liu L, Mondal MM, Idris MA, Lokman HS, Rajapakse PJ, Satrija F, Diaz JL, Upatham ES, Attwood SW 2010. The phylogeography of *Indoplanorbis exustus* (Gastropoda: Planorbidae) in Asia. *Parasite Vector 3*: 57.

Malek EA 1985. *Snail hosts of schistosomiasis and other snail-transmitted diseases in tropical America: a manual*, Scientific Publication 478, Pan American Health Organization, Washington, 325 pp.

Mas-Coma S 2005. Epidemiology of fascioliasis in human endemic areas. *J Helminthol 79*: 207-216.

Mas-Coma S, Bargues MD 2009. Populations, hybrids and the systematic concepts of species and subspecies in Chagas disease triatomine vectors inferred from nuclear ribosomal and mitochondrial DNA. *Acta Trop 110*: 112-136.

Mas-Coma S, Funatsu IR, Bargues MD 2001. *Fasciola hepatica* and lymnaeid snails occurring at very high altitude in South America. *Parasitology 123*: S115-S127.

Mas-Coma S, Valero MA, Bargues MD 2008. Effects of climate change on animal and zoonotic helminthiasis. *Rev Sci Tech 27*: 443-457.

Mas-Coma S, Valero MA, Bargues MD 2009a. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Adv Parasitol 69*: 41-146.

Mas-Coma S, Valero MA, Bargues MD 2009b. Climate change effects on trematodiases, with emphasis on zoonotic fascioliasis and schistosomiasis. *Vet Parasitol 163*: 264-280.

Mera y Sierra R, Agramunt VH, Cuervo P, Mas-Coma S 2011. Human fascioliasis in Argentina: retrospective overview, critical analysis and baseline for future research. *Parasit Vectors 4*: 104.

Mera y Sierra R, Artigas P, Cuervo P, Deis E, Sidoti L, Mas-Coma S, Bargues MD 2009. Fascioliasis transmission by *Lymnaea neotropica* confirmed by nuclear rDNA and mtDNA sequencing in Argentina. *Vet Parasitol 166*: 73-79.

Morales MA, Luengo J, Vasquez O 2000. Distribución y tendencia de la fasciolasis en el ganado de abasto en Chile, 1989-1995. *Parasitol Dia 24*: 115-118.

Olaechea FV 1994. Epidemiologia y control de *Fasciola hepatica* en la Argentina. In A Nari, C Fiel, *Enfermedades parasitarias de importancia económica en bovinos*, Editorial Agropecuaria, Montevideo, p. 213-232.

Paraense WL 1976. *Lymnaea viatrix*: a study of topotypic specimens (Mollusca: Lymnaeidae). *Rev Bras Biol 36*: 419-428.

Paraense WL 1984. *Lymnaea diaphana*: a study of topotypic specimens (Pulmonata: Lymnaeidae). *Mem Inst Oswaldo Cruz 79*:75-81.

Pilsbry HA 1911. Non-marine Mollusca of Patagonia. In *Reports of the Princeton University expeditions to Patagonia, 1896-1899*, vol. 3, Part 5, p. 513-633.

Posada D 2008. jModeltest: phylogenetic model averaging. *Mol Biol Evol 25*: 1253-1256.

Posada D, Buckley TR 2004. Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. *Syst Biol 53*: 793-808.

Remigio EA 2002. Molecular phylogenetic relationships in the aquatic snail genus *Lymnaea*, the intermediate host of the causative agent of fascioliasis: insights from broader taxon sampling. *Parasitol Res 88*: 687-696.

Remigio EA, Blair D 1997a. Molecular systematics of the freshwater snail family Lymnaeidae (Pulmonata: Basommatophora) utilising mitochondrial ribosomal DNA sequences. *J Moll Stud 63*: 173-185.

Remigio EA, Blair D 1997b. Relationships among problematic North American stagnicoline snails (Pulmonata: Lymnaeidae) reinvestigated using nuclear ribosomal DNA internal transcribed spacer sequences. *Can J Zool 75*: 1540-1545.

Remigio EA, Hebert PD 2003. Testing the utility of partial COI sequences for phylogenetic estimates of gastropod relationships. *Mol Phylogenet Evol 29*: 641-647.

Rochebrune AT, Mabille J 1885. Diagnoses de mollusques nouveaux, recueillis par les membres de la mission du Cap Horn et M. Lebrun, Préparateur au Muséum, chargé d'une mission à Santa-Cruz de Patagonie. *Bull Soc Philomat Paris 7*: 100-111.

Ronquist F, Huelsenbeck JP 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics 19*: 1572-1574.

Rubel D, Prepelitchi L, Kleiman F, Carnevale S, Wisnivesky-Colli C 2005. Estudio del foco en un caso de fasciolosis humana en Neuquén. *Medicina Buenos Aires 65*: 207-212.

Saitou N, Nei M 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol 4*: 406-425.

Samadi S, Roumegoux A, Barges MD, Mas-Coma S, Yong M, Pointier JP 2000. Morphological studies of lymnaeid snails from the human fascioliasis endemic zone of Bolivia. *J Moll Stud 66*: 31-44.

Sanger F, Nicklen S, Coulson AR 1977. DNA sequencing with chain-terminating inhibitors. *Proc Nat Acad Sci USA 74*: 5463-5467.

Sielfeld W 2001. *Phylum Mollusca. Guías de identificación y biodiversidad. Fauna Chilena,* Universidad Arturo Prat, Iquique, 15 pp.

Simon C, Franke A, Martin A 1991. The polymerase chain reaction: DNA extraction and amplification. In GM Hewitt, AWB Johnston, JPW Young, *Molecular techniques in taxonomy*, Springer-Verlag, Berlin, p. 329-355.

Spithill TW, Smooker PM, Copeman DB 1999. *Fasciola gigantica*: epidemiology, control, immunology and molecular biology. In JP Dalton, *Fasciolosis*, CAB International Publishing, Wallingford, p. 465-525.

Swofford DL 2002. PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.01b, Sinauer Associates, Sunderland, Massachusetts.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S 2011. MEGA5: Molecular Evolutionary Genetic Analyses using likelihood, distance and parsimony methods. *Mol Biol Evol 28*: 2731-2739.

Tantalean M, Huiza A, Capuñay R 1974. Los hospederos intermediarios de *Fasciola hepatica* en el Perú. I. Estudio de la infección natural y experimental de *Lymnaea viator*, *L. diaphana* y *Physa venustula*. *Biota 10*: 243-250.

Thompson JD, Higins DG, Gibson TJ 1994. CLUSTALW: improving the sensitivity and progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucl Acids Res 22*: 4673-4680.

Torgerson P, Claxton J 1999. Epidemiology and control. In JP Dalton, *Fasciolosis*, CAB International Publishing, Wallingford, p. 113-149.

Valdovinos C 2006. Estado de conocimiento de los gastropodos dulceaquícolas de Chile. *Gayana 70*: 88-95.

Valero MA, Darce NA, Panova M, Mas-Coma S 2001. Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the northern Bolivian Altiplano hyperendemic region. *Vet Parasitol 102*: 85-100.

Valero MA, Gironés N, García-Bodelón MA, Periago MV, Chico-Calero I, Khoubbane M, Fresno M, Mas-Coma S 2008. Anaemia in advanced chronic fasciolosis. *Acta Trop 108*: 35-43.

Valero MA, Mas-Coma S 2000. Comparative infectivity of *Fasciola hepatica* metacercariae from isolates of the main and secondary reservoir animal host species in the Bolivian Altiplano high human endemic region. *Folia Parasitol 47*: 17-22.

Valero MA, Navarro M, García-Bodelón MA, Marcilla A, Morales M, García JE, Hernandez JL, Mas-Coma S 2006. High risk of bacterobilia in advanced experimental chronic fasciolosis. *Acta Trop 100*: 17-23.

Valero MA, Santana M, Morales M, Hernandez JL, Mas-Coma S 2003. Risk of gallstone disease in advanced chronic phase of fascioliasis: an experimental study in a rat model. *J Infect Dis 188*: 787-793.

Wethington AR, Lydeard C 2007. Molecular phylogeny of Physidae (Gastropoda: Basommatophora) based on mitochondrial DNA sequences. *J Moll Stud 73*: 241-257.

Winnepennickx B, Backeljau T, Van de Peer Y, De Wachter R 1992. Structure of the small ribosomal subunit RNA of the pulmonate snail, *Limicolaria kambeul*, and phylogenetic analysis of the Metazoa. *FEBS Lett 309*: 123-126.

zilch A 1959-1960. Gastropoda (Euthyneura). In O Schindewolf, *Handbuch der paleozoologie*, Borntraeger, Berlin, p. 91-102.

Nucleotide differences found in the complete 18S ribosomal DNA (rDNA) sequence of the lymnaeid species compared and their location in the secondary structure

Variable areas	VVVVV V	VVVVVVVVVV	VVVVVVVVVV	VVVVVVVVVV	V	VV	VV	VV
	11111 2	2222222222	2222222222	2222222222	2	45	79	99
Helix	666668E999	EEEEEEEEEE	EEEEEEEEEE	EEEEEEEEEE	E11111511E	EEEE	22334444	
	8	1111111111	1111111111	1111111111	122222 882	2222782513		47
		0000000000	0000000000	0000000000	1 1	11111		
		----------	----------	----------	-	-----		
		1111111111	1111111111	1111111111	1	25777		
Position							111111	11
	11112	2222222222	2222222222	2222222222	3333334557	7788123347		
	6777925550	3333333444	4444445555	5555556666	1999996882	4935530133		59
	9234892786	3456789012	3456790123	4567890126	2145784025	7496643763		23
Lymnaea (Lymnaea) stagnalis	G TG - T----------C	CG ---------- TGC	CGGGGGAC T C	GTGC ------- GC	- CG T AC - CC -	AACG T - GC TG		T A
Lymnaea (Stagnicola) palustris	. . . - . CA - - .	. . - - - - - . . .	. . . . .	. . . . - - - - . .	- . . . . . - . . -			. .
Omphiscola glabra	T AAC . - - - - .	. . - - - - - . . .	. . . . .	. . . . - - - - . .	- . . . . . - . . -			. .
Galba truncatula	. . . - . AA - - .	T - - - - - - - - -	. C T T T . CGAG	. - - - - - - - . T	- . . . . . - T T -	CG . A . - . - C .		. G
Lymnaea cubensis	. . . - . AA - - .	T . T CG TGCCG	. . . T . A . GC .	. . . G T CGC . .	- ..... C T T G	CG . . . - T - C .		CG
Lymnaea viatrix = Lymnaea neotropica	. . . - . AA - - .	T . TGCC T CCG	. . . T . A . GC .	. . . G T CGC . .	- ..... C T T -	CG . . . C T - C .		CG
Lymnaea humilis	. . . - . AA - - T	T---------- CG	. . . C . AGGC .	. A . G - - - - - .	- ..... C T T -	CG . . . C T - CC		CG
Pseudosuccinea columella	. . . - . AA - - .	T----------C . G	T CCC . . - - G	. G . . CG - - . T	- ..... C T T -	CG . . . C T - C .		. .
Lymnaea diaphana	. . . - . AA - - .	- - - - - - - CCG	. . . C T . C - - -	. CCG TG - - . .	- ..... C T T -	TG . . . C T - C .		. .
Radix auricularia	. . . - CAA - - .	. . ---------- TG	. T C T T . CGGG	. . . - - - - . T	CG T AC T - T AG	CG . AC - T - C .		. .
Radix balthica	. . . - . CAT T .	. . ---------- TG	. T C T T . CGGG	. . . - - - - . T	CG T AC T - T T -	CG . AC - . - C .		. .
Total variable positions (n = 62)	1	1111111111	2222222222	3333333333	4444444444	5555555555		66
	1234567890	1234567890	1234567890	1234567890	1234567890	1234567890		12

numbers (to be read in vertical) refer to positions obtained in the alignment made with MEGA 5.0. Shaded area corresponds to variable area V2 and helix E10-1 where Lymnaeidae differences in the 18S rRNA gene are concentrated. ·: identical; -: indel.

Polymorphic sites (n = 108), including parsimony informative, singleton sites and gapped or ambiguous characters, detected in the mitochondrial DNA and 16S ribosomal DNA gene sequence alignment of the lymnaeid species compared according to MEGA 5.0

a: AF485657; *b*: EU038315; ·: identical; -: indel**?**.

Rev. Bras. Parasitol. Vet., Jaboticabal, v. 22, n. 1, p. 53-57, jan.-mar. 2013
ISSN 0103-846X (impresso) / ISSN 1984-2961 (eletrônico)

Fasciola hepatica infection and association with gastrointestinal parasites in Creole goats from western Argentina

Fasciola hepatica infecção e associação com parasitas gastrintestinais em caprinos crioulos do oeste da Argentina

Pablo Cuervo[1]; Laura Sidoti[1]; Cecilia Fantozzi[1]; Gisela Neira[1]; Leticia Gerbeno[1]; Roberto Mera y Sierra[1]*

[1]Regional Parasitology Research Center – CIPAR, Faculty of Veterinary Sciences, University Juan A. Maza, Mendoza, Argentina

Received May 3, 2012
Accepted July 2, 2012

Abstract

Goats, called "the cow of the poor", are the livestock species with the most significant population growth worldwide in recent years. Gastrointestinal parasitism constitutes one of the main constraints to its outdoor and extensive breeding in temperate and tropical countries. Despite a Creole goat population of nearly 4 million heads, local reports on parasitological prevalence are scarce, and while *Fasciola hepatica* infection is spread all over Argentina, the goat is usually neglected as a reservoir and economic losses are not considered. To evaluate gastrointestinal parasitism prevalence and associations between parasite genera and species, with emphasis on fascioliasis, Creole goats from the plateau and Andean regions from western Argentina were investigated by coprological techniques, and associations were statistically assessed. Eighty-five percent (85%) of the animals harbored one or more parasite types, while 46% showed mixed infections. Significant positive associations between *F. hepatica* + Strongyle eggs, *Eimeria* sp. + *Nematodirus* sp. and *Nematodirus* sp. + *Trichuris ovis* were detected. Further studies are required to define the causality of these associations and their relevance in epidemiology. *F.hepatica* is rarely considered as goat parasite in the country, but a 33% prevalence poses an interrogation on the role goats play on the transmission and dissemination of this zoonotic trematode.

Keywords: Caprine, fascioliasis, Wertern Argentina, *Eimeria* sp., *Nematodirus*, *Trichuris ovis*.

Resumo

As cabras, nomeadas como "a vaca dos pobres", são as espécies de gado com o crescimento populacional mais significativo nos últimos anos em todo o mundo. O parasitismo gastrintestinal constitui uma das principais limitações à sua criação extensiva em clima temperado e tropical. Na Argentina, apesar de uma população de caprinos crioulos de cerca de quatro milhões de cabeças, são escassos os relatórios locais de prevalências parasitológicas. Embora a infecção por *Fasciola hepatica* esteja espalhada em todo o país, as cabras são geralmente negligenciadas como um reservatório, e as perdas econômicas não são consideradas. Para avaliar a prevalência do parasitismo gastrintestinal e associações entre os gêneros e espécies de parasitos, com ênfase na fasciolose, caprinos crioulos da região andina e do planalto do oeste de Argentina foram avaliados por meio de técnicas coprológicas. Oitenta e cinco por cento dos animais hospedaram um ou mais tipos de parasitos, enquanto 46% hospedaram infecções mistas. Foram encontradas associações significativas entre *F.hepatica* + ovos de estrongilídeos, *Eimeria* sp. + *Nematodirus* sp. e *Nematodirus* sp. + *Trichuris ovis*. Mais estudos são necessários para definir a causalidade dessas associações e sua relevância na epidemiologia. Raramente *F.hepatica* é considerada como um parasito de cabra no país, mas uma prevalência de 33% suscita uma interrogação sobre o papel dos caprinos na transmissão e disseminação desse trematódeo zoonótico.

Palavras-chave: Caprinos, fasciolose, Oeste da Argentina, *Eimeria* sp., *Nematodirus*, *Trichuris ovis*.

Introduction

Goat exploitation is an example of a sustainable production that is fully integrated within the local rural development, being the domestic livestock species with the most significant population growth worldwide in recent years, mainly in developing countries (MORAND-FEHR et al., 2004; BOYAZOGLU et al., 2005). Its current success appears to be related with two characteristics: i) goats are efficient converters of low-quality forages into quality products, and ii) constitute a source of high quality protein; both of them high valuable properties in farming systems with limited resources (LEBBIE, 2004; BOYAZOGLU et al., 2005), such as those present in most developing countries (LEBBIE, 2004). ftese characteristics may explain the frequent labeling of goats as "the

*Corresponding author: Roberto Mera y Sierra
Facultad de Ciencias Veterinarias y Ambientales, Universidad Juan A. Maza, Av. Acceso Este, Lat. Sur, 2245, Mendoza, Argentina
e-mail: cipar.umaza@gmail.com

cow of the poor", highlighting its importance in small farming systems (BOYAZOGLU et al., 2005; HOSTE et al., 2010).

Gastrointestinal parasitism constitutes one of the main constraints to the outdoor and extensive breeding of goats in temperate and tropical countries (HOSTE et al., 2005, 2008; COELHO et al., 2012). For years, it has been considered that data obtained on parasite infections in sheep could be directly transferred to goats (HOSTE et al., 2010), and thus disregarding specific studies on goat parasitism and its consequences. However, in recent years, accumulated information have underlined the fact that gastrointestinal parasitism in goats differed in many aspects to that in sheep (HOSTE et al., 2008), and thus studies in the area are encouraged (SAHLU; GOETSCH, 2005).

fte goat population in Argentina reaches about 4 million heads, mainly distributed in the harsh and dry environments of the central and western regions. fte dominant flocks are of Creole goats, derived from introductions during the Spanish colonial times, and mainly owned by small holders, usually belonging to the most marginalized sector of the population. Animals are raised extensively, grazing rangelands during the day and housed during the nights in rustic corrals near the households (DEGEA et al., 2005).

