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FENOTIPAJE DE INDIVIDUOS DEL GENERO *Fasciola* Linnaeus, 1758 (Trematoda: Fasciolidae) DE AREAS ENDEMICAS CON CARACTERISTICAS EPIDEMIOLOGICAS HETEROGENEUS

PHENOTYPING OF INDIVIDUALS OF THE GENUS *Fasciola* FROM HETEROGENEUS ENDEMIC AREAS

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Los abajo firmantes, Dra. MARÍA ADELA VALERO ALEIXANDRE, Profesora Titular del Departament de Biologia Cel.lular i Parasitologia de la Facultat de Farmàcia de la Universitat de València y el Prof. Dr. Dr.h.c. SANTIAGO MAS-COMA, Catedrático de Parasitología del citado Departamento, por la presente:

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Que Doña Raquel Vieira Peixoto ha realizado íntegramente el trabajo titulado "Fenotipaje de individuos del género *Fasciola* Linnaeus, 1758 (Trematoda: Fasciolidae) de áreas endémicas con características epidemiológicas heterogéneas" en el laboratorio del Departamento antedicho de la Facultat de Farmàcia de la Universitat de València bajo su dirección y con el fin de optar al Grado de Doctor.

Y para así conste a los efectos oportunos, firman la presente

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O amor de Deus transcende qualquer conhecimento humano.

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PEIXOTO (R.V.), 2015.- Fenotipaje de individuos del género *Fasciola* Linnaeus, 1758 (Trematoda: Fasciolidae) de áreas endémicas con características epidemiológicas heterogéneas. Tesis doctoral (Dirs. M.A. Valero y S. Mas-Coma), Facultat de Farmàcia, Universitat de València, pp. 152.

Abstract

Fascioliasis is a zoonotic disease emerging in numerous parts of the world. Fascioliasis is an important food-borne parasitic disease caused by the two trematode species, *Fasciola hepatica* and *Fasciola gigantica*. The characterisation and differentiation of *Fasciola* populations is crucial to control the disease, given the different transmission, epidemiology and pathology characteristics of the two species. Furthermore, in any endemic area, the characterisation of scenarios and patterns of infection must always be considered the starting point before implementing any control measure. Morphometric analyses were made with a computer image analysis system (CIAS) applied on the basis of standardised measurements and the logistic model of the body growth and development of fasciolids in the different host groups. The specific results have been grouped into three parts. A) The phenotypic features of fasciolid adults infecting bovines inhabiting Pakistan and Bangladesh have been studied to characterize fasciolid populations involved. Since it is the first time that such a study has been performed, the results are compared to pure fasciolid populations, (i) *F. hepatica* from the European Mediterranean area (Spain and France) and (ii) *F. gigantica* from Burkina Faso, i.e. geographical areas where both species do not co-exist. Only parasites obtained from bovines were used. The multivariate analysis used showed that the characteristics of fasciolids from Pakistan and Bangladesh are between *F. hepatica* and *F. gigantica* standard populations. These results demonstrate the existence of fasciolid intermediate forms in the endemic area studied in Pakistan and Bangladesh, respectively. These results are analysed by considering the present emergence of animal fascioliasis, the local lymnaeid fauna, the impact of climate change, and the risk of human infection in the country. B) Fascioliasis caused by *F. hepatica*, *F. gigantica* and intermediate forms is present in Guilan province, a complicated epidemiological situation where the highest human infection rates have been

described in Iran. Morphometric tools were used to analyse the possible relationship between liver-fluke metric traits and geographical and altitudinal distribution. This is the first study in which a detailed distribution of both *Fasciola* species is analysed in a human fascioliasis endemic area with a zonal overlap transmission pattern. An accurate analysis was conducted to phenotypically discriminate between fasciolids from naturally infected livestock (cattle, buffaloes, sheep and goats). The distribution of the *F. hepatica*-like (F.h.) and *F. gigantica*-like (F.g.) flukes detected in each liver versus altitude in each group (m) was analysed. The presence of (F.g.) specimens mainly in locations below sea level (average: 11.23 % (F.h.) , 88.77 % (F.g.), the presence of both species with similar intensity at 1-99 m (average: 56.95 % (F.h.) , 43.05 % (F.g.) and the presence of F.h. specimens mainly from 100-999 m (average: 71.69 % F.h., 28.31 % F.g.) as well as in locations with an altitude above 1,000 m (average: 97.48 % F.h., 2.52 % F.g.) are noteworthy. A significant positive correlation was obtained between altitude and % F.h., and a significant negative correlation was obtained between altitude and % F.g. The results show that F.g. populations in cattle, buffaloes and sheep share larger size values, but smaller specimens are present mainly in lowland populations located below sea level, independently of the host species (cattle, buffalo). F.g. from lowland cattle presented a larger worm size variability. Four different fascioliasis transmission areas may be distinguished in Guilan: a) lowland coastal areas neighbouring the Caspian Sea shore, below sea level, where basically *F. gigantica*-like specimens are found; b) a coastal plain with an altitude between 1-100 m where both species co-exist; c) areas with altitude values of 100-999 m where mainly *F. hepatica*-like specimens are found; d) highland mountainous areas, where basically *F. hepatica*-like specimens are found. The study of the influence of the host species on the liver fluke was also carried out by a size-out analysis. This is the first report concerning the decisive influence exercised by the host species on the metric traits of *F. gigantica* adults. C) The phenotypic features of *F. hepatica* adults infecting cattle, sheep and wild boars inhabiting Galicia (Spain) have been studied. Specimens presents a similar body development and gravidity. Our study shows for the first time that the *F. hepatica* uterus from the wild boar presents an intermediate size between that found in primary reservoir hosts such as cattle and sheep, i.e., the individual potential egg output capacity of the wild boar does not greatly differ from that detected in Galician live-stock. These results show