Fascioliasis, caused in the Americas by the trematode *Fasciola hepatica* (MAS-COMA et al., 2009), produces a serious economic impact (reduction in milk and meat production, liver condemnation, reproductive failure and mortality) in the livestock industry (KAPLAN, 2001; SCHWEIZER et al., 2005). Despite the mentioned economic impact and an emergent, potentially important, goat production in South America (DUBEUF et al., 2004), goat fascioliasis is mainly neglected in the sub-continent. In Argentina, according to official slaughterhouse records, animal fascioliasis covers the whole country (MERA Y SIERRA et al., 2011), but this information only comprises cattle and sheep, and rarely involves goats. Despite goat fascioliasis in the country not being even considered (ROSSANIGO, 2007), there are few, usually opportunistic, reports of infection, but demonstrating high local prevalence rates (AGUIRRE et al., 2005; RUBEL et al., 2005; ISSIA et al., 2009).

fte aim of this study was to determine i) the prevalence rates of gastrointestinal parasites, ii) single and multiple infections, and iii) statistical associations, with special emphasis on liver fluke *F.hepatica*, in Creole goats from the plateau and Andean regions of western Argentina

Materials and Methods

1. *Feces collection and laboratory methods*

Six hundred sixty-three (663) Creole goats born in the plateau and Andean regions of Mendoza (623), San Juan (16) and La Rioja (24) provinces (western Argentina) were surveyed between June 2006 and December 2011. Samples were sent by private practitioners, who requested coproparasitological analysis. Individuals were selected by convenience and fecal samples were directly collected from the rectum. fte collected samples were labeled, refrigerated and transported to the laboratory for examination. Coproparasitological exams were systematically performed by means of three methods: Sheater's flotation technique (SHEATHER, 1923), Ritchie's formol-ether concentration technique (RITCHIE, 1948) and Lumbreras technique (LUMBRERAS et al., 1962). Fecal culture and larvae identification were performed when sufficient material from positive samples was available (NIEC, 1968).

2. *Statistical analysis*

Statistix® for Windows 7.0 and SPSS® for Windows 17.0 software programs were used for statistical analysis, comparison of categorical variables and chi-square test. Chi-square test was applied for comparison between observed and expected prevalence.

Associations between *F.hepatica* and other parasite types were investigated by 2 × 2 contingency tables, from which the chi-square was calculated. First, each parasite type occurrence was compared to each other; and then, *F.hepatica* occurrence was compared with pairs of parasite types (in each threesome, all combinations possible were tested). Due to small *N* and loss of statistical power, no further comparisons were investigated. Values of $P < 0.05$ were taken as significant. Odds Ratio (OR), OR confidence interval (95%) and Relative Risk (RR) were calculated in cases where significant positive association was detected. Considering that, unlike OR, the calculation of RR is influenced by the position of the variable in the 2 × 2 contingency table, both combinations were tested.

Results

Out of the total 663 goats examined, 84.8% (562) were found to host one or more parasite types (as described in Table 1). Among the infected animals, 46% hosted mixed infections. 64.7% of the animals were infected with a protozoan species and 63.49% with at least one helminth species. Two hundred and seventeen (32.9%) of the examined animals (659) were positive for *F.hepatica*, 344 (51.88%) were positive for nematodes, while 422 (64.72%) were positive for *Eimeria* sp. oocysts (Table 1).

Following the few reports available (AGUIRRE et al., 2005; RUBEL et al., 2005; ISSIA et al., 2009), expected prevalence could only be determined for *F.hepatica* infection (94.7%), being the observed prevalence (32.9%) significantly lower than expected ($P<0.05$).

fte prevalence rates of single and multiple parasite type infections are shown in Table 2. Considering presence of parasite types, single presence (38.61%) was the most common, with *Eimeria* sp. as the predominant type (21.27%), followed by double presence (31.82%). fte most frequent combinations were *Eimeria* sp. + *Nematodirus* sp. (15.38%) and *Fasciola hepatica* + *Eimeria* sp. (11.01%).

F.hepatica occurred mainly as a co-infection with another parasite, while only 22.12% (48) of the total cases of fascioliasis (217) occurred as monoparasitism (Table 2). It was most frequently combined with *Eimeria* sp. (11.01%), followed by the duet *F.hepatica* + *Nematodirus* sp. (2.11%), and lastly combined with Strongyle eggs (0.90%).

Table 1. Observed prevalence rates of parasite infection among 663 Creole goats in the plateau and Andean regions, Argentina (overall and per province surveyed).

Parasites	*N*	% Infect. (CI 95)	Mza (%)	S. Juan (%)	La Rioja (%)
All	663	84.8 (82.2-87.3)	623	16	24
Strongyle eggs	652	9 (6.9-11)	42 (6.74)	0	17 (70.83)
Nematodirus sp.	663	40.6 (37.1-44.1)	265 (42.54)	2 (12.5)	2 (8.33)
T. ovis	663	2.3 (1.2-3.4)	15 (5.7)	0	0
F. hepatica	659	32.9 (29.6-36.2)	208 (33.39)	0	9 (37.5)
Eimeria sp.	652	64.7 (61.3-68.1)	414 (66.45)	1 (6.25)	7 (29.17)

% Infect.: % of infection; Mza: Mendoza province; S. Juan: San Juan province.

Table 2. Combination of infecting parasites among 663 Creole goats in the plateau and Andean regions, Argentina (combinations with less than 0.5% are not shown).

Parasites of combination	Nº (%)
Any single type	256 (38.61)
Strongyle eggs	8 (1.21)
Nematodirus sp.	59 (8.90)
F. hepatica	48 (7.24)
Eimeria sp.	141 (21.27)
Double combination	211 (31.82)
F. hepatica + *Eimeria* sp.	73 (11.01)
F. hepatica + Strongyle eggs	6 (0.90)
F. hepatica + *Nematodirus* sp.	14 (2.11)
Eimeria sp. + Strongyle eggs	11 (1.66)
Eimeria sp. + *Nematodirus* sp.	102 (15.38)
Triple combination	77 (11.61)
F. hepatica + *Eimeria* sp. + *Nematodirus* sp.	53 (7.99)
Eimeria sp. + Strongyle eggs + *Nematodirus* sp.	10 (1.51)
Eimeria sp. + *Nematodirus* sp. + *T. ovis*	8 (1.21)
Quadruple combination	17 (2.56)
F. hepatica + *Eimeria* sp. + Strongyle eggs + *Nematodirus* sp.	16 (2.41)

Due to insufficient material (feces) and low eggs per gram (mean 158.4 epg), results from fecal culture for larvae identification were obtained only with the positive samples from La Rioja province. *Trichostrongylus* sp. were identified in every sample cultured (15), while 9 samples were positive to *Haemonchus* sp., 2 to *Oesophagostomum* sp. and 1 to *Ostertagia* sp. fte overall prevalence rates found was: *Trichostrongylus* sp. 95.81%, *Haemonchus* sp. 3.11%, *Oesophagostomum* sp. 0.95% and *Ostertagia* sp. 0.14%.

Chi-square association tests revealed significant positive associations between *F. hepatica* and Strongyle eggs, *Eimeria* sp. and *Nematodirus* sp., and *Nematodirus* sp. and *T. ovis* (Table 3).

When *F. hepatica* occurrence was compared with pairs of parasite types and possible combinations, significant positive associations were found in the following threesomes: *F. hepatica* + *Eimeria* sp. + *Nematodirus* sp., *F. hepatica* + *Eimeria* sp. + Strongyle eggs, and *F. hepatica* + Strongyle eggs + *Nematodirus* sp. (See Table 3 for details).

Discussion

To the authors' knowledge, this study is the first to report precise goat parasites prevalence rates in western Argentina. As usual, and in accordance with previous world tendencies, goat parasitism in the country has been frequently disregarded, and such valuable information not informed due to general disinterest. Most of the available local studies only informed about seasonality of gastrointestinal parasites fecal counts and treatment results, but gave no information about local prevalence that allowed further epidemiological comparison.

ftis study reveals a high overall prevalence of gastrointestinal parasites, reaching almost 85% - maximum for *Eimeria* sp. (64.7%) and minimum for *Trichuris ovis* (2.3%). Meanwhile, the high prevalence (32.9%) of *F. hepatica* is remarkable.

Due to the mentioned scarcity of local data regarding prevalence of goat gastrointestinal parasitism, observed and expected values could only be compared in the case of infection by *F. hepatica*, which was significantly lower than expected ($P < 0.05$) (Table 1). In the few national reports where *F. hepatica* infection in goats was described (AGUIRRE et al., 2005; RUBEL et al., 2005; ISSIA et al., 2009), studies were developed in response to outbreaks, sample numbers were small, and individuals sampled belonged to the same herd, thus explaining the very high local prevalence and the consequent difference between observed and expected values.

fte status of polyparasitism using coproscopy as the method indicated that almost half (46.1%) of these animals harbored 2-5 different parasite eggs. Despite the numerous parasite combinations observed (Table 2), significant positive associations ($P < 0.05$) were detected only between *F. hepatica* + Strongyle eggs, *Eimeria* sp. + *Nematodirus* sp. and *Nematodirus* sp. + *T. ovis* (Table 3).

In the first case, *F. hepatica* + Strongyle eggs, the association could be non-causal (confounding factor), possibly due to favorable environmental characteristics for the development of larvae and infection in the same places where infection with *F. hepatica* occurs (e.g. swamps and waterlogged areas). On the other hand, the OR and RR analysis suggests that infection by *F. hepatica* may act as a contributing factor for Strongyle infection, enhancing almost twice (1.96 and 1.83 respectively) the odds, probably due to the host immunosuppression attributed to the trematode (CERVI et al., 1996; SIDOTI, 2011) or just due to a more vulnerable host (a host's weakened state) (BEGON et al, 2006). Considering that most of the larvae identified belonged to the family Trichostrongylidae, association could also be attributed to the arrested larvae released by the suggested immunosuppression (ANDERSON, 2000).

Table 3. Associations between gastrointestinal parasite among 663 Creole goats in the plateau and Andean regions, Argentina.

Parasite sp.	χ^2	*P*-value	OR (CI 95%)
*F. hepatica** Strongyle eggs	6.11	0.0134	1.96 (1.14-3.36)
Eimeria sp.* *Nematodirus* sp.	7.91	0.0049	1.61 (1.15-2.25)
Nematodirus sp.* *T. ovis*	9.89	0.0017	6.09 (1.70-21.78)
Eimeria sp.* *F. hepatica* + *Nematodirus* sp.	12.54	0.0004	2.70 (1.53-4.78)
Nematodirus sp.* *F. hepatica* + *Eimeria* sp.	4.68	0.0306	1.50 (1.04-2.17)
*F. hepatica** *Eimeria* sp. + Strongyle eggs	4.38	0.0363	1.94 (1.03-3.63)
Strongyle eggs* *F. hepatica* + *Eimeria* sp.	4.79	0.0287	1.88 (1.06-3.34)
*F. hepatica** Strongyle eggs + *Nematodirus* sp.	13.2	0.0003	3.76 (1.76-8.05)
Strongyle eggs* *F. hepatica* + *Nematodirus* sp.	19.9	0.0000	3.67 (2.01-6.69)
Nematodirus sp.* *F. hepatica* + Strongyle eggs	9.03	0.0027	3.25 (1.45-7.30)

* Significant associations.

The significant association observed between *Eimeria* sp. + *Nematodirus* sp. and *Nematodirus* sp. + *T. ovis* could be a consequence of infective stages (oocysts and eggs) resistant to extreme environmental conditions or related to housing conditions, with animals returning to corrals every day and manure being accumulated during months.

When complex associations were evaluated, significant positive associations were detected in three sets of combinations: *F. hepatica* + *Eimeria* sp. + *Nematodirus* sp., *F. hepatica* + *Eimeria* sp. + Strongyle eggs, and *F. hepatica* + Strongyle eggs + *Nematodirus* sp. (Table 3). In the first two threesomes, the association observed is attributed to the significant associations detected when analyzing by pairs (*Eimeria* sp. + *Nematodirus* sp. and *F. hepatica* + Strongyle eggs), even more when the third parasite type (*F. hepatica* and *Eimeria* sp., respectively) is tested against the associated pair and no significant association is detected. It is worth noting the last threesome, where, following the previous criteria, the presence of eggs of *Nematodirus* sp. is significantly associated with the combination *F. hepatica* + Strongyle eggs ($\chi^2 = 9.03$, $P = 0.0027$).

Further studies are required to define whether these associations are causal or not, and their relevance in the epidemiology of the parasite types implicated. Future studies should also analyze the economic losses generated by goat gastrointestinal multiparasitism, especially considering its impact on local extensive small farming systems.

As highlighted before, *F. hepatica* is rarely considered as a parasite of goats in the country. Consequently, not only the recognized economic losses are not analyzed, but also this ruminant is neglected as an important reservoir of this zoonosis. Furthermore, the stunning 33% prevalence detected poses an interrogation and alerts about the role goats play on the transmission and dissemination of this zoonotic trematode.

Acknowledgements

ftis research was funded by "Área de Ciencia y Técnica, Universidad Juan A. Maza", Mendoza, Argentina.

References

Aguirre DH, Cafrune MM, Salatin AO, Abeyá AA. Fasciolosis clínica en cabras de Metán (Salta, Argentina). *Rev FAVE – Cienc Vet* 2005; 4(1-2): 17-21.

Anderson RC. *Nematode parasites of vertebrates:* fteir development and transmission. 2nd ed. Wallingford: CAB International; 2000. http://dx.doi.org/10.1079/9780851994215.0000

Begon M, Townsend CR, Harper JL. *Ecology:* from individuals to ecosystems. 4th ed. Oxford: Blackwell Publishing; 2006.

Boyazoglu J, Hatziminaoglou I, Morand-Fehr P. The role of the goat in society: past, present and perspectives for the future. *Small Ruminant Res* 2005; 60(1-2): 13-23. http://dx.doi.org/10.1016/j.smallrumres.2005.06.003

Cervi L, Rubinstein H, Masih DT. Involvement of excretion-secretion products from *Fasciola hepatica* inducing suppression of the cellular immune responses. *Vet Parasitol* 1996; 61(1-2): 97-111. http://dx.doi.org/10.1016/0304-4017(95)00816-0

Coelho WM, Amarante AF, Bresciani KD. Ocurrence of gastrointestinal parasites in goat kids. *Rev Bras Parasitol Vet* 2012; 21(1): 65-67. PMid:22534948. http://dx.doi.org/10.1590/S1984-29612012000100013

De Gea G, Petryna AM, Mellano A, Bonvillani A, Turiello P. *El ganado caprino en la Argentina:* Antecedentes para su estudio. Córdoba: Universidad Nacional de Río Cuarto; 2005.

Dubeuf JP, Morand-Fehr P, Rubino R. Situation, changes, and future of goat industry around the world. *Small Ruminant Res* 2004; 51(2): 165-173. http://dx.doi.org/10.1016/j.smallrumres.2003.08.007

Hoste H, Torres-Acosta JF, Paolini V, Aguilar-Caballero A, Etter E, Lefrileux Y, et al. Interactions between nutrition and gastrointestinal infections with parasitic nematodes in goats. *Small Ruminant Res* 2005; 60(1-2): 141-151. http://dx.doi.org/10.1016/j.smallrumres.2005.06.008

Hoste H, Torres-Acosta JF, Aguilar-Caballero AJ. Nutrition-parasite interactions in goats: is immunoregulation involved in the control of gastrointestinal nematodes? *Parasite Immunol* 2008; 30(2): 79-88. PMid:18186768.

Hoste H, Sotiraki S, Yan Landau S, Jackson F, Beveridge I. Goat-nematode interactions: think differently. *Trends Parasitol* 2010; 26(8): 376-381. PMid:20488752. http://dx.doi.org/10.1016/j.pt.2010.04.007

Issia L, Pietrokovsky S, Sousa-Figueiredo J, Russell Stothard J, Wisnivesky-Colli C. *Fasciola hepatica* infection in livestock flocks, guanacos and coypus in two wildlife reserves in Argentina. *Vet Parasitol* 2009; 165(3-4): 341-344. PMid:19700244. http://dx.doi.org/10.1016/j.vetpar.2009.07.011

Kaplan RM. *Fasciola hepatica*: a review of the economic impact in cattle and considerations for control. *Vet Ther* 2001; 2(1): 40-50. PMid:19753697.

Lebbie SHB. Goats under household conditions. *Small Ruminant Res* 2004; 51(2): 131-136. http://dx.doi.org/10.1016/j.smallrumres.2003.08.015

Lumbreras H, Cantella R, Burga R. Acerca de un procedimiento de sedimentación rápida para investigar huevos de *Fasciola hepatica* en las heces, su evaluación y uso en el campo. *Rev Med Peru* 1962; 31(332): 167-174.

Mas-Coma S, Valero MA, Bargues MD. Chapter 2. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Adv Parasitol* 2009; 69: 41-146. http://dx.doi.org/10.1016/S0065-308X(09)69002-3

Mera y Sierra R, Agramunt VH, Cuervo P, Mas-Coma S. Human fascioliasis in Argentina: retrospective overview, critical analysis and baseline for future research. *Parasite Vectors* 2011; 4: 104. PMid:21663691 PMCid:3141741. http://dx.doi.org/10.1186/1756-3305-4-104

Morand-Fehr P, Boutonnet JP, Devendra C, Dubeuf JP, Haenlein GFW, Holst P, et al. Strategy for goat farming in the 21st century. *Small Ruminant Res* 2004; 51(2): 175-183. http://dx.doi.org/10.1016/j.smallrumres.2003.08.013

Niec R. *Cultivo e identificación de larvas infectantes de nemátodes gastrointestinales del bovino y ovino*. Buenos Aires: INTA; 1968. Manual técnico n. 3.

Ritchie LS. An ether sedimentation technique for routine stool examination. *Bull US Army Med Dep* 1948; 8(4): 326.

Rossanigo CE. Parasitosis de las cabras. In: Suárez VH, Olaechea FV, Rossanigo CE, Romero JR. *Enfermedades parasitarias de los ovinos y otros rumiantes menores en el cono sur de América*. EEA Anguil, INTA; 2007. p. 247-270.

Rubel D, Prepelitchi L, Kleiman F, Carnevale S, Wisnivesky-Colli C. Estudio del foco en un caso de fasciolosis humana en Neuquén. *Medicina* 2005; 65(3): 207-212.

Sahlu T, Goetsch AL. A foresight in goat research. *Small Ruminant Res* 2005; 60(1-2): 7-12. http://dx.doi.org/10.1016/j.smallrumres.2005.06.002

Schweizer G, Braun U, Deplazes P, Torgerson PS. Estimating the financial losses due to bovine fasciolosis in Switzerland. *Vet Rec* 2005; 157(7): 188-193. PMid:16100368.

Sheather AL. fte detection of intestinal protozoa and mange parasites by a flotation technique. *J Comp Pathol* 1923; 36: 266-275. http://dx.doi.org/10.1016/S0368-1742(23)80052-2

Sidoti LE. *Diagnóstico de Fascioliasis bovina mediante coprología y serología, y relación con parámetros hematológicos y bioquímicos* [ftesis]. Mendoza: Facultad de Ciencias Veterinarias y Ambientales, Universidad Juan A. Maza; 2011.

a otras zonas endémicas del mundo, no concuerda debido a que siempre se encuentra una correlación positiva con el factor pluviosidad. Esto puede deberse a que el caudal de agua en los cauces de zonas de montaña en Mendoza no depende de la precipitación puntual de la zona en que se encuentra sino de las precipitaciones que ocurren en alta montaña, separado generalmente a cientos de kilómetros de las áreas endémicas e incluso separados por meses del momento en que ocurre la precipitación y el momento que se ve favorecido el caudal debido al derretimiento de la nieve en primavera y verano. Pueden ser factores micro o nano meteorológicos los determinantes principales para la supervivencia de los hospedadores intermediarios y las formas libres del parásito.

Tabla 1: Análisis de Pearson entre prevalencia de *F. hepatica* y variables climáticas

	T MIN	T MAX	T MED	PRECIP
Sur	-0.302	-0.327	-0.316	0.439
Noreste	0.399	0.355	0.382	0.462
Valle de Uco	0.880	0.873	0.876	-0.876

Bibliografía

1. Van Dijk J, Sargison ND, Kenyon F, Skuce PJ. Climate change and infectious disease: helminthological challenges to farmed ruminants in temperate regions. Animal 2010; 4(3):377–92.
2. Rossanigo CE Actualización sobre las parasitosis del ganado caprino. Vet Arg 2003; 20(193):188-204 .
3. Levine ND, Todd Jr. KS (1975) Micrometeorological Factors Involved in Development and Survival of Free-Living Stages of the Sheep Nematodes *Haemonchus contortus* and *Trichostrongylus columbriformis*. A Review. Int J Biometeo 1975; 19(3):174-183.
4. Mera y Sierra R, Artigas P, Cuervo P, Deis E, Sidoti L, Mas-Coma S, Bargues MD. Fascioliasis transmission by *Lymnaea neotropica* confirmed by nuclear rDNA and mtDNA sequencing in Argentina. Vet Parasitol 2009; 166: 73-79.

Palabras clave: *Fasciola hepatica*, cabras, clima.

(I) Centro de Investigación en Parasitología Regional (CIPAR), Universidad Juan Agustín Maza. Mendoza, Argentina.
cipar.umaza@gmail.com

Fasciola hepatica en guanacos y llamas en un establecimiento de Malargüe, provincia de Mendoza

Fasciola hepatica **in guanacos and llamas in a farm of Malargüe, Mendoza province**

Roberto Mera y Sierra[1,2], Fernando Cantero[3] , Mariana González[1]

Fasciola hepatica es un trematodo que afecta animales herbívoros y omnívoros, no sólo domésticos, sino también silvestres; es además un serio problema de salud pública con millones de personas afectadas a nivel mundial con las mayores prevalencias en zonas andinas. Ingresó a América desde Europa durante la colonización, siendo descripta por primera vez en Argentina, en ovinos, en 1867. *F. hepatica* está distribuida en todo el país exceptuando el sur de la Patagonia. En la provincia de Mendoza se han reportado muy altas prevalencias del parásito en el ganado domestico y hay 27 casos humanos publicados. En Mendoza se han identificado tres especies de lymnaeidos: *Lymnaea viatrix*, *Lymnaea neotropica* y *Galba truncatula.* El guanaco (*Lama gaunicoe*) el mayor de lo camélidos sudamericanos de Argentina, tiene una distribución actual en las zonas de estepa cordilleranas y precordilleranas del país, con una población de 550 000 animales aproximadamente. En la provincia de Mendoza la presencia de estos camélidos abarca toda la zona de la cordillera, en poblaciones en áreas protegidas, reservas provinciales y privadas. La población de estos animales es de aproximadamente 16 000 animales. En establecimientos productivos particulares se pueden encontrar debido a la extensión de los mismos a las zonas de cordillera. Existen diversos proyectos en Argentina orientados al uso sustentable de esta especie, particularmente a la explotación de la fibra. En cuanto a la llama (*Lama glama*), existen muy pocos ejemplares en la provincia de Mendoza, no existen establecimientos dedicados a la producción de llamas y los escasos ejemplares cumplen un rol más bien ornamental o de colecciones privadas. Se han reportado casos de *F. hepatica* en llamas y son escasos los reportes en guanacos, tanto en animales en semicautividad como en guanacos silvestres. Se ha reportado en guanacos también la presencia de otros parásitos gastrointestinales (*Nematodirus sp, Eimeria sp, Marshallagia, Trichuris sp*). La fascioliasis puede comprometer la salud de los camélidos, y los mismos pueden ser un potencial reservorio para el ganado domestico, y los humanos. *F. hepatica* afecta el potencial productivo de los animales. En camélidos sudamericanos, dicho potencial se ha estudiado ampliamente, pudiendo usarse las especies para la producción de lana de fibra fina, cuero y carne, con un alto valor socio-económico para los pobladores rurales. El objetivo del presente trabajo es la de describir la presentación de un caso de fascioliasis en dos especies de camélidos sudamericanos en estado de cautividad.

Materiales y Métodos

Se presenta un estudio de tipo descriptivo transversal ante la presencia de un cuadro clínico de diarrea y muerte de un guanaco en un establecimiento que se dedica al manejo con la intención de reintroducción de guanacos en determinadas áreas del departamento de Malargüe, provincia de Mendoza. Se tomaron y remitieron muestras de materia fecal de seis camélidos sudamericanos, dos llamas (*Lama glama*) y cuatro guanacos (*L. guanicoe*) de una población de 38 guanacos de un año de edad y 6 llamas adultas. Los animales se encuentran bajo un sistema de cría a base de pastizal natural, alfalfa y abrevan en un cauce artificial de agua. Las muestras se remitieron refrigeradas al laboratorio donde se procesaron mediante las técnicas de flotación simple, sedimentación rápida de Lumbreras, y se cuantificó la carga parasitaria, expresándose en huevos por gramo (HPG) u ooquistes por gramo (opg). También se llevó a cabo la necropsia del animal muerto y se remitieron muestras para histopatología.

Resultados

En el guanaco que se murió, el estudio coprológico fue positivo para huevos de *F. hepatica*, hallándose una carga de 1HPG y también ooquistes de *Eimeria* spp (4 opg). Al estudio histopatológico se observó hepatosis leve, peritonitis supurativa, inflamación supurativa del bazo y congestión venosa renal. Los restantes tres guanacos resultaron negativos a los estudios parasitológicos. Ambas llamas presentaron huevos de *F. hepatica* con una carga de 6 y 38 HPG respectivamente, y un animal fue positivo a *Nematodirus* con una carga de *2* HPG.

Discusión

A pesar de que no se puede atribuir la muerte del guanaco a la presencia de *F. hepatica*, la fascioliasis ha sido descripta como causa de enfermedad grave y muerte en los demás camélidos sudamericanos (vicuña, llama y alpaca). El otro parásito hallado, (*Eimeria* spp) puede producir cuadros graves pero generalmente se observan muy altas cargas parasitarias que suelen evidenciarse por el alto conteo de opg. El hallazgo de *F. hepatica* es un llamado de atención al rol patógeno que puede estar cumpliendo este trematódo en el guanaco. Si tenemos en cuenta las muy altas prevalencias de fascioliasis en animales domésticos de la región y el impacto que producen sobre su productividad, esta parasitosis también pude ser un factor determinante en la conservación y uso sustentable de este camélido autóctono. El coprológico positivo para *F. hepatica* en dos de dos llamas estudiadas es también llamativo y alerta no solo sobre el efecto que puede tener este parasito sobre las llamas sino también su potencial rol como reservorio para otros animales silvestres y domésticos . Es llamativa la ausencia de cauces de agua naturales o surgentes, donde el hospedador intermediario de *F. hepatica* suele encontrarse. Puede suponerse que los lymnaeidos vectores se han adaptado a los cauces de riego artificiales, lo cual demuestra su adaptabilidad a diversos ambientes. Estos hallazgos ameritan que se continúen los estudios en camélidos, particularmente en guanacos, para dilcidar, cuál es el efecto que tiene *F. hepatica* sobre su salud y como afecta su producción. Esta información es fundamental para poder llevar a cabo proyectos de conservación y de uso sustentable de esta especie.

Bibliografía

1. Baldomenico PM, Uhart M, Bono MF, Marull C, Baldi R, Peralta JL. Internal parasites of free-ranging guanacos from Patagonia. Vet Parasitol 2003; 18:71–77
2. Cuervo P, Mera y Sierra R, Deis E, Zarco A. La introducción de *Fasciola hepatica* en América: ¿un posible riesgo para la conservación de especies en peligro de extinción de Argentina? III Congreso Nacional de Conservación de la Biodiversidad. Buenos Aires Argentina.11 a 14 de Agosto 2008. pp. 123
3. Issia L, Pietrokovsky S, Sousa-Figueiredo J, Stothard JR, Wisnivesky-Colli C. *Fasciola hepatica* infections in livestock flock, guanacos and coypus in two wildlife reserves in Argentina. Vet Parasitol. 2009 Nov 12;165(3-4):341-4
4. Olaechea, F.V, Abad, M. An outbreak of fascioliasis in semi-captive guanacos (Lama guanicoe) in Patagonia (Argentina). First report. XX international Conference, World Association for the Advancement of Veterinary Parasitology. Christchurch, New Zeland. 17 al 20 de Octubre 2005.

Palabras clave: Camélidos sudamericanos, *Fasciola hepatica*, Mendoza.

(1) Centro de Investigación en Parasitología Regional (CIPAR), Universidad Juan Agustín Maza. Mendoza, Argentina. *cipar.umaza@gmail.com.* (2) Area Parasitología, Departamento de Patología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo. Mendoza. Argentina. (3) Veterinaria Anay, Mendoza, Argentina. *vetanay@yahoo.com.ar*

Factores climáticos y su relación con la presencia de *Fasciola hepatica* en caprinos de las distintas regiones productivas de la provincia de Mendoza, Argentina

Climatic factors and their relation to the presence of *Fasciola hepatica* in goats from different productive regions of the province of Mendoza, Argentina

Sophia Di Cataldo[1], Noelia Sohaefer[1], Pablo Cuervo[1], Mariana González[1], Gisela Neira[1], Laura Sidoti[1], Roberto Mera y Sierra[1]

La producción caprina suele asociarse con áreas degradadas, poca disponibilidad de alimento y escaso manejo y desarrollo tecnológico. En la provincia de Mendoza los caprinos son el ganado más abundante y con 776 169 cabezas, es la segunda provincia en el país. En la misma, se reconocen tres regiones productivas caprinas: Noreste, Valle de Uco, y Zona Sur. Además de la escasez de nutrientes, se considera que los parásitos del aparato digestivo son una de las principales causas de disminución de la productividad. La fascioliasis es una enfermedad producida en el continente americano por el trematodo *Fasciola hepatica* y es el causante de considerables pérdidas económicas en el ganado. Es además, una importante zoonosis en franca expansión a nivel mundial. La mayor cantidad de casos humanos se concentran en zonas andinas. Los factores climáticos, tal como la temperatura y las precipitaciones, son determinantes de la persistencia del parásito en el ambiente y particularmente de su hospedador intermediario, moluscos gasterópodos acuáticos de la familia Lymnaeidae. Es por esto que, aumentos o disminuciones leves de temperatura, pueden llegar a alterar el desarrollo, transmisión o subsistencia de éste trematodo y de los lymnaeidos. En la provincia de Mendoza, se ha detectado la presencia de lymnaeidos vectores previamente no reportados para la región: *Galba truncatula* y *Lymnaea neotropica*. El objetivo de este trabajo fue determinar la prevalencia de *F. hepatica* en el ganado caprino de las regiones productivas de Mendoza, y correlacionarla con las precipitaciones y temperaturas.

Materiales y métodos

Se trata de un diseño descriptivo correlacional. Se tomaron, entre los años 2006 al 2013, muestras de materia fecal del recto de 1141 caprinos de 36 establecimientos de Mendoza (10 en el Noreste, 6 en el Valle de Uco, y 20 en el Sur). Para el muestreo se incluyeron sólo individuos adultos. Se realizó la técnica de sedimentación rápida de Lumbreras, considerándose positivos los animales en que se observaron huevos de *F. hepatica*. Los datos de precipitación, temperatura máxima y mínima fueron obtenidos de la base de datos WorldClim. Se determinó la correlación entre las variables climáticas y las prevalencias parasitarias mediante el coeficiente de Pearson y se compararon las prevalencias mediante la prueba de Chi^2.

Resultados

En la región Noreste se hallaron huevos de *F. hepatica* en 9 de 184 animales (4.9%, IC 1.8-8); en la región Valle de Uco en 99 de 303 animales (32.7% IC 27.4-38); y en la región Sur en 136 de 574 animales (23.7%, IC 20.2-27.2). Las diferencias entre las prevalencias de las distintas regiones fue significativa ($p<0.01$) según la prueba de de Chi^2. El análisis de Pearson para la correlación entre las variables climáticas y la prevalencia de *F. hepatica*, determinó: Región Sur: Temperatura mínima -0.302, Temperatura máxima -0.327, Temperatura media -0.316, Precipitaciones 0.439; Región de Valle de Uco: Temperatura mínima 0.880, Temperatura máxima 0.873, Temperatura media 0.876, Precipitaciones -0.876; Región Noreste: Temperatura mínima 0.399, Temperatura máxima 0.355, Temperatura media 0.382, Precipitaciones 0.462.

Discusión

La menor prevalencia de *F. hepatica* en la región Noreste, puede deberse a las escasas y transitorias fuentes naturales de agua, lo cual dificulta la supervivencia de los lymnaeidos hospedadores intermediarios y las formas libres del parásito. Además, los cauces naturales se ven prácticamente anulados debido a la extracción del agua para riego del oasis río arriba. Regiones como el Valle de Uco y los departamentos del Sur, presentan ambientes más húmedos, siendo más propicios para el desarrollo del caracol intermediario y generando prevalencias más altas. En cuanto a su relación con factores climáticos, pareciera haber una leve correlación con las precipitaciones, para las regiones Sur y Noreste; esto podría deberse a que un aumento en las lluvias podría modificar los cauces de agua en donde habita el caracol, favoreciendo su desarrollo y permanencia. A su vez, la correlación negativa para la región de Valle de Uco correspondería a que al tratarse de una zona de grandes pendientes, podría generar un efecto de arrastre del caracol intermediario. Sin embargo, se puede observar claramente resultados discordantes, desde correlaciones netamente negativas como el caso de precipitación en el Valle de Uco siendo la misma positiva en la zona Sur y Noreste. Comparado

a otras zonas endémicas del mundo, no concuerda debido a que siempre se encuentra una correlación positiva con el factor pluviosidad. Esto puede deberse a que el caudal de agua en los cauces de zonas de montaña en Mendoza no depende de la precipitación puntual de la zona en que se encuentra sino de las precipitaciones que ocurren en alta montaña, separado generalmente a cientos de kilómetros de las áreas endémicas e incluso separados por meses del momento en que ocurre la precipitación y el momento que se ve favorecido el caudal debido al derretimiento de la nieve en primavera y verano. Pueden ser factores micro o nano meteorológicos los determinantes principales para la supervivencia de los hospedadores intermediarios y las formas libres del parásito.

Tabla 1: Análisis de Pearson entre prevalencia de *F. hepatica* y variables climáticas

	T MIN	T MAX	T MED	PRECIP
Sur	-0.302	-0.327	-0.316	0.439
Noreste	0.399	0.355	0.382	0.462
Valle de Uco	0.880	0.873	0.876	-0.876

Bibliografía

1. Van Dijk J, Sargison ND, Kenyon F, Skuce PJ. Climate change and infectious disease: helminthological challenges to farmed ruminants in temperate regions. Animal 2010; 4(3):377–92.
2. Rossanigo CE Actualización sobre las parasitosis del ganado caprino. Vet Arg 2003; 20(193):188-204 .
3. Levine ND, Todd Jr. KS (1975) Micrometeorological Factors Involved in Development and Survival of Free-Living Stages of the Sheep Nematodes *Haemonchus contortus* and *Trichostrongylus columbriformis*. A Review. Int J Biometeo 1975; 19(3):174-183.
4. Mera y Sierra R, Artigas P, Cuervo P, Deis E, Sidoti L, Mas-Coma S, Bargues MD. Fascioliasis transmission by *Lymnaea neotropica* confirmed by nuclear rDNA and mtDNA sequencing in Argentina. Vet Parasitol 2009; 166: 73-79.

Palabras clave: *Fasciola hepatica*, cabras, clima.

(1) Centro de Investigación en Parasitología Regional (CIPAR), Universidad Juan Agustín Maza. Mendoza, Argentina.
cipar.umaza@gmail.com

Fasciola hepatica en guanacos y llamas en un establecimiento de Malargüe, provincia de Mendoza

Fasciola hepatica **in guanacos and llamas in a farm of Malargüe, Mendoza province**

Roberto Mera y Sierra[1,2], Fernando Cantero[3] , Mariana González[1]

Fasciola hepatica es un trematodo que afecta animales herbívoros y omnívoros, no sólo domésticos, sino también silvestres; es además un serio problema de salud pública con millones de personas afectadas a nivel mundial con las mayores prevalencias en zonas andinas. Ingresó a América desde Europa durante la colonización, siendo descripta por primera vez en Argentina, en ovinos, en 1867. *F. hepatica* está distribuida en todo el país exceptuando el sur de la Patagonia. En la provincia de Mendoza se han reportado muy altas prevalencias del parásito en el ganado domestico y hay 27 casos humanos publicados. En Mendoza se han identificado tres especies de lymnaeidos: *Lymnaea viatrix*, *Lymnaea neotropica* y *Galba truncatula.* El guanaco (*Lama gaunicoe*) el mayor de lo camélidos sudamericanos de Argentina, tiene una distribución actual en las zonas de estepa cordilleranas y precordilleranas del país, con una población de 550 000 animales aproximadamente. En la provincia de Mendoza la presencia de estos camélidos abarca toda la zona de la cordillera, en poblaciones en áreas protegidas, reservas provinciales y privadas. La población de estos animales es de aproximadamente 16 000 animales. En establecimientos productivos particulares se pueden encontrar debido a la extensión de los mismos a las zonas de cordillera. Existen diversos proyectos en Argentina orientados al uso sustentable de esta especie, particularmente a la explotación de la fibra. En cuanto a la llama (*Lama glama*), existen muy pocos ejemplares en la provincia de Mendoza, no existen establecimientos dedicados a la producción de llamas y los escasos ejemplares cumplen un rol más bien ornamental o de colecciones privadas. Se han reportado casos de *F. hepatica* en llamas y son escasos los reportes en guanacos, tanto en animales en semicautividad como en guanacos silvestres. Se ha reportado en guanacos también la presencia de otros parásitos gastrointestinales (*Nematodirus sp, Eimeria sp, Marshallagia, Trichuris sp*). La fascioliasis puede comprometer la salud de los camélidos, y los mismos pueden ser un potencial reservorio para el ganado domestico, y los humanos. *F. hepatica* afecta el potencial productivo de los animales. En camélidos sudamericanos, dicho potencial se ha estudiado ampliamente, pudiendo usarse las especies para la producción de lana de fibra fina, cuero y carne, con un alto valor socio-económico para los pobladores rurales. El objetivo del presente trabajo es la de describir la presentación de un caso de fascioliasis en dos especies de camélidos sudamericanos en estado de cautividad.

DOI: 10.1515/ap-2015-00

Acta Parasitologica, 2015, 60(4), 000–000; ISSN 1230-2821

DE GRUYTER

Liver fluke (*Fasciola hepatica*) naturally infecting introduced European brown hare (*Lepus europaeus*) in northern Patagonia: phenotype, prevalence and potential risk

Pablo F. Cuervo[1]*, Sophia Di Cataldo[1], M. Cecilia Fantozzi[1], Erika Deis[1], Gabriela Díaz[2], Gabriela Viberti[3], Patricio Artigas[4], Raquel Peixoto[4], M. Adela Valero[4], Roberto Mera y Sierra[1] and Santiago Mas-Coma[4]

[1]Centro de Investigación en Parasitología Regional, Facultad de Ciencias Veterinarias y Ambientales, Universidad Juan A. Maza. Av. Acceso Este, Lat. Sur 2245, Guaymallén, Mendoza, Argentina. Postal Code 5519; [2]Instituto de Ciencias Básicas. Universidad Nacional de Cuyo. Campus Educativo Muncipal Malargüe. International Center for Earth Sciences. Malargüe, Mendoza, Argentina; [3]Instituto Superior del Profesorado San Pedro Nolasco, Universidad del Aconcagua, Mendoza, Argentina; [4]Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia. Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain

Abstract

Fascioliasis has recently been included in the WHO list of Neglected Zoonotic Diseases. Besides being a major veterinary health problem, fascioliasis has large underdeveloping effects on the human communities affected. Though scarcely considered in fascioliasis epidemiology, it is well recognized that both native and introduced wildlife species may play a significant role as reservoirs of the disease. The objectives are to study the morphological characteristics of *Fasciola hepatica* adults and eggs in a population of *Lepus europaeus*, to assess liver fluke prevalence, and to analyze the potential reservoir role of the European brown hare in northern Patagonia, Argentina, where fascioliasis is endemic. Measures of *F. hepatica* found in *L. europaeus* from northern Patagonia demonstrate that the liver fluke is able to fully develop in wild hares and to shed normal eggs through their faeces. Egg shedding to the environment is close to the lower limit obtained for pigs, a domestic animal whose epidemiological importance in endemic areas has already been highlighted. The former, combined with the high prevalence found (14.28%), suggest an even more important role in the transmission cycle than previously considered. The results obtained do not only remark the extraordinary plasticity and adaptability of this trematode species to different host species, but also highlight the role of the European brown hare, and other NIS, as reservoirs capable for parasite spillback to domestic and native cycle, representing a potentially important, but hitherto neglected, cause of disease emergence.

Keywords

Fasciola hepatica, *Lepus europaeus*, introduced species, reservoir

Introduction

Fascioliasis, traditionally considered as a veterinary health problem (Kaplan 2001), has recently been included in the World Health Organization list of Neglected Zoonotic Diseases (NZDs). This consideration is due to its emergence and re-emergence worldwide, affecting an estimated 17 million people (Mas-Coma *et al.* 2009), in a phenomenon which has partly been related to climate change (Mas-Coma *et al.* 2008; Afshan *et al.* 2014), and to the long-term pathogenic impact of this disease (Mas-Coma *et al.* 2014a). True human endemic areas have recently been described in which fascioliasis chronicity and superimposed repetitive infections pose pathological complications, indicating this disease to have large underdeveloping effects on the human communities affected (Valero *et al.* 2003, 2006a, 2008).

Fasciolid flukes follow a two-host life cycle, including a less specific adult stage which develops in many species of

*Corresponding author: pablofcuervo@gmail.com

herbivorous mammals and even in a few omnivorous ones, and highly specific larval stages which only develop in given freshwater snail species of the family Lymnaeidae (Bargues and Mas-Coma 2005). With regard to the infection of animal reservoirs, the infectivity of the metacercarial infective stage from different animal species isolates has experimentally shown to be similar (Valero and Mas-Coma 2000; Valero *et al.* 2001a, 2011). Hence, the importance of ascertaining which animal species, including both domestic and sylvatic, develop a reservoir role in an endemic area.

Argentina presents a very widely distributed veterinary problem of fascioliasis in livestock (Olaechea 2007). Additionally, a recent analysis highlights that human fascioliasis in the country may have been overlooked in the past and its real epidemiological situation may currently be underestimated (Mera y Sierra *et al.* 2011). Surprisingly, geographical distribution of human infection does not fit that of fascioliasis in livestock, suggesting other transmission and epidemiological factors to be involved (Mera y Sierra *et al.* 2011).

Though scarcely considered, it is known that wildlife species may play a significant role as reservoirs of fascioliasis (Mas-Coma *et al.* 1988; Daszak *et al.* 2000; Bengis *et al.* 2004; Kruse *et al.* 2004; Polley 2005; Gayo *et al.* 2011; Mezo *et al.* 2013). Introduced non-indigenous species (NIS) are widely recognized as a source of disease (Daszak *et al.* 2000; Kelly *et al.* 2009). The importance and consequences of the introduction of NIS in fascioliasis has been the subject of several analyses, concerning both lymnaeids (Mas-Coma *et al.* 2003, 2005, 2009; Bargues and Mas-Coma 2005) and animal reservoirs (Mas-Coma *et al.* 2009).

Introduced into South America at the end of 19th century, the European brown hare (*Lepus europaeus*) represents one of the most widespread species of mammals (Bonino *et al.* 2010). The species has invaded almost all the extension of Argentina, Chile and Uruguay, and southern regions of Peru, Bolivia, Paraguay, and Brazil (Bonino *et al.* 2010). Despite old reports of *F. hepatica* in lagomorphs in general (Arru *et al.* 1967) and especifically infecting the hare in its original home range (Tropilo 1964; Kutzer and Frey 1976; Nickel and Gottwald 1979; Shimalov 2001; Ziege *et al.* 2009; Walker *et al.* 2011), the latter has been rarely considered in the epidemiology of the disease, particularly with regard to South American introduced populations. Additionally, phenotypic descriptions of adults and eggs of *F. hepatica* infecting natural populations of *L. europaeus* are lacking in the Neotropical region and even scarce worldwide.

The aims of the present article are to study the morphological characteristics of parasite adults and eggs in a population of *L. europaeus* in the northernmost part of Patagonia region (Argentina), to assess liver fluke prevalence and to analyze the potential reservoir role of this wild lagomorph in an area where fascioliasis is known to occur in livestock (Sidoti *et al.* 2009).

Materials and Methods

Host materials

Specimens of the European brown hare were obtained from the outskirts of Malargüe city (Mendoza province, Argentina), within the northernmost unit (Payunia district) of the Central Patagonia biogeographic province (Morrone 2006). Animals were captured by local hunters between August and September 2010, in an area of a mean altitude around 1500 m.a.s.l. A total of 35 refrigerated intestinal tracts and 27 livers were received for parasitological examination.

Parasitological techniques

Refrigerated intestinal tracts were immediately inspected for helminths, while faeces and livers were preserved in formaldehyde 4% for later examination. The content from the gas-

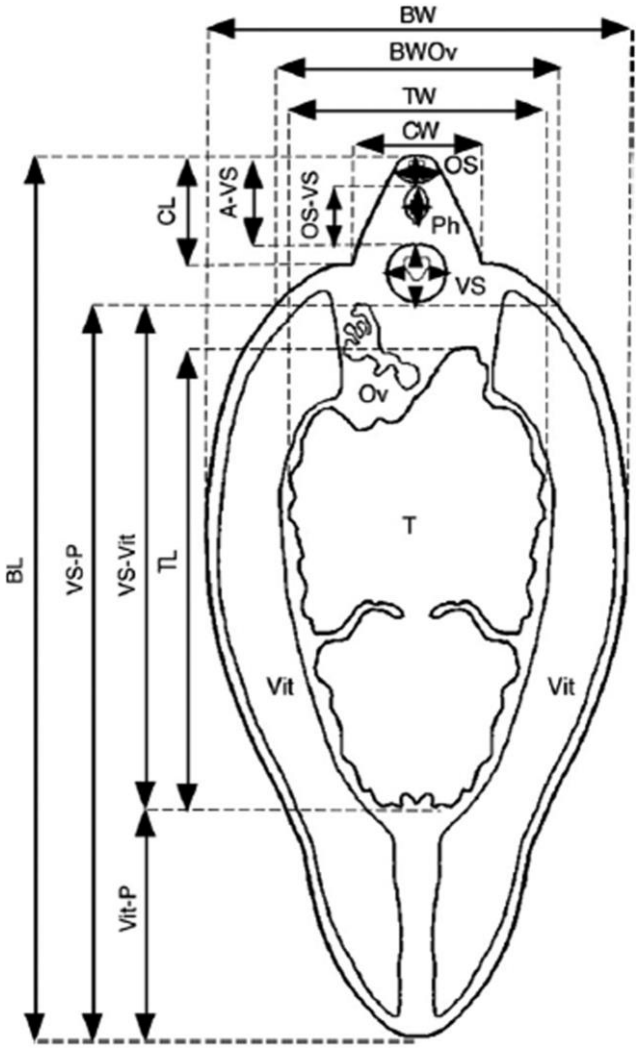


Fig. 1. Standardised measurements in gravid adults of *Fasciola hepatica*

Table I. Comparative morphometrics of 6 adult liver flukes in European brown hare (*Lepus europaeus*) (after Periago *et al*., 2006). Data from liver flukes naturally obtained in domestic species by a) Valero *et al*. (2001a). Data from liver flukes experimentally obtained in Black rat (*Rattus rattus*) and Wistar rat (*Rattus norvegicus*) by a) Valero *et al*. (2001a), and b) Valero *et al*. (1998). All values are shown as range, with the mean and standard deviation (SD) in parentheses

	Natural infection				Experimental infection	
	European brown hare	Sheep[a]	Cattle[a]	Pig[a]	Wistar rat[a]	Black rat (50d.p.i.)[b]
Body length, BL	7.47–11.35 (9.13 ± 1.54)	4.90–31.11 (16.10 ± 4.80)	8.92–28.74 (19.07 ± 3.45)	10.03–24.87 (16.91 ± 3.00)	4.26–23.37 (14.24 ± 4.59)	5.7–13.1 (9.4)
Body width, BW	3.23–4.53 (4.01 ± 0.47)	1.58–12.55 (7.11 ± 2.27)	3.16–12.95 (8.39 ± 1.51)	5.21–11.45 (8.50 ± 1.55)	1.90–12.95 (8.11 ± 3.08)	2.0–5.1 (3.5)
BW at ovary level, BWOv	2.18–3.52 (2.94 ± 0.58)	–	–	–	–	–
Body perimeter, BP	19.64–24.79 (22.37 ± 2.09)	12.39–68.35 (43.13 ± 12.54)	23.60–67.73 (52.03 ± 8.33)	30.23–66.39 (47.32 ± 7.79)	12.28–62.91 (40.33 ± 13.12)	–
Body roundness, BR	1.35–2.19 (1.70 ±0.34)	–	–	–	–	–
Cone length, CL	0.85–1.49 (1.06 ± 0.26)	0.80–3.04 (2.06 ± 0.31)	1.38–3.06 (2.11 ± 0.31)	1.60–3.38 (2.33 ± 0.33)	0.85–3.08 (1.95 ± 0.42)	1.1–2.3 (1.7)
Cone width, CW	1.23–2.32 (1.59 ± 0.39)	1.20–3.90 (2.46 ± 0.43)	1.48–3.75 (2.76 ± 0.41)	2.03–4.47 (3.18 ± 0.47)	1.03–3.73 (2.60 ± 0.72)	–
Maximum diameter of the oral sucker, OSmax	0.61–0.69 (0.63 ± 0.04)	0.37–1.06 (0.72 ± 0.10)	0.54–0.92 (0.73 ± 0.07)	0.57–1.15 (0.84 ± 0.10)	0.32–0.86 (0.63 ± 0.14)	–
Minimum diameter of the oral sucker, OSmin	0.500.56 (0.52 ± .02)	–	–	–	–	–
Maximum diameter of the ventral sucker, VSmax	0.32–0.49 (0.41 ± 0.07)	0.43–1.25 (0.94 ± 0.14)	0.69–1.88 (1.02 ± 0.11)	0.75–1.29 (1.07 ± 0.10)	0.40–1.20 (0.88 ± 0.20)	0.5–0.8 (0.6)
Minimum diameter of the ventral sucker, VSmin	0.17–0.24 (0.20 ± 0.03)	–	–	–	–	–
Distance between the anterior end of the and the ventral sucker, A-VS	0.65–1.23 (0.99 ± 0.24)	0.79–3.35 (2.11 ± 0.36)	1.49–2.98 (2.27 ± 0.28)	1.62–3.35 (2.36 ± 0.35)	0.90–2.80 (1.97 ± 0.45)	1.1–2.1 (1.6)
Distance between the oral sucker and the ventral sucker, OS-VS	0.33–0.80 (0.65 ± 0.24)	0.06–2.56 (1.51 ± 0.31)	0.86–2.29 (1.63 ± 0.27)	1.03–2.55 (1.70 ± 0.33)	0.60–2.26 (1.51 ± 0.39)	0.6–1.4 (1.0
Distance between the ventral sucker and the union of the vitelline glands, VS-Vit	4.22–6.55 (5.69 ± 1.05)	–	–	–	–	–
Distance between the union of the vitelline glands and the posterior end of the body, Vit-P	1.29–2.74 (2.17 ± 0.55)	–	–	–	–	–
Distance between the ventral sucker and the posterior end of the body, VS-P	6.73–9.26 (7.86 ± 0.94)	3.16–27.39 (13.03 ± 4.45)	6.63–24.95 (15.81 ± 3.19)	7.58–20.18 (13.52 ± 2.64)	2.76–19.58 (11.41 ± 4.03)	4.2–9.8 (7.0)

Pharynx length, PhL	0.48–0.68 (0.55 ± 0.12)	–	–	–	–	–
Pharynx width, PhW	0.30–0.32 (0.31 ± 0.01)	–	–	–	–	–
Testicular length, TL	2.85–4.08 (3.56 ± 0.57)	–	–	–	–	–
Testicular width, TW	1.872.72 (2.09 ± 0.36)	–	–	–	–	–
Testicular perimeter, TP	12.16–7.96 (10.84 ± 1.69)	–	–	–	–	–
Body area, BA	22.7–32.4 (25.7 ± 3.9)	6.08–216.77 (84.70 ± 46.96)	19.06–196.35 (114.31 ± 34.25)	42.47–182.03 (101.69 ± 32.65)	6.90–191.79 (89.76 ± 50.03)	–
Oral sucker area, OSA	0.27–0.32 (0.3 ± 0.02)	0.08–0.66 (0.34 ± 0.09)	0.18–0.57 (0.37 ± 0.07)	0.22–0.68 (0.43 ± 0.09)	0.07–0.39 (0.25 ± 0.09)	
Ventral sucker area, VSA	0.6–0.7 (0.6 ± 0.05)	0.15–1.23 (0.71 ± 0.19)	0.37–1.70 (0.80 ± 0.15)	0.45–1.25 (0.88 ± 0.17)	0.12–1.06 (0.63 ± 0.26)	–
Pharynx area, PhA	0.11–0.16 (0.13 ± 0.03)	–	–	–	–	–
Testicular area, TA	5.57–6.78 (6.24 ± 0.49)	–	–	–	–	–
BL/BW ratio	1.68–3.22 (2.33 ± 0.63)	1.33–4.17 (2.33 ± 0.44)	1.40–3.48 (2.30 ± 0.37)	1.50–2.68 (2.02 ± 0.27)	1.18–2.99 (1.85 ± 0.36)	–
BWOv/CW ratio	1.39–2.79 (1.92 ± 0.52)	–	–	–	–	–
OSA/VSA ratio	0.43–0.57 (0.50 ± 0.05)	0.25–0.70 (0.49 ± 0.08)	0.22–0.74 (0.47 ± 0.08)	0.30–0.70 (0.49 ± 0.08)	0.19–0.71 (0.42 ± 0.09)	–
BL/VS-P ratio	1.32–1.07 (1.16 ± 0.09)	1.13–1.55 1.25 ± 0.06	1.00–1.36 1.21 ± 0.04	1.18–1.37 1.26 ± 0.03	1.06–1.60 1.27 ± 0.08	–

50d.p.i., 50 days post-infection

Table II. Measurements taken from 280 eggs of *Fasciola hepatica*, recovered from faeces of European brown hare (*Lepus europaeus*). All values are shown as range, with the mean and standard deviation (SD) in parentheses. EL, egg length (µm); EW, egg width (µm); EA, egg area (µm^2), ER, egg ratio; n.i., natural infection; e.i., experimental infection. Data from rodents and other domestic species by a) Valero *et al*. (2002), and b) Valero *et al*. (2001a)

Host	Geographical location	EL	EW	EA	ER
Brown Hare (*Lepus europaeus*), n.i.	Northern Patagonia, Argentina	90.5–143.7 (120.0 ± 8.9)	56.6–86.2 (68.9 ± 4.9)	6142.4–11408.7 (8275.1 ± 919.3)	1.3–2.3 (1.7 ± 0.2)
Mouse (*Mus musculus*), n.i.[a]	Corsica island, Mediterranean Sea	117–122 (119 ± 2)	60–83 (74 ± 7)	7158–9887 (8836 ± 809)	–
Black rat (*Rattus rattus*), n.i.[a]	Corsica island, Mediterranean Sea	122–148 (133 ± 8)	60–74 (67 ± 3)	7148–10344 (9011 ± 685)	–
Cattle (*Bos Taurus*), n.i.[a]	Corsica island, Mediterranean Sea	125–149 (136 ± 9)	68–83 (74 ± 6)	9128–11300 (10114 ± 801)	–
Cattle (*Bos Taurus*), n.i.[b]	Northern Bolivian Altiplano	105.3–155.9 (132.0 ± 10.5)	61.7–82.5 (71.1 ± 4.4)	5286.5–9676.8 (7170.2 ± 802.5)	1.6–2.3 (1.9 ± 0.2)
Sheep (*Ovis aries*), n.i.[b]	Northern Bolivian Altiplano	114.8–151.2 (130.8 ± 7.1)	65.5–81.4 (72.6 ± 3.9)	5998.2–8608.5 (7238.0 ± 532.8)	1.5–2.2 (1.8 ± 0.1)
Pig (*Sus scrofa domestica*), n.i.[b]	Northern Bolivian Altiplano	73.8–148.6 (123.8 ± 11.3)	58.1–82.5 (71.8 ± 4.4)	3988.7–8626.9 (6836.0 ± 820.4)	1.1–2.1 (1.7 ± 0.2)
Donkey (*Equus asinus*), n.i.[b]	Northern Bolivian Altiplano	96.4–140.8 (125.4 ± 8.3)	63.3–84.7 (75.0 ± 3.7)	5562.6–8686.2 (7177.4 ± 646.1)	1.3–2.0 (1.7 ± 0.1)
Wistar rat (*Rattus norvegicus*), e.i.[a]	Corsica island, Mediterranean Sea	122–148 (134 ± 6)	63–80 (70 ± 4)	7681–11841 9376 ± 866)	–
Wistar rat (*Rattus norvegicus*), e.i.[b]	Northern Bolivian Altiplano	98.1–144.2 (124.6 ± 7.8)	56.9–80.8 (67.6 ± 3.4)	4836.2–7982.3 (6380.1 ± 510.8)	1.4–2.2 (1.9 ± 0.2)

trointestinal tracts from each hare were thoroughly examined following standard methods (Egerton *et al.* 1979).

Previously preserved faecal samples were analyzed by means of two methods: Sheather's sucrose flotation technique (MacPherson and McQueen, 1993) and Lumbreras' rapid sedimentation technique (Lumbreras *et al.* 1962). Sediment obtained from Lumbrera's technique was subsequently passed through a 140 µm sieve. Both techniques were performed with three grams of material. Slides from Sheather's technique and filtered sediment from Lumbrera's technique were microscopically examined. Faecal counts (eggs per gram = epg; oocysts per gram = opg) were determined in every sample.

Liver fluke adults were recovered from preserved livers, while eggs were concentrated by means of sedimentation and filtration from the remaining faecal material previously found 'positive'. Adult worms were stained with Grenacher's borax carmine and mounted in Canada balsam between slide and coverglass but without coverglass pressure (Valero *et al.* 2005, 2012).

Measurement techniques and data analysis

Egg characteristics studied were length (EL) and width (EW) in µm. The product of these 2 dimensions was used as a measure of egg size (EL × EW = ES µm^2), and the ratio as a measure of shape (EL/EW = ER) (Poulin 1997; Abrous *et al.* 1998; Valero *et al.* 1998, 2001a, 2002). For egg classification, egg size was considered according to recent updates on this characteristic (Valero *et al*. 2009; Mas-Coma *et al.* 2014b), and by taking into account the influence of the host species (Valero *et al.* 1998, 2001a, 2002),

For adult fasciolids, the following standardized measurements were taken (Valero *et al.* 2005; Periago *et al.* 2006) (Fig. 1): (i) lineal biometric characters (mm): body length (BL), maximum body width (BW), body width at ovary level (BWOv), body perimeter (BP), body roundness (BR), cone length (CL), cone width (CW), maximum diameter of oral sucker (OS max), minimum diameter of oral sucker (OS min), maximum diameter of ventral sucker (VS max), minimum diameter of ventral sucker (VS min), distance between the anterior end of the body and ventral sucker (A–VS), distance between the oral sucker and ventral sucker (OS–VS), distance between the oral sucker and the union of the vitelline glands (VS–Vit), distance between the union of the vitelline glands and the posterior end of the body (Vit–P), distance between the ventral sucker and the posterior end of the body (VS–P), pharynx length (PhL), pharynx width (PhW), testicular space (taking both testes together) length (TL), testicular space width (TW), testicular space perimeter (TP); (ii) areas (mm^2): body area (BA), oral sucker area (OSA), ventral sucker area (VSA), pharynx area (PhA), testicular space area (taking both testes together, TA); (iii) ratios: body length over body width (BL/BW), body width at ovary level over cone width (BWOv/CW), oral sucker area over ventral sucker area

(OSA/VSA), and body length over the distance between the ventral sucker and the posterior end of the body (BL/VS–P).

Morphometric measurements used for *F. hepatica* adults follow a logistic growth model with respect to time (Valero *et al.* 2001a,b, 2005). This implies that the morphometric development of the fasciolid adult is not limited but 'damped' and does not exceed certain characteristic maximum (Valero *et al.* 1998, 2006b). Since the morphometric maximum values are characteristic for each population, they are considered the comparative base of this study (Table I).

Results

Five faecal samples were detected positive to *Fasciola hepatica* (14.28%, 2.7–25.8% CI 0.95), while 33 showed *Eimeria* sp. oocysts (94.28%, 86.61–100% CI 0.95). No nematode and cestode eggs or adults were observed. Faecal counts showed between 1 and 3 epg for liver fluke (mean 2.08 epg, ± 1.25), and 91.73 mean opg (± 155.84) for *Eimeria* sp.

Twenty-two liver fluke adults were recovered from a single liver, but only six of them could be measured (Table I), while a total of 280 eggs were recovered from faeces and measured (Table II).

Discussion

Each trematode species has its own adult and egg phenotype, generally within a specific range (Valero *et al.* 2009). However, small host body mass offers limited microhabitat (e.g. liver) and places a physical constraint upon the trematode body size and number of flukes that can fit in (Poulin 1997; Valero *et al.* 2001a, 2005); while it has been associated with diminished egg size (Valero *et al.* 2002). Consequently, the final host species decisively influences the size of adults and eggs of *F. hepatica* (Valero *et al.* 2001a,b, 2005, 2009).

Measures of *F. hepatica* found in *L. europaeus* from Malargüe department proved to be among the smaller described in adults and eggs recovered from naturally and experimentally infected murid rodents, lagomorphs and domestic species (see Tables I and II) (Abrous *et al.* 1998; Valero *et al.* 1998, 2001a, 2002). With regard to adult liver flukes, it shall be considered that samples were preserved in formaldehyde 4% during, at least, two months, which might have slightly decreased measures. Size of the fasciolids from the European brown hare appears similar to fasciolids of 50 days of age experimentally obtained in the Black rat (Valero *et al.* 1998) (Table I). However, the size of *F. hepatica* eggs found in faecal samples of the hares fully overlap not only with those of natural and experimental infections in murid rodents, but also with those of natural infections in cattle and other domestic animals (Valero *et al.* 2001a, 2002). All in all, the data obtained indicates that the liver fluke is able to fully develop in wild hares and to shed normal eggs through their faeces.

Additionally, the heavy parasite burden observed (22 liver flukes in a single liver) and the small adult size described strongly suggest an effect of crowding, a phenomenon reflected in a decreased adult development when the number of flukes is high (Valero *et al.* 2006b). Meanwhile, due to experimental evidence of a direct relation between uterus size and the numbers of eggs shed per gram of faeces (Valero *et al.* 2001b, 2011), the reduced uterus development as consequence of smaller adults (Poulin 1997) may explain the low epg observed.

Although *F. hepatica* infection in wild *L. europaeus* has been detected before in its original European range, to the best of our knowledge only one report deals with that aspect in South America (Kleinman *et al.* 2004). Unfortunately, the information provided is only restricted to the local prevalence found. The high prevalence found in our study (14.28%, 2.7–25.8% CI 0.95) strongly contrasts with the very low one registered (<1‰) in the aforementioned study (Kleiman *et al.* 2004). Our results suggest an even more important role in the transmission cycle than previously considered, at least in given areas.

Considering a daily defaecation rate of 410 faecal pellets per hare (Novaro *et al.* 1992), a pellet weight between 1 and 1.4 gr (Kleiman *et al.* 2004), and the epg here obtained, each hare could shed to the environment a daily rate of 410–1,722 eggs of *F. hepatica*. This result is close to the lower limit obtained for pigs (2,000–195,000 eggs/individual/day), a domestic animal whose epidemiological importance in endemic areas has already been highlighted (Mas-Coma *et al.* 1997, 2005).

Parasites tend to have threshold levels of host populations size below which they are unable to persist (Tompkins and Poulin 2006). The population dynamics of the European brown hare, as a competent host for liver fluke (i.e. hosts in which the parasites can develop normally), may allow parasite spillback by amplifying the total number of infective stages and increasing the infection burdens in populations of other susceptible hosts (native or domestic) (Kelly *et al.* 2009; Poulin *et al.* 2011). This situation set the stage for the European brown hare, a NIS, to alter local parasite dynamics in ways that could lead to disease emergence and an outbreak (Rachowicz *et al.* 2005; Thieltges *et al.* 2009; Poulin *et al.* 2011).

The results obtained do not only remark the extraordinary plasticity and adaptability of this trematode species to different host species, but also highlight the role of the European brown hare, and other NIS, as reservoirs capable for parasite spillback to domestic and native cycle, representing a potentially important, but hitherto neglected, cause of disease emergence. The present finding of *F. hepatica* in hares indicates that the geography of the populations of this lagomorph will be in need to be considered when analysing the distribution and extent of fascioliasis infection risk areas (Fuentes *et al.* 1999, 2001; Afshan *et al.* 2014) in Argentina and also in other South American endemic countries where the European hare has been introduced.

References

Abrous M., Comes A.M., Gasnier N., Rondelaud D., Dreyfuss G., Chauvin A., Ménard A., Agoulon A., Cabaret, J. 1998. Morphological variability in *Fasciola hepatica* eggs in ruminants, rodents and lagomorphs. *Journal of Helminthology* 72, 313–317. DOI: 10.1017/S0022149X00016667

Afshan K., Fortes-Lima C.A., Artigas P., Valero M.A., Qayyum M., Mas-Coma, S. 2014. Impact of climate change and man-made irrigation systems on the transmission risk, long-term trend and seasonality of human and animal fascioliasis in Pakistan. *Geospatial Health* 8, 317–334. DOI: 24893010

Arru E., Deiana S., Nuvole A. 1967. Distomatosi epatica nei leporidi selvatici in Sardegna. *Atti della Societa Italiana delle Scienze Veterinaria* 21, 762–766

Bargues M.D., Mas-Coma S. 2005. Reviewing lymnaeid vectors of fascioliasis by ribosomal DNA sequence analyses. *Journal of Helminthology* 79, 257–267. DOI: 10.1079/JOH2005297

Bengis R.G., Leighton F.A., Fischer J.R., Artois M., Mörner T., Tate C. M. 2004. The role of wildlife in emerging and re-emerging zoonoses. *Revue Scientifique et Technique – Office International des Epizooties* 23, 497–511

Bonino N., Cossios D., Meneghetti J. 2010. Dispersal of the European hare, *Lepus europaeus* in South America. *Folia Zoologica* 59, 9–15

Daszak P., Cunningham A.A., Hyatt A.D. 2000. Emerging infectious diseases of wildlife – Threats to biodiversity and human health. *Science* 287, 443–449. DOI: 10.1126/science.287.5452.443

Egerton J.R., Ostlind D.A., Blair L.S., Eary C.H., Suhayda D., Cifelli S., Riek R.F., Campbell W.C. 1979. Avermectins, new family of potent anthelmintic agents: efficacy of the B1a component. *Antimicrobial Agents and Chemotherapy* 15, 372–378

Fuentes M.V., Valero M.A., Bargues M.D., Esteban J.G., Angles R., Mas-Coma S. 1999. Analysis of climatic data and forecast indices for human fascioliasis at very high altitude. *Annals of Tropical Medicine and Parasitology* 93, 835–850

Fuentes M.V., Malone J.B., Mas-Coma S. 2001. Validation of a mapping and predicting model for human fasciolosis transmission in Andean very high altitude endemic areas using remote sensing data. *Acta Tropica* 79, 87–95. DOI: 10.1016/S0001-706X(01)00106-1

Gayo V., Cuervo P., Rosadilla D., Birriel S., Dell'Oca L., Trelles A., Cuore U., Mera y Sierra R. 2011. Natural *Fasciola hepatica* infection in "Nutria" (*Myocastor coypus*), in Uruguay. *Journal of Zoo and Wildlife Medicine* 42, 354–356. DOI: 10.1638/2010-0226.1

Kaplan R.M. 2001. *Fasciola hepatica*: a review of the economic impact in cattle and considerations for control. *Veterinary Therapeutics* 2, 40–50

Kelly D.W., Paterson R.A., Townsend C.R., Poulin R., Tompkins D.M. 2009. Parasite spillback: a neglected concept in invasion ecology? *Ecology* 90, 2047–2056. DOI: 10.1890/08-1085.1

Kleinman F., González N., Rubel D., Wisnivesky C. 2004. *Fasciola hepatica* (Linnaeus, 1758) (Trematoda, Digenea) en liebres europeas (*Lepus europaeus*, Pallas 1778) (Lagomorpha, Leporidae) en la región Cordillerana Patagónica, Chubut, Argentina. *Parasitología Latinoamericana* 59, 68–71. DOI: 10.4067/S0717-77122004000100014

Kruse H., Kirkemo A., Handeland K. 2004. Wildlife as source of zoonotic infections. *Emerging Infectious Diseases* 10, 2067–2072. DOI: 10.3201/eid1012.040707.

Kutzer E., Frey H. 1976. Die Parasiten der Feldhasen (*Lepus europaeus*) in Österreich. (Parasites of the European brown hare in Austria). *Berliner und Münchener Tierärztliche Wochenschrift* 89, 480–483

Lumbreras H., Cantella R., Burga R. 1962. Acerca de un procedimiento de sedimentación rápida para investigar huevos de *Fasciola hepatica* en las heces, su evaluación y uso en el campo. *Revista Médica Peruana* 31, 167–174

MacPherson D.W., McQueen R. 1993. Cryptosporidiosis: multiattribute evaluation of six diagnostic methods. *Journal of Clinical Microbiology* 31, 198–202

Mas-Coma S., Fons R., Feliu C., Bargues M.D., Valero M.A., Galan-Puchades M.T. 1988. Small mammals as natural definitive hosts of the liver fluke, *Fasciola hepatica* Linnaeus, 1758 (Trematoda: Fasciolidae): a review and two new records of epidemiologic interest on the island of Corsica. *Rivista di Parasitologia* 5, 73–78

Mas-Coma S., Rodriguez A., Bargues M.D., Valero M.A., Coello J.R., Angles R. 1997. Secondary reservoir role of domestic animals other than sheep and cattle in fascioliasis transmission in the Northern Bolivian Altiplano. *Research and Reviews in Parasitology* 57, 39–46

Mas-Coma S., Bargues M.D., Valero M.A., Fuentes M.V. 2003. Adaptation capacities of *Fasciola hepatica* and their relationships with human fascioliasis: from below sea level up to the very high altitude. In: (Eds. C. Combes and J. Jourdane) *Taxonomy, Ecology and Evolution of Metazoan Parasites*, Perpignan, France, Presses Universitaires de Perpignan, 81–123

Mas-Coma S., Bargues M.D., Valero M. A. 2005. Fascioliasis and other plant-borne trematode zoonoses. *International Journal for Parasitology* 35, 1255–1278. DOI:10.1016/j.ijpara.2005.07.010

Mas-Coma S., Valero MA., Bargues MD. 2008. Effects of climate change on animal and zoonotic helminthiases. *Revue Scientifique et Technique – Office International des Epizooties* 27,443–457

Mas-Coma S., Valero M. A., Bargues M. D. 2009. *Fasciola*, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Advances in Parasitology* 69, 41–146. DOI: 10.1016/S0065-308X(09)69002-3

Mas-Coma S., Agramunt V.H., Valero M.A. 2014a. Neurological and ocular fascioliasis in humans. *Advances in Parasitology* 84, 27–149. DOI: 10.1016/B978-0-12-800099-1.00002-8

Mas-Coma S., Bargues M.D., Valero M.A. 2014b. Diagnosis of human fascioliasis by stool and blood techniques: Update for the present global scenario. *Parasitology*, 141 (Special Issue), 1918–1946. DOI: 10.1017/S0031182014000869

Mera y Sierra R., Agramunt V.H., Cuervo P., Mas-Coma S. 2011. Human fascioliasis in Argentina: retrospective overview, critical analysis and baseline for future research. *Parasites and Vectors* 4, 104. DOI: 10.1186/1756-3305-4-104

Mezo M., González-Warleta M., Castro-Hermida J.A., Manga-González M.Y., Peixoto R., Mas-Coma S., Valero M.A. 2013. The wild boar (*Sus scrofa* Linnaeus, 1758) as secondary reservoir of *Fasciola hepatica* in Galicia (NW Spain). *Veterinary Parasitology* 198, 274–283. DOI: 10.1016/j.vetpar.2013.09.009

Morrone J.J. 2006. Biogeographic areas and transition zones of Latin America and the Caribbean islands based on pangeographic and cladistic analyses of the entomofauna. *Annual Review of Entomology* 51, 467–494. DOI: 10.1146/annurev.ento.50.071803.130447

Nickel S., Gottwald A. 1979. Parasites of the GDR. 3. Endoparasites of the hare (*Lepus europeus*). *Angewandte Parasitologie*, 20, 57–62

Novaro A.J., Capurro A.F., Travaini A., Funes M.C., Rabinovich J.E. 1992. Pellet-count sampling based on spatial distribution: a case study of the European hare in Patagonia. *Ecología Austral* 2, 11–18

Olaechea F. V. 2007. Tremátodes y Céstodes: *Fasciola hepatica*. In: (Eds. V. H. Suárez, F. V. Olaechea, C. E. Rossanigo, and J. R. Romero), *Enfermedades parasitarias de los ovinos y otros rumiantes menores en el cono sur de América*, Buenos Aires, Argentina, Instituto Nacional de Tecnología Agropecuaria – INTA-, 159–168

Periago M.V., Valero M.A., Panova M., Mas-Coma S. 2006. Phenotypic comparison of allopatric populations of *Fasciola hepatica* and *Fasciola gigantica* from European and African bovines using a computer image analysis system (CIAS). *Parasitology Research* 99, 368–378. DOI: 10.1007/s00436-006-0174-3

Polley L. 2005. Navigating parasite webs and parasite flow: Emerging and re-emerging parasitic zoonoses of wildlife origin. *International Journal for Parasitology* 35, 1279–1294. DOI: 10.1016/j.ijpara.2005.07.003

Poulin R. 1997. Egg production in adult trematodes: adaptation or constraint. *Parasitology* 11, 195–204. DOI: 10.1017/S00 31182096008372

Poulin R., Paterson R.A., Townsend C.R., Tompkins D.M., Kelly D.W. 2011. Biological invasions and the dynamics of endemic diseases in freshwater ecosystems. *Freshwater Biology* 56, 676–688. DOI: 10.1111/j.1365-2427.2010.02425.x

Rachowicz L.J., Hero J.M., Alford R.A., Taylor J.W., Morgan J.A.T., Vredenburg V.T., Collins J.P., Briggs C.J. 2005. The novel and endemic pathogen hypotheses: competing explanations for the origin of emerging infectious diseases of wildlife. *Conservation Biology* 19, 1441–1448. DOI: 10.1111/j.1523-1739. 2005.00255.x

Shimalov V.V. 2001. Helminth fauna of the hare (*Lepus europaeus* Pallas, 1778) in the southern part of Belarus. *Parasitology Research* 87, 85. DOI: 10.1007/s004360000259

Sidoti L., Mera y Sierra R.L., Cuervo P., Micó M., Bargues M.D., Mas-Coma S., Artigas P. 2009. Sheep and goat fascioliasis in the Mendoza province, Argentina. *Acta Parasitologica Portuguesa* 16, 112–113

Thieltges D.W., Reise K., Prinz K., Jensen K.T. 2009. Invaders interfere with native parasite–host interactions. *Biological Invasions* 11, 1421–1429. DOI: 10.1007/s10530-008-9350-y

Tompkins D.M., Poulin R. 2006. Parasites and biological invasions. In: (Eds. R. B. Allen, and W. G. Lee), *Biological Invasions in New Zealand*, Heidelberg, Berlin, Germany, Springer, 67–86

Tropilo J. 1964. Intensity and extensity of invasion by *Cysticercus pisiformis* and *Fasciola hepatica* in hares (in Polish). *Medycyna Weterynaryjna Warszawa* 20, 259–269

Valero M.A., Mas-Coma S. 2000. Comparative infectivity of *Fasciola hepatica* metacercariae from isolates of the main and secondary reservoir animal host species in the Bolivian Altiplano high human endemic region. *Folia Parasitologica* 47, 17–22

Valero M.A., Marcos M.D., Fons R., Mas-Coma S. 1998. *Fasciola hepatica* development in the experimentally infected black rat *Rattus rattus*. *Parasitology Research* 84, 188–194. DOI: 10.1007/s004360050381

Valero M.A., Darce N.A., Panova M., Mas-Coma S. 2001a. Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the northern Bolivian Altiplano hyperendemic region. *Veterinary Parasitology* 102, 85–100. DOI: 10.1016/S0304-4017(01)00499-X

Valero M.A., Panova M., Mas-Coma S. 2001b. Developmental differences in the uterus of *Fasciola hepatica* between livestock liver fluke populations from Bolivian Highlands and European lowlands. *Parasitology Research* 87, 337–342. DOI: 10.1007/PL00008588

Valero M.A., Panova M., Comes A.M., Fons R., Mas-Coma S. 2002. Patterns in size and shedding of *Fasciola hepatica* eggs by naturally and experimentally infected murid rodents. *Journal of Parasitology* 88, 308–313. DOI: 10.1645/0022-3395(2002) 088[0308:PISASO]2.0.CO;2

Valero M. A., Santana M., Morales M., Hernandez J. L., Mas-Coma S. 2003. Risk of gallstone disease in advanced chronic phase of fascioliasis: an experimental study in a rat model. *Journal of Infectious Diseases* 188, 787–793. DOI: 10.1086/377281

Valero M. A., Panova M., Mas-Coma S. 2005. Phenotypic analysis of adults and eggs of *Fasciola hepatica* by computer image analysis system. *Journal of Helminthology* 79, 217–225. DOI: 10.1079/JOH2005301

Valero M.A., Navarro M., Garcia-Bodelon M.A., Marcilla A., Morales M., Garcia J.E., Hernandez J. L., Mas-Coma S. 2006a. High risk of bacterobilia in advanced experimental chronic fasciolosis. *Acta Tropica* 100, 17–23. DOI: 10.1016/j.actatropica.2006.09.002

Valero M. A., De Renzi M., Panova M., García-Bodelon M. A., Periago M. V., Ordoñez D., Mas-Coma S. 2006b. Crowding effect on adult growth, pre-patent period and egg sheding of *Fasciola hepatica*. *Parasitology* 133, 453–463. DOI: 10.1017/S003118200600059X

Valero M.A., Girones N., Garcia-Bodelon M.A., Periago M.V., Chico-Calero I., Khoubbane M., Fresno M., Mas-Coma S. 2008. Anaemia in advanced chronic fasciolosis. *Acta Tropica* 108, 35–43. DOI: 10.1016/j.actatropica.2008.08.007.

Valero M.A., Perez-Crespo I., Periago M.V., Khoubbane M., Mas-Coma S. 2009. Fluke eggs characteristics for the diagnosis of human and animal fascioliasis by *Fasciola hepatica* and *Fasciola gigantica*. *Acta Tropica* 111, 150–159. DOI: 10.1016/ j.actatropica.2009.04.005

Valero M.A., Panova M., Pérez-Crespo I., Khoubbane M., Mas-Coma S. 2011. Correlation between egg-shedding and uterus development in *Fasciola hepatica* human and animal isolates: applied implications. *Veterinary Parasitology* 183, 79–86. DOI: 10.1016/j.vetpar.2011.07.003

Valero M.A., Perez-Crespo I., Khoubbane M., Artigas P., Panova M., Ortiz P., Maco V., Espinoza J. R., Mas-Coma S. 2012. *Fasciola hepatica* phenotypic characterization in Andean human endemic areas: valley versus altiplanic patterns analysed in liver flukes from sheep from Cajamarca and Mantaro, Peru. *Infection, Genetics and Evolution* 12, 403–410. DOI: 10.1016/j.meegid.2012.01.009

Walker S.M., Johnston C., Hoey E.M., Fairweather I., Borgsteede F.H.M., Gaasenbeek C.P.H., Prodohl P.A., Trudgett A. 2011. Potential role of hares in the spread of liver fluke in the Netherlands. *Veterinary Parasitology* 177, 179–181. DOI: 10.1016/j.vetpar.2010.11.043

Ziege S., Brauneis M., von Keyserlingk M., Wohlsein P. 2009. Fasziolose beim Europäischen Feldhasen (*Lepus europaeus*) in Nordwestdeutschland. *Deutsche Tierarztliche Wochenschrifte* 116, 60–63

Received: December 13, 2014
Revised: February 24, 2015
Accepted for publication: April 17, 2015

ICU Investigación, Ciencia y Universidad Año 2016 Vol 1 / Nº 1

Distribución Altitudinal de Fasciola hepatica en Bovinos de la Provincia de Mendoza, Argentina

Altitudinal Distribution of Fasciola hepatica in Cattle of Mendoza Province, Argentina

R. Mera y Sierra, M. González, G. Neira
Centro de Investigación en Parasitología Regional
Universidad Juan Agustín Maza

Contacto: cipar.umaza@gmail.com

Palabras claves: altitud, *Fasciola* hepatica, bovinos, Mendoza
Keywords: *altitude, Fasciola hepatica, cattle, Mendoza*

Resumen

La fascioliasis se encuentra en franca expansión. *Fasciola hepatica* parasita a millones de personas en los cinco continentes y existen zonas andinas hiperendémicas. Afecta a la ganadería ocasionando pérdidas de hasta 3.000 millones de dólares. En Argentina, salvo Tierra del Fuego, la fascioliasis animal es endémica en todas las provincias y los casos humanos publicados superan los 600. En Cuyo existen altas prevalencias en ganado y animales silvestres, además de reportes de casos humanos. Las moluscos vectores en dicha región son *Lymnaea viator, Lymnaea neotropica y Galba truncatula*. En Mendoza se han descrito prevalencias de hasta el 60% en bovinos. Los patrones epidemiológicos de transmisión según la altitud han sido descritos para la fascioliasis humana. Estudios demuestran que estos patrones epidemiológicos pueden aplicarse a ganadería. El conocimiento de la epidemiología de esta enfermedad es fundamental, ya que es una parasitosis con un intrincado ciclo biológico en el cuál intervienen fases de desarrollo en hospedadores invertebrados, adquiriendo el ambiente un rol determinante en su evolución. Este estudio se llevó a cabo con el objetivo de describir la distribución de la fascioliasis bovina según la altitud en Mendoza. Se tomaron muestras de 325 bovinos de 18 establecimientos ganaderos, de los cuales resultaron positivos a *F. hepatica* al examen coprológico el 20,31% de los animales. Se establecieron rangos de altitud: < 800msnm, de 800-1500msnm y >1500msnm. Las prevalencias más altas se hallaron en los animales de establecimientos ubicados a más de 1500 msnm. Se hallaron diferencias significativas entre los animales de los distintos rangos. Los resultados encontrados coinciden con los patrones de altiplano y valle descritos para humanos. Se demuestra que la fascioliasis en bovinos en la provincia de Mendoza sigue un gradiente altitudinal ascendente, lo cual debe ser tenido en cuenta al momento de desarrollar medidas de control.

Abstract

Fasciola hepatica has a worldwide distribution, is currently expanding and can be accounted across 5 continents. Fascioliasis represents a worldwide burden for animals and humans. South America has hyperendemic human areas at the Andean regions. Financial loss in animal production due to Fasciola hepatica is estimated on 3 billion USD per year. Animal fascioliasis is widespread throughout all the provinces of Argentina, except for Tierra del Fuego and over 600 human cases have been published. Liver fluke prevalence is high in domestic and wild animals of Cuyo region. Mollusks described as vectors in Mendoza are *Lymnaea viator, Lymnaea neotropica and Galba truncatula*. *F. hepatica* occurrence in cattle from Mendoza reaches up to 60%. The altitude related epidemiological transmission patterns had been described in human fascioliasis in other Andean countries. Recent surveys have proved that these patterns can be applied to study animal fascioliasis. The knowledge of the epidemiology of liver fluke disease is very important. Due to the high intricacy of its life cycle, and the fact that this trematode needs to evolve inside an invertebrate host, makes it very sensitive to environmental characteristics. The aim of this study was to describe the altitude related prevalence of cattle fascioliasis in Mendoza province. Samples of 325 bovines from eighteen cattle farms were analyzed. The coprological study was positive for *F. hepatica* in 20,31% animals. Altitude ranges were established: < 800m a.s.l., 800-1500m a.s.l. and >1500m a.s.l. The highest prevalence was found in cattle over the 1500m a.s.l. Significative differences were found between the prevalence in cattle of different altitudinal ranges. These findings show that cattle fascioliasis in Mendoza follows an ascendant gradient of altitude. This knowledge should be taken into account when control measures are developed.

Introducción

La fascioliasis es una zoonosis de distribución mundial representada en nuestro continente por *Fasciola hepatica* (Mas-Coma et al., 2009; WHO, 2016). En una iniciativa de la Organización Mundial de la Salud por estimar la importancia de las trematodiasis transmitidas por alimentos en salud humana, Fürst y colaboradores, realizaron una revisión y metanálisis de la información a nivel mundial. Ellos determinaron que la fascioliasis ocasiona a nivel global, 35206 años ajustados de discapacidad (DALY´S) en el humano. De estos, 17318 DALY´S corresponden a Latinoamérica (Fürst et al., 2012).

Esta trematodiasis, conocida por los veterinarios hace mucho tiempo, es una importante fuente de pérdidas económicas en producción bovina, estimada por la FAO en 3000 millones de dólares anuales (FAO, 1994). Los detrimentos que origina han sido descritos como reducción del 3-5% en producción láctea y de aproximadamente 3,8kg (0,5-0,7%) en el peso final de la res. En terneros se ha reportado un 9% menos de ganancia de peso en ganado parasitado. Además se comprobó un retraso de 39 días del primer celo en terneras infectados experimentalmente. En bovinos adultos extiende el periodo de servicio 13 días y aumenta la cuota de servicio en una proporción de 0.75 más por concepción (Charlier et al., 2013).

La fascioliasis es la enfermedad parasitaria transmitida por vectores con mayor distribución latitudinal, longitudinal y altitudinal (Mas-Coma et al., 2003). En Argentina las mayores prevalencias en humanos se han reportado en zonas andinas y serranas, encontrando reportes publicados de más de 600 casos (Mera y Sierra et al., 2011). En nuestro país la distribución de la parasitosis en animales domésticos abarca todas las provincias excepto Tierra del Fuego, con prevalencias elevadas en zonas del Litoral y del centro-oeste (Rebak et al., 2005; Moriena et al., 2004; Issia et al., 2007; Dwinger et al., 1982). En Mendoza existen tres especies de moluscos vectores, *Lymnaea viator, Lymnaea neotropica y Galba truncatula*, siendo este último el vector más eficiente de fascioliasis (Bargués et al., 2006; Mera y Sierra et al., 2009; Standley et al., 2013). A pesar de no ser en humanos una enfermedad de declaración obligatoria, hay reportes para la provincia de Mendoza; existen 28 casos publicados en la literatura científica (Mera y Sierra et al., 2010).

La ocurrencia de fascioliasis bovina en nuestra provincia ha sido estimada tanto mediante estudios post mortem como así también y mediante coproparasitología. Se hallaron prevalencias a la faena del 67,5% y 34% (Mera y Sierra et al., 2005a; González et al., 2006). En los estudios coprológicos se halló prevalencias de hasta el 57,6% (Sidoti, 2011).

En fascioliasis humana existen distintos patrones epidemiológicos según la altitud. El patrón de gran altitud, dividido a su vez en patrón de altiplano relacionado con el vector *Galba truncatula* y con transmisión durante todo el año. Dentro de esta categoría se desglosan dos subpatrones; el patrón de altiplano (con transmisión durante todo el año) y el patrón de valle (con estacionalidad en la transmisión). Además existe el patrón insular caribeño, que presenta otras especies de vectores además del más eficiente y brotes epidémicos en zonas hipo endémicas. Otro patrón africano-mediterráneo de zonas bajas en el cual los hospedadores intermediarios son lymnaeidos del grupo Galba/Fossaria, Radix y con Pseudosuccinea como vector secundario. Asimismo en este patrón existe solapamiento entre Fasciola hepatica y Fasciola gigantica con marcada estacionalidad. Por último el patrón de las zonas aledañas al Mar Caspio, en el cual se solapan F. hepatica y F. gigantica, teniendo como vectores caracoles del grupo Galba/Fossaria, Radix y lymnaeidos estagnicolinos. En este último se producen importantes brotes de fascioliasis humana (Mas-Coma, 2005). En estudios realizados en Irán, en zonas donde se presenta el patrón de zonas aledañas al Mar Caspio, se observo mayor prevalencia de Fasciola hepatica en bovinos, búfalos, caprinos y ovinos cuando la altitud superaba los 100msnm.

Dada las diversas especies de lymnaeidos presentes en la provincia de Mendoza, es necesario conocer si la distribución de la fascioliasis sigue algún patrón epidemiológico de transmisión relacionado con rangos de altitud. El objetivo del presente estudio es describir la prevalencia de la fascioliasis bovina en Mendoza según la altitud del establecimiento ganadero donde se encuentran los animales. Esta información es necesaria para una descripción de la epidemiologia de la fascioliasis en Mendoza, imprescindible para el desarrollo de medidas de control.

Metodología

Se procesaron muestras de un total de 325 bovinos pertenecientes a 18 establecimientos ganaderos (Tabla 1), dedicados tanto a la producción bovina como así también a la de otro ganado (caprino u ovino).

Departamento	Número de Animales	Número de Establecimientos
Lavalle	15	2
La Paz	32	2
Lujan de Cuyo	4	1
Tunuyán	92	6
Tupungato	49	3
Malargüe	24	2
San Carlos	76	1
Las Heras	33	1
Total	325	18

Tabla 1. Número de bovinos muestreados según departamento y establecimiento de origen

Los establecimientos de Lavalle se encuentran en una región de llanura, sumamente árida, donde no se realiza la práctica de la trashumancia, por lo tanto los animales permanecen en un rango altitudinal acotado. El establecimiento de San Carlos realiza cría extensiva sobre pastura natural en valles con régimen de veranada, siendo llevados los animales durante el verano a pasturas altas de hasta 3.000 metros sobre el nivel del mar (msnm), para bajar con el comienzo de las nevadas, situación similar ocurre en el establecimiento de Malargüe.

En cada sitio de muestreo, se registró la altitud mediante el uso de un GPS (GARMIN Etrex Vista®). Los establecimientos se agruparon, según la altitud, en tres categorias: altitud menor a 800 mnsnm, altitud entre 800 y 1.500 msnm y altitud superior a los 1.500 msn. Las muestras de materia fecal fueron recolectadas directamente del recto y colocadas en bolsas de polietileno, vaciando la mayor parte de aire, y se mantuvieron refrigeradas a 4°C hasta su procesamiento en el laboratorio (Fiel et al., 2011). En el laboratorio, se realizò la técnica de sedimentación rápida de Lumbreras (Lumbreras et al., 1962).

Se comparó la proporción de animales en cada categoría mediante la prueba de x2 , se consideraron estadísticamente significativas diferencias con una p<0,05. En todas las maniobras y procedimientos que involucraron el uso de animales vivos se siguieron las pautas estipuladas en la Guía y Cuidado para el Uso de Animales de Laboratorio (NRC, 1996). Este trabajo fue evaluado y aprobado por el Comité Institucional de Cuidado y Uso de Animales en Investigación y Docencia de la Facultad de Ciencias Veterinarias u Ambientales de la Universidad Juan Agustín Maza.

Resultados

Según la altitud, 4 establecimientos (2 de Lavalle y 2 de La Paz) se encuentran por debajo de los 800 msnm, de los cuales se tomaron muestras de 47 animales. Entre los 800 y 1.500 msnm se ubicaron 8 establecimientos, (1 de Lujan de Cuyo, 1 de Tupungato y 6 de Tunuyán) de los cuales se tomaron muestras a 127 animales. En altitudes superiores a los 1.500 msnm se encuentran 6 establecimientos (2 de Malargüe, 1 de San Carlos, 2 de Tupungato, 1 de Las Heras), de los cuales se tomaron muestras a 151 animales. Al examen coprológico, se observaron huevos de Fasciola hepatica en 66 (20,31%) de los bovinos muestreados.

Departamento	Total	Negativo a *F. hepatica*	Positivo a *F. hepatica*	Prevalencia
Tunuyán	92	86	6	6,52%
San Carlos	76	59	17	22,37%
Lavalle	15	13	2	13,33%
Lujan de Cuyo	4	0	4	100,00%
Malargüe	24	20	4	16,67%
Tupungato	36	35	1	2,78%
La Paz	32	32	0	0,00%
Las Heras	46	14	32	69,56%
Total	325	259	66	20,31%

Tabla 2. Prevalencia de *F. hepatica* a los estudios coprológicos en bovinos de Mendoza según departamento de origen.

Altitud msnm	Total	Negativo *F. hepatica*	Positivo *F. hepatica*	Prevalencia
<800	47	45	2	4,44%
800-1.500	127	117	10	7,87%
>1.500	151	97	54	35,76%
Total	325	259	66	20,31%

Tabla 3. Prevalencia de *F. hepatica* a los estudios coprológicos en bovinos de Mendoza según distribución altitudinal.

Según el departamento de origen, fueron positivos la siguiente cantidad de bovinos: Lavalle 2 (13,33%), Luján de Cuyo 4 (100%), Tunuyán 6 (6,52%), San Carlos 17(22,37%), Malargüe 4 (20,00%) y Tupungato 1 (2,78%), Las Heras 32 (69,56%), no hallándose animales positivos en La Paz (Tabla 2). Las diferencias en las prevalencias en los departamentos donde se encontraron animales positivas son significativas (x2=65,57; p<0.0001).

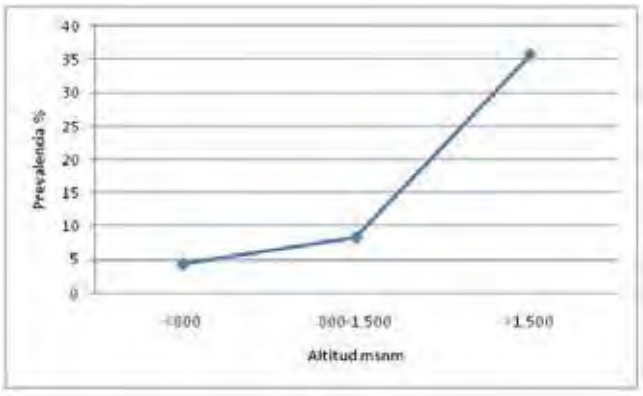


Figura 1. Prevalencia de Fasciola hepatica en bovinos según distribución altitudinal.

Según la distribución altitudinal, la más alta prevalencia correspondio a la zona de cordillera (>1.500 msnm) con una prevalencia de 35,76%, seguido por la de los animales en el rango de 800-1.500 msnm (zona de precordillera, piedemonte) con una prevalencia de 7,87% (Figura 1). Por debajo de los 800 msnm (zona de travesías) la prevalencia hallada fue del 4,44% (Tabla 3). Se hallaron diferencias significativas entre las prevalencias de los distintos grupos (x2=28,19; p<0,0001). No se hallaron diferencias

significativas entre las prevalencias de los animales a <800 msnm y los animales entre 800-1.500 msnm (x2=0,60; p=0,4377).

Discusión

Según el presente estudio, la prevalencia de fascioliasis bovina es significativamente mayor en zonas de cordillera. Se observa un gradiente ascendente a medida que aumenta la altitud (Figura 1). Si tenemos en cuenta que en el resto del país, existen zonas endémicas en regiones de muy baja altitud, tal como es el litoral e incluso en zonas costeras de la provincia de Buenos Aires (Olaechea 1994; Moriena et al., 2004; Rebak et al., 2005), la altitud de por sí, no explica la distribución hallada. En zonas andinas de Perú y Bolivia, donde existen zonas endémicas e incluso hiper endémicas, la presencia del lymnaeido *Galba truncatula*, el cual se adapta a grandes altitudes, podría estar explicando esta distribución. Incluso se ha demostrado una mayor capacidad de emisión de cercarias a mayores altitudes (Mas-Coma et al., 2001). Estudios previos de distribución realizados en la provincia de Mendoza que incluyeron las cuencas del Rio Mendoza, Rio Tunuyan, Rio Diamante, Rio Atuel y la cuenca endorreica de Llancanelo hallaron lymnaeidos en un rango altitudinal desde los 1.526 hasta los 2.638 msnm (Mera y Sierra, 2001; Mera y Sierra et al., 2005). No se hallaron lymnaeidos por debajo de los 1.500 msnm. Esta distribución, juntamente con la presencia de *Galba truncatula* en Mendoza (Bargues et al., 2006), explicaría la distribución de casos de fascioliasis bovina hallada en el presente estudio a grandes alturas. Sin embargo, surge el interrogante, y más aun luego de la descripción de *Lymnaea neotropica* transmitiendo *Fasciola hepatica* en zonas de menor altitud en el piedemonte (Mera y Sierra et al., 2009), si la presencia de este vector es una introducción reciente que explicaría la prevalencia hallada a menor altitud en el presente estudio.

Las altas prevalencias halladas en altitud, coincidirían con el patrón de transmisión andino descrito para zonas donde se encuentra el lymnaeido importado, *Galba truncatula*. Se diferencian en patrón de valle, con estacionalidad, y patrón de altiplano, sin estacionalidad (Mas-Coma et al., 2009). A la fecha se desconoce si en Mendoza la transmisión de fascioliasis es estacional, por lo cual serán necesarios estudios longitudinales para determinarlo.

Conclusiones

La fascioliasis en bovinos de la provincia de Mendoza tiene una prevalencia significativamente mayor por encima de los 1.500 msnm y prevalencias muy bajas o nulas en zonas de baja altitud en la llanura. Esta información debe de ser tenida en cuenta al momento de implementar medidas de control. Son necesarios futuros estudios, tanto de monitoreo de la presencia y prevalencia de fascioliasis en bovinos a distintas altitudes, como así también para conocer cuales son los factores que determinan la distribución de la fascioliasis en la provincia de Mendoza.

Bibliografía

Bargués, M.D., Mera y Sierra, R., Gómez, H.G., Artigas, P., & Mas-Coma S. (2006). Ribosomal DNA ITS-1 sequencing of *Galba truncatula* (Gastropoda: Lymnaeidae) and its potential impact on fascioliasis transmission in Mendoza, Argentina. *Animal Biodiversity and Conservation*, 29, pp. 191-194.

Charlier, J., Vercruysse, J., Morgan, E., Van Dijk, J., & Williams, D. J. L. (2013). Recent advances in the diagnosis, impact on production and prediction of Fasciola hepatica in cattle. *Parasitology* (2014), 141, pp. 326–335.

Dwinger, R. H., Le Riche, P.D., & Kühne, G. I. (1982). Fasciolosis in beef cattle in North-west Argentina. *Tropical Animal Health and Production*, 14, pp. 167-171.

FAO (1994). *Diseases of domestic animals caused by liver flukes: epidemiology, diagnosis and control of Fasciola, paramphistome, Dicroceoelium, Eurytrema and schistosome infections of ruminants in developing countries*. FAO: Rome.

Fiel, C. A., Steffan, P. E., y Ferreyra, D., (2011). *Diagnóstico de las Parasitosis más Frecuentes de los Rumiantes: Técnicas de Laboratorio e Interpretación de Resultados.* (1ra ed.) Tandil: Abad Benjamín.

Fürst, T., Keiser, J., & Utzinger, J. (2012). Global burden of human food-borne trematodiasis: a systematic review and meta-analysis. *Lancet Infectious Diseases*; 12, pp. 210–21.

González, M. S., Di Nucci, D., Sidoti, L., y Mera y Sierra, R.L. (2006). Decomiso en frigorífico de hígados provenientes de Mendoza debido a Fasciola hepatica. Presentado en *XII Jornadas de Microbiología, I Jornadas Conjuntas de Microbiología, Infectología y Alergia e Inmunología de Cuyo, III Jornadas Mendocinas de Zoonosis.* Mendoza, Argentina.

Issia, L., Pietrokovsky, S., Racioppi, O., Alvarez, D., Moriena, R. A. y Wisnivesky, C. (2007). Fasciolosis en un pastoreo mixto ovino-bovino en la provincia de Corrientes. Resumen n° 48. Presentado en *XXVIII Sesión de Comunicaciones Científicas de la Facultad de Ciencias Veterinarias,* Universidad del Nordeste.

Lumbreras, H., Cantella, R. y Burga, R. (1962). Acerca de un procedimiento de sedimentación rápida para investigar huevos de Fasciola hepatica en las heces, su evaluación y uso en el campo. *Revista de Medicina Peruana*, 31: pp.167–174.

Mas-Coma, S. (2005). Epidemiology of fascioliasis in human endemic areas. *Journal of Helminthology*. 79(3), pp. 207-16.

Mas-Coma, S., Funatsu, I.R., & Bargues, M.D. (2001). Fasciola hepatica and lymnaeid snails occurring at very high altitude in South America. *Parasitology* 123: pp. 115–127.

Mas-Coma, S., Bargues, M. D., Valero, M. A., & Fuentes, M.V. (2003) Adaptation capacities of Fasciola hepatica and their relationships with human fascioliasis: from below sea level up to the very high altitude. pp. 81 – 123 in Combes, C. & Jourdane, J. (Eds) *Taxonomy, ecology and evolution of metazoan parasites. Vol. 2*, Perpignan, Presses Universitaires de Perpignan.

Mas-Coma, S., Valero, M. A., & Bargues, M. (2009). Fasciola, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. *Advances in Parasitology*, 69, pp. 41-146.

Mera y Sierra, R. (2001). *Distribución de Lymnaeidos en el sistema hidrográfico andino de la provincia de Mendoza, Argentina*. [Tesis de Maestría]. Valencia: Departamento de Parasitología, Facultad de Farmacia, Universidad de Valencia.

Mera y Sierra, R. L., Scibilia, C., Pasbt, A., Irrazabal, G., & Senar, M. (2005a). Abattoir condemnation of bovine livers due to *Fasciola hepatica* in Tupungato, Mendoza. Presentado en *XXIII Reunión Científica Anual, Sociedad Biológica de Cuyo. Biocell, 29* (3), Abstract 92, 317.

Mera y Sierra, R., Morales, J. J., Guiñazu, M., Sidoti, L., & Artigas, P. (2005b). Altitudinal distribution of Lymnaeid molluscs in Mendoza province, Argentina. Presentado en *XXIII Reunión Científica Anual, Sociedad Biológica de Cuyo. Biocell, 29* (3), Abstract 91, 371.

Mera y Sierra, R. L., Artigas, P., Cuervo, P., Deis, E., Sidoti, L., Mas-Coma, S., & Bargues, M.D. (2009). Fascioliasis transmission by *Lymnaea neotropica* in Argentina, confirmed by nuclear rDNA and mtDNA sequencing. *Veterinary Parasitology*, 166: pp. 73-79.

Mera y Sierra, R. L., Fantozzi, C., Sidoti, L., Deis, E., & Cuervo, P. (2010). Fascioliasis humana en Cuyo: revisión y análisis de casos publicados. Presentado en el *I Congreso Nacional de Enfermedades Tropicales. San Salvador de Jujuy, Mayo 2010*. Libro de Resúmenes.

Mera y Sierra, R. L., Agramunt, V. H., Cuervo, P. & Mas-Coma, S. (2011). Human Fascioliasis in Argentina: retrospective overview, critical analysis and baseline for future research. *Parasites & Vectors*, 4: 104.

Moriena, R. A., Racioppi, O., y Alvarez, J.D. (2004). Fasciolosis en bovinos del nordeste argentino. Prevalencia según edad. Revista Veterinaria, 15(1), pp. 3–4.

National Research Council, Institute of Laboratory Animal Resources, Commission on Life Sciences (1996). *Guía para el Cuidado y Uso de los Animales de Laboratorio*. Edición Mexicana. Washington DC: National Academia Press.

Olaechea, F.V. (1994). Epidemiología y control de Fasciola hepatica en la Argentina. En: Nari, A., Fiel, C. *Enfermedades Parasitarias de Importancia Económica en Bovinos*. Montevideo: Editorial Agropecuaria, Hemisferio Sur SRL. pp. 213-232.

Olaechea, F.V. (2007). Trematodes y Céstodes: *Fasciola hepatica*. En: (Eds. Suárez V.H., Olaechea F.V., Rossanigo, C. E., y Romero, J. R.), *Enfermedades parasitarias de los ovinos y otros rumiantes menores en el cono sur de América*, Buenos Aires, Argentina, Instituto Nacional de Tecnología Agropecuaria –INTA-, pp. 159–168.

Rebak, G.I., Brenn, G., Sánchez, S., Molina, K., y Cedrés, J.F. (2005). Hallazgos de distomatosis hepatica (Fasciolosis) post mortem en Corrientes. Presentado en *XXVI Sesión de Comunicaciones Científicas 85º Aniversario,* Facultad de Ciencias Veterinarias, Universidad Nacional del Nordeste. Resumen 48.

Standley, C. J., Prepelitchi, L., Pietrovoski, S. M., Issia, L., Russell Slothard, J., & Wisnivesky-Coli, C. (2013). Molecular characterization of cryptic and sympatric lymnaeid species from the Galba/Fossaria group in Mendoza Province, Northern Patagonia, Argentina. *Parasites & Vectors* 6:304.

WHO, (2015). *Third WHO report on neglected tropical diseases: Investing to overcome the global impact of neglected tropical diseases*. Geneva: World Health Organization. Recuperado de: apps.who.int/iris/bitstream/10665/152781/1/9789241564861_eng.pdfua=1.

Experimental Parasitology 160 (2016) 49–53

Revista electrónica de difusión científica / Universidad Juan Agustín Maza

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/ yexpr

Full length article

In vivo assessment of closantel ovicidal activity in *Fasciola hepatica* eggs

María Victoria Solana [a, *], Roberto Mera y Sierra [b], Silvana Scarcella [a], Gisela Neira [b], Hugo Daniel Solana [a]

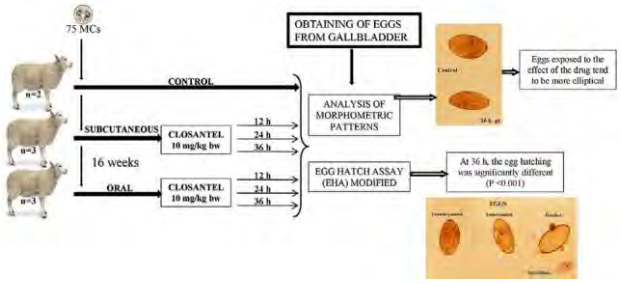
[a] Laboratorio de Biología Celular y Molecular, Centro de Investigación Veterinaria de Tandil (CIVETAN), CONICET, Facultad de Ciencias Veterinarias, UNCPBA, Tandil, Argentina

[b] Centro de Investigación en Parasitología Regional (CIPAR), Universidad J.A. Maza, Guaymallén, Argentina

highlights

- The in vivo ovicidal activity of closantel, an option to triclabendazole in fasciolasis treatment, is yet unknown.
- Sheep were treated with closantel and eggs collected at different times for morphometric studies and eggs hatch assay.
- Significant differences were observed in the morphology and hatchability between control and treated sheep.
- 67,5% of recovered eggs post closantel treatment was unembryonated differing significatively with the control group.
- Results obtained confirm that closantel affects in vivo the normal development of the eggs.

graphical abstract



articleinfo

Article history:
Received 31 March 2015
Received in revised form
1 September 2015
Accepted 29 October 2015
Available online 6 November 2015

Keywords:
Ovicidal activity
Fasciola hepatica
Closantel
Egg hatch assay

abstract

Anthelmintic resistance in livestock parasites is currently a worldwide problem.

Fasciola hepatica is a cosmopolitan parasite which causes considerable loss in sheep and cattle production systems all over the world. Chemotherapy is currently the main tool available for its control. The intensive use of triclabendazole, the drug of choice for more than 20 years, has resulted in the development of resistant strains. The therapeutic options are adulticides such as closantel (salicylanilide anthelmintic that binds extensively to plasma albumin) to treat chronic fascioliasis in sheep, and cattle. In the present work, an Egg Hatch Assay (EHA) and morphometric studies were used to evaluate in vivo the ovicidal activity and morphology *F. hepatica* eggs, recovered from closantel treated sheep collected at different time intervals post treatment.

Statistically significant differences ($p < 0.0001$) were observed in egg morphometry between the control and the treated groups in all the parameters studied. Eggs recovered from treated animals tend to be narrower and longer. Significant differences were found in the embryonation and hatching of eggs between 36 h post treatment (32, 5%) vs. approximately 85% in control, 12 h and 24 h post treatment. Our results confirm that closantel affects in vivo the normal development of the eggs.

* Corresponding author. Int. Campus Universitario, 7000 Tandil, Buenos Aires, Argentina. Tel: þ54-9-2494385850.
E-mail address: mvsolana@vet.unicen.edu.ar (M.V. Solana).

http://dx.doi.org/10.1016/j.exppara.2015.10.010

As one of the first effects, this drug affects the performance of the trematode's reproductive physiology. Even though closantel treated animals may still eliminate eggs in the first days post treatment, these are not viable.

1. Introduction

Fasciola hepatica is a cosmopolitan parasite which causes considerable loss in sheep and cattle production systems all over the world estimated at USD 2000–3000 billion (Boray, 1994; Fairweather, 2011c). Since its introduction in 1983, the fasciolicide triclabendazole (TCBZ) has been extensively used worldwide to control liver fluke infections in sheep and cattle, mainly because it possesses a uniquely wide spectrum of activity, killing not only adult *Fasciola* spp., but also immature and juvenile flukes as young as 2 days post-infection (Boray et al., 1983; Fairweather et al., 1999). In the absence of an effective vaccine against the fluke, control is achieved mainly by chemotherapy.

Triclabendazole (TCBZ) has been the drug of choice for treating liver fluke infections in livestock for over 20 years. More recently, it has been used successfully to treat cases of human fascioliasis (WHO, 2006). Anthelmintic resistance in livestock parasites is currently a worldwide problem. The intensive use of TCBZ has resulted in the development of resistant liver flukes, which was first described in farm animals in Australia in the mid-1990s (Overend and Bowen, 1995) and since then has been reported in, Europe and South America (Fairweather, 2011a; Olaechea et al., 2011). On those farms where the local fluke population is resistant to TCBZ, the therapeutic options are to use adulticides such as closantel to control chronic fascioliasis in sheep and cattle, in an attempt to minimize pasture contamination with fluke eggs and reduce the risk of acute infection in the next (Hanna et al., 2015). Closantel is a salicylanilide anthelmintic that binds extensively to plasma albumin (Michiels et al., 1987). As a result, its activity is mainly directed against blood-feeding internal parasites such as *F. hepatica*, *Haemonchus contortus*, *Oestrus ovis* and *Oesophagostomum* larvae.

The primary action of salicylanilides has been associated with the uncoupling of oxidative phosphorylation in mitochondria. Early *in vitro* studies, using houseflies as well as rat liver mitochondria, showed that several salicylanilides were potent inhibitors of electron transport-associated phosphorylation (Williamson and Metcalf, 1967). *In vitro* inhibition of electron transport-associated phosphorylation in *F. hepatica* and *Ascaris lumbricoides* was later reported for oxyclozanide, rafoxanide and closantel (Corbett and Goose, 1971; Kane et al., 1980).

Several forecasting systems exist for disease surveillance and will assist in monitoring any changes in the pattern of fascioliasis (Fox et al., 2011; McCann et al., 2010). Diagnosis has to operate at two other levels: diagnosis of drug efficacy and diagnosis of drug resistance. There is no standard protocol for the determination of drug efficacy, so interpretation of results can be difficult and there are fewer tests available. The Faecal Egg Count Reduction Test (FECRT) is probably most often used, with drug treatment being regarded as successful if there is a 95% reduction in fluke egg counts by 14 days post-treatment (pt). However, it is known that eggs can be stored in the gall bladder for several weeks (Chowaniec and Darski, 1970), so they may still be present, even though the flukes have been successfully removed; this can lead to false positive results.

The Egg Hatch Assay (EHA) test (Coles et al., 2006; von Samson-Himmelstjerna et al., 2009). It could be applied to fluke as well. Flukicides can affect not only egg formation and production, but also egg development and hatching (i.e. viability) (Maes et al., 1988; Malone et al., 1984; Toner et al., 2011). The EHA may have some potential to detect anthelmintic resistance in flukes. This test, used as a diagnostic method for the detection of benzimidazole resistance in nematodes (Coles et al., 2006).

In the present work, a modified EHA was used to evaluate in vivo the ovicidal activity and morphology in fluke eggs, recovered from closantel treated sheep collected at different time intervals post treatment, and immediately incubated without further addition of the drug.

2. Materials & methods

2.1. Experimental infection

Animal procedures and management protocols were approved by the Ethics Committee according to the Animal Welfare Policy (Act 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar), and to internationally accepted Animal Welfare Guidelines (A.V.M.A, 2001). Eight parasite-free Corriedale weaned lambs were inoculated orally with 200 metacercariae of *F. hepatica* contained in a gelatin capsule. The isolate used for this experiment was the Cullompton isolate, which is TCBZ-susceptible. Details of its provenance were reviewed by Fairweather (Fairweather, 2011b). The presence of liver fluke in the lambs was confirmed 16 weeks after infection by the finding of eggs in the faeces, and liver damage was estimated indirectly by measurement of serum Glutamate Dehydrogenase and Gamma Glutamyl Transferase activities, as described previously (Solana et al., 2001). Sixteen weeks after oral inoculation, the animals were assigned to three experimental groups, based on their clinical condition and body weight, and were treated orally or by subcutaneous injection with closantel as detailed in Table 1.

2.2. Collection of F. hepatica eggs

Treated animals in groups 2 and 3 were stunned and exsanguinated at 12 h, 24 h and 36 h post-treatment, following the W.A.A.V.P. guidelines for evaluating antiparasitic treatments in ruminants (Wood et al., 1995). The eggs were directly recovered from the bile of each infected sheep by puncture of the gallbladder. After several washes with tap water, eggs were suspended in water (500 eggs/ml) and conserved in darkness at 4 °C until used.

2.3. Analysis of morphometric patterns

Eggs (45–60 per group) were measured using an ocular micrometer attached to 10X optical microscope with 10X objective. In all analyzed eggs the following measurements were made: I) WIDTH, the central area perpendicular to the axial axis, II) LENGTH, a centerline from one pole to the other, III) SIZE, was determined by multiplying the length by the width of each egg and IV) SHAPE was

Table 1
Summary of treatments administered to groups of experimental animals.

Group	Number of animals	Dose	Days post-infection before treatment
1	2	No treatment	–
2	3	10 mg/kg b.w. oral	90
3	3	10 mg/kg b.w. subcutaneous	90

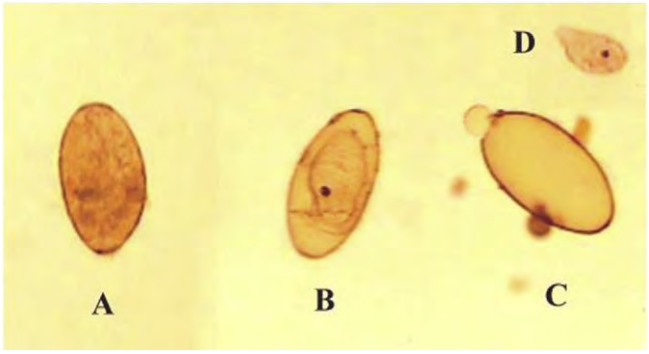


Fig. 1. *F. hepatica* Eggs. A: Unembryonated eggs; B: Unhatched embryonated eggs; C: Hatched eggs; D: Unstained miracidium (100X), direct observation.

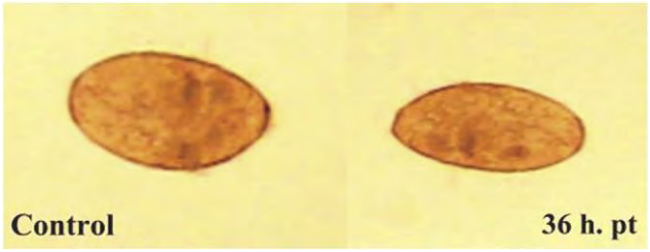


Fig. 2. *F. hepatica* Eggs. Effect of closantel (10 mg/kg bw) 36 h post treatment. Eggs recovered from treated animals tend to be more elliptical.

obtained by dividing the length by the width (Abrous et al., 1998; Poulin, 1997; Valero et al., 2001). Data processing was carried out with Infostat®. From the collected data, arithmetic means and standard deviations were obtained. The comparison of the egg measurements from the different groups was carried out with the one way Analyses of Variance and Tukey test. Values were considered significant if $p < 0.05$.

2.4. Egg hatch assay (EHA)

Untreated and treated eggs were kept in darkness at 25 °C for 15 days. After this period, the trematode eggs were exposed to daylight for 2 h. Afterwards, 1 ml of 10% (v/v) buffered formalin was added to each tube in order to stop egg hatching. Eggs were evaluated using an optical microscope (40× magnification). Approximately 80–90 eggs were counted to estimate the proportion of hatched eggs in each tube. Upon observation, eggs were classified in two groups:

1) Embryonated eggs (E): including hatched and unhatched embryonated eggs, with inner miracidium identified by the eyespots.
2) Unembryonated eggs (U): with no identifiable miracidium or increase in the number of cells, similar to freshly collected eggs (Abrous et al., 1998) (Fig. 1). The percentages of egg hatch are reported as the arithmetic mean ± standard deviation (SD). One way Analyses of Variance and Tukey test was used for the statistical comparison of the egg hatch data obtained from each experiment. A value of $P < 0.05$ was considered statistically significant. The statistical analysis was performed using Infostat®.

3. Results

3.1. Morphometric analysis

Results of the morphometric analysis of eggs can be seen in Table 2. Statistically significant differences ($p < 0.0001$) were observed between the eggs of the control group and the rest in all the parameters evaluated. Eggs exposed to the effect of closantel tend to be narrower and longer (Fig. 2).

3.2. EHA

The test (n = 3) was expressed as percentage of total eggs analyzed. The result obtained for control was 89, 5% ± 2.12 for (E) and 10.5% ± 2.12 (U). The results obtained of the oral and subcutaneous (SC) routes were not significantly different (data not shown), so it was decided to use the mean of both as a unique result for each group. The mean of both ways of administration at 12 h pt was 84.5% ± 0.71 (E) and 15.5% ± 0.71 (U); at 24 h pt was 86.75 ± 3.18 (E) and 12.75% ± 3.18 (U) and 36 h pt was 32.5% ± 3.54 (E) and 67.5% ± 3.54 (U) (Table 3). At 36 h, the egg embryonation and hatching was significantly different ($p < 0.001$).

Table 2
Morphometric analysis of *F. hepatica* eggs. Effect of closantel (10 mg/kg bw) subcutaneous (SC) and oral at 12, 24 and 36 h post treatment.

		Mean ± SD			
Eggs		Length (μm)	Width (μm)	Size (μm²)	Shape
Control (n = 44)		149.78 ± 6.98	94.66 ± 10.67	14,204.95 ± 1917.58	1.60 ± 0.18
12 h	SC(n = 50)	152.68 ± 6.80	88.74 ± 7.10	13,561.04 ± 1379.62	1.73 ± 0.14
	Oral(n = 61)	151.88 ± 5.46	91.36 ± 8.61	13,875.66 ± 1420.21	1.68 ± 0.16
24 h	SC(n = 60)	154.55 ± 4.60	88.38 ± 3.61	13,655.61 ± 628.11	1.75 ± 0.10
	Oral(n = 59)	148.48 ± 5.36	86.18 ± 5.19	12,798.44 ± 932.35	1.73 ± 0.12
36 h	SC(n = 62)	152.99 ± 7.34	89.36 ± 5.63	13,677.98 ± 1174.60	1.72 ± 0.13
	Oral(n = 62)	153.12 ± 6.70	87.75 ± 5.94	13,442.10 ± 1152.01	1.75 ± 0.13

Table 3
Percentage (%) of embryonated and hatched eggs (E) and unembryonated eggs (U). Effect of closantel (10 mg/kg bw) subcutaneous (SC) and Oral at 12, 24 and 36 h post treatment (pt).***: Significantly different.

Stage	Eggs hatch assay (EHA) (%)								
	Control	Experimental							
		12 h pt			24 h pt			36 h pt***	
		Oral	Sc	Mean ± SD	Oral	Sc	Mean ± SD	Oral	Sc	Mean ± SD
E	89.5 ± 2.12	86.5	82.5	84.5 ± 0.71	86.5	87	86.75 ± 3.18	34	31	32.5 ± 3.54
U	10.5 ± 2.12	13.5	17.5	15.5 ± 0.71	13.5	13	13.25 ± 3.18	66	69	67.5 ± 3.54

4. Discussion

The basic aim of this study was to determine ovicidal activity and morphology in fluke eggs recovered from closantel treated sheep. The results showed clearly that the aim of the study was achieved.

The effect of closantel on the fluke's reproductive system can explain the observed changes in egg embryonation and hatching. Bearing in mind that each egg assembled in the ootype of *F. hepatica* incorporates about 30 vitelline cells, and that each fluke produces some 25,000 eggs each day, cell division and differentiation in the vitelline follicles consumes the majority, possibly the largest, proportion of the energy generated by intermediary metabolism. Therefore it is not surprising that drug induced restriction of available ATP had a marked effect on the functional activity in the vitelline tissue, which causes a decrease production and quality of eggs (Hanna et al., 2006). It is likely that defects in the quality of the vitelline cells would prevent successful shell formation and therefore render the eggs non-viable. The release of fertile eggs of *F. hepatica* into the environment is one of the main ways available to the fluke to perpetuate their species and maintain their active life cycle. Our results confirm that closantel affects in vivo the normal development of the eggs. As one of the first effects, this drug affects the performance of the trematode reproductive physiology.

The results of the time-course studies, reinforce the idea that any eggs present in successful efficacy trials represent eggs stored in the gall bladder prior to drug treatment and may not properly reflect what is happening to the actual flukes themselves, which are eliminated within 3–4 days pt ((Toner, E. et al. 2011)). Our results confirm that closantel affects in vivo the normal development of the eggs. As one of the first effects, this drug affects the performance of the trematode's reproductive physiology.

On the other hand, these results have an impact on the interpretation of the Faecal Egg Counts in efficacy studies and may be useful to further understand the mechanisms underlying the drug activity in target helminths parasites. Finally a curious data was obtained from de morphometric analysis, the size of the Cullompton strain is considerably greater than descriptions of field isolates described in the literature (Abrous et al., 1998; Fantozzi et al., 2011, 2012; Larroza and Olaechea, 2008; Valero et al., 2001). These reports describe eggs of approximately 130μm in length and 72μm in width, quite smaller than what we have observed (Table 2).

References

A.V.M.A, 2001. 2000 report of the A.V.M.A panel on Euthanasia. J. Am. Vet. Med. Assoc. 218, 669–696.

Abrous, M., Comes, A., Gasnier, N., Rondelaud, D., Dreyfuss, G., Chauvin, A., Menard, A., Agoulon, A., Cabaret, J., 1998. Morphological variability in *Fasciola hepatica* eggs in ruminants, rodents and lagomorphs. J. Helminthol. 72, 313–317.

Boray, J.C., 1994. Disease of Domestic Animals Caused by Flukes (Rome, FAO), pp. 1–49.

Boray, J.C., Crowfoot, P.D., Strong, M.B., Allison, J.R., Schellenbaum, M., Von Orelli, M., Sarasin, G., 1983. Treatment of immature and mature *Fasciola hepatica* infections in sheep with triclabendazole. Vet. Rec. 113, 315–317.

Coles, G., Jackson, F., Pomroy, W., Prichard, R., von Samson-Himmelstjerna, G., Silvestre, A., Taylor, M., Vercruysse, J., 2006. The detection of anthelmintic resistance in nematodes of veterinary importance. Vet. Parasitol. 136, 167–185.

Corbett, J.R., Goose, J., 1971. A possible biochemical mode of action of the fasciolicides nitroxynil, hexachlorophene and oxyclozanide. Pestic. Sci. 2, 119–121.

Chowaniec, W., Darski, J., 1970. Investigations on excretion time of liver fluke eggs after killing the parasite. Bull. Vet. Inst. Pulaway 14, 108–110.

Fairweather, I., 2011a. Liver fluke isolates: a question of provenance. Vet. Parasitol. 176, 1–8.

Fairweather, I., 2011b. Liver fluke isolates: a question of provenance. Vet. Parasitol. 176, 1–8.

Fairweather, I., 2011c. Reducing the future threat from (liver) fluke: realistic prospect or quixotic fantasy? Vet. Parasitol. 180, 133–143.

Fairweather, I., Boray, J.C., Dalton, J.P. (Eds.), 1999. Mechanisms of Fasciolicide Action and Drug Resistance in *Fasciola Hepatica*. CABI Publishing, Wallingford, Ox, pp. 225–276.

Fantozzi, C., Sidoti, L., Cuervo, P., Mera y Sierra, R., 2011. Análisis morfométrico de huevos de *Fasciola hepatica* en huéspedes domésticos del oeste argentino. In: Congreso Internacional de Zoonosis y Enfermedades Emergentes y VII Congreso Argentino de Zoonosis, p. 149. Buenos Aires, Argentina.

Fantozzi, C., Sidoti, L., Neira, G., Mercado, C., Cuervo, P., Mera y Sierra, R., 2012. Phenotypic analysis of eggs of *Fasciola hepatica* recovered from Creole goats, in western Argentina. In: XI European Multicolloquium of Parasitology, pp. 518–519. Cluj-Napoca, Rumania.

Fox, N.J., White, P.C., McClean, C.J., Marion, G., Evans, A., Hutchings, M.R., 2011. Predicting impacts of climate change on *Fasciola hepatica* risk. Plos One 6, e16126.

Hanna, R.E.B., Cromie, L., Taylor, S.M., Couper, A., 2006. The effect of a parenteral ivermectin/closantel injection on the growth and reproductive development of early immature *Fasciola hepatica* in cattle. Vet. Parasitol. 142, 78–90.

Hanna, R.E.B., McMahon, C., Ellison, S., Edgar, H.W., Kajugu, P.E., Gordon, A., Irwin, D., Barley, J.P., Malone, F.E., Brennan, G.P., 2015. *Fasciola hepatica*: a comparative survey of adult fluke resistance to triclabendazole, nitroxynil and closantel on selected upland and lowland sheep farms in Northern Ireland using faecal egg counting, coproantigen ELISA testing and fluke histology. Vet. Parasitol. 207, 34–43.

Kane, H.J., Behm, C.A., Bryant, C., 1980. Metabolic studies on the new fasciolicidal drug, closantel. Mol. Biochem. Parasitol. 1, 347–355.

Larroza, M., Olaechea, F., 2008. Morfología y viabilidad de los huevos de *Fasciola hepatica* en distintos hospedadores en Patagonia. In: XVII Reunión Científico Técnica de la Asociación Argentina de Veterinarios de Laboratorios de Diagnóstico (Santa Fe, Argentina).

Maes, L., Lauwers, H., Deckers, W., Vanparijs, O., 1988. Flukicidal action of closantel against immature and mature *Fasciola hepatica* in experimentally infected rats and sheep. Res. Vet. Sci. 44, 229–232.

Malone, J., Ramsey, R., Loyacano, A., 1984. Efficacy of clorsulon for treatment of mature naturally acquired and 8-week-old experimentally induced *Fasciola hepatica* infections in cattle. Am. J. Vet. Res. 45, 851–854.

McCann, C.M., Baylis, M., Williams, D.J., 2010. The development of linear regression models using environmental variables to explain the spatial distribution of *Fasciola hepatica* infection in dairy herds in England and Wales. Int. J. Parasitol. 40, 1021–1028.

Michiels, M., Meuldermans, W., Heykants, J., 1987. The metabolism and fate of closantel (Flukiver) in sheep and cattle. Drug Metab. Rev. 18, 235–251.

Olaechea, F., Lovera, V., Larroza, M., Raffo, F., Cabrera, R., 2011. Resistance of *Fasciola hepatica* against triclabendazole in cattle in Patagonia (Argentina). Vet. Parasitol. 178, 364–366.

Overend, D.J., Bowen, F.L., 1995. Resistance of *Fasciola hepatica* to triclabendazole. Aust. Vet. J. 22, 275–276.

Poulin, R., 1997. Egg production in adult trematodes: adaptation or constraint? Parasitology 114, 195–204.

Solana, H.D., Rodriguez, J.A., Lanusse, C.E., 2001. Comparative metabolism of albendazole and albendazole sulphoxide by different helminth parasites. Parasitol. Res. 87, 275–280.

Toner, E., Brennan, G.P., Hanna, R.E.B., Edgar, H.W.J., Fairweather, I., 2011. Disruption of egg formation by Fasciola hepatica following treatment in vivo with triclabendazole in the sheep host. Veterinary parasitology 177 (1), 79–89.

Toner, E., Brennan, G.P., Hanna, R.E., Edgar, H.W., Fairweather, I., 2011. *Fasciola hepatica*: time-dependent disruption of spermatogenesis following in vivo treatment with triclabendazole. Parasitol. Res. 109, 1035–1043.

Valero, M.A., Darce, N.A.N., Panova, M., Mas-Coma, S., 2001. Relationships between host species and morphometric patterns in *Fasciola hepatica* adults and eggs from the northern Bolivian Altiplano hyperendemic region. Vet. Parasitol. 102, 85–100.

von Samson-Himmelstjerna, G., Coles, G.C., Jackson, F., Bauer, C., Borgsteede, F., Cirak, V.Y., Demeler, J., Donnan, A., Dorny, P., Epe, C., 2009. Standardization of the egg hatch test for the detection of benzimidazole resistance in parasitic nematodes. Parasitol. Res. 105, 825–834.

WHO, 2006. Report of the WHO Informal Meeting on Use of Triclabendazole in Fascioliasis Control. World Health Organization, Headquarters Geneva, pp. 17–18.

Williamson, R.L., Metcalf, R.L., 1967. Salicylanilides: a new group of active uncouplers of oxidative phosphorylation. Science 158, 1694–1695.

Wood, I.B., Amaral, N.K., Bairden, K., Duncan, J.L., Kassai, T., Malone, J.B., Pankavich, J.A., Reinecke, R.K., Slocombe, O., Taylor, S.M., Vercruysse, J., 1995. World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) Second edition of guidelines for evaluating the efficacy of anthelmintics in ruminants (bovine, ovine, caprine). Vet. Parasitol. 58, 181–213.

María Victoria Solana Veterinary; PhD Student, and Professor of Biology Cell and Systemic in Veterinary Medicine at the National University of Central Prov. Bs. As (UNCPBA).

Roberto Mera y Sierra Master in tropical diseases; Veterinary and Professor of Parasitology and Parasitic Diseases, in Faculty of Veterinary Science, University JA Maza (UMAZA), Guaymallen, Argentina. Actually he work as researcher in the Regional Centre for Research in Parasitology (CIPAR) at UMAZA.

Silvana Scarcella PhD in Animal Science; Veterinary and Professor of Biology Cell and Systemic in Veterinary Medicine at the National University of Central Prov. Bs. As (UNCPBA). Actually work as researcher in the National Scientific and Technical Research Council (CONICET).

Neira Gisela Veterinary Student in Faculty of Veterinary Science, University JA Maza (UMAZA), Guaymallen, Argentina. Actually she work as scholarship holder in the Regional Centre for Research in Parasitology (CIPAR) at UMAZA.

Hugo D. Solana PhD in Animal Science Veterinary and Professor of Biology Cell and Systemic in Veterinary Medicine at the National University of Central Prov. Bs. As (UNCPBA). Actually, he is the Director of the Laboratory of Biology Cellular and Molecular UNCPBA.