that *F. hepatica* in Galicia has a normal development in wild boars, presenting its own characteristics in shape and size in comparison with other host species. The normal fluke development in the liver, suggest a possible role of this species as a secondary reservoir in this region.

Key words: *Fasciola hepatica*, *Fasciola gigantica*, intermediate forms, morphometric analysis, altitudinal relationships, geographical distribution, host influence, cattle, buffaloes, zebu, sheep, goats, wild boars, Pakistan, Bangladesh, Guilan, Iran, Spain, France, Burkina Faso.

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Capítulo 1

Introducción

Introduction

La fascioliasis es una enfermedad parasitaria humana y animal causada por dos especies de trematodos pertenecientes a la familia Fasciolidae: *Fasciola hepatica* Linnaeus, 1758 y *Fasciola gigantica* Cobbold, 1856. El aumento de zonas endémicas humanas en Europa, las Américas, Asia y África en las últimas décadas (CHEN & MOTT, 1990; MAS-COMA et al., 1999, 2009a, 2014a) ha cambiado su estado de enfermedad zoonótica secundaria a enfermedad zoonótica emergente o re-emergente. Se estima que 17 millones de personas presentan fascioliasis por todo el mundo (MAS-COMA et al., 2014c). Estas cifras están todavía lejos de la realidad, pues la enfermedad no está debidamente diagnosticada en países en vías de desarrollo, países que más sufren la enfermedad. El número de citas de casos humanos ha aumentado en muchos países de los cinco continentes. Resultados recientes de estudios sobre patogenicidad e inmunidad están en la base de la decisión de considerar a la fascioliasis como una enfermedad parasitaria humana importante (MAS-COMA et al. 1999) e incluirla como una trematodiasis transmitida por alimentos de prioridad dentro de la agenda de la Organización Mundial de la Salud (WHO 2013). La patogenia ocurre principalmente por la migración de las formas juveniles de los fasciólidos hasta su llegada a los conductos biliares. Los signos clínicos que desencadenan son reacciones alérgicas e inmunológicas. En la fascioliasis humana, las complicaciones que podemos encontrar son hemorragias y cirrosis biliar, las cuales pueden ser las principales causas de muerte, si bien la muerte es rara en esta infección (CHEN & MOTT, 1990). Sin embargo, la fascioliasis ha demostrado ser altamente patógena, no sólo durante la fase aguda como también en su fase crónica. Este aspecto es de gran importancia en las zonas endémicas humanas de los países en desarrollo, donde la mayoría de los sujetos infectados, principalmente niños, se detectan en la etapa crónica (VALERO et al., 2003, 2006a, 2008). Hay descritos casos de fascioliasis ectópica en el tracto gastrointestinal, pared abdominal, páncreas, bazo, tejido subcutáneo, corazón, vasos sanguíneos, pulmón y cavidad pleural, músculo esquelético, apéndice y epidídimo (MAS-COMA & BARGUES, 1997).

1.1. Sistemática y morfología de los agentes etiológicos implicados

Según los trabajos de YAMAGUTI (1958), PANTELOURIS (1965), TAYLOR (1964), SCHELL (1970), ODENING (1971), BORCHET (1981), BORAY (1982), BOCH & SUPPERER (1982), EUZEBY (1984); URQUHART et al., (1987), la clasificación taxonómica de las especies de Trematodos en estudio quedaría como sigue:

Phylum: Platyhelminthes, 1872
Clase: Trematoda, 1808
Subclase: Digenea, 1858
Orden Echinostomatida, 1957
Suborden Prosostomata Odhner, 1905
Familia Fasciolidae Railliet, 1985
Género *Fasciola* Linnaeus, 1758
Especie *F. hepatica* Linnaeus, 1758
Especie *F. gigantica* Cobbold, 1855

La fase adulta de *F. hepatica* presenta un cuerpo foliáceo, aplanado dorso ventralmente, con una longitud comprendida entre 20 y 30 mm y una anchura variable entre 6 y 13 mm (MAS-COMA & BARGUES, 1997). Por otra parte, *F. gigantica* presenta un tamaño de 24 a 76 mm de largo y de 5 a 13 mm de ancho. Por lo tanto, *F. gigantica* es más alargada y estrecha, con paredes laterales, tendiendo a ser paralelas y con los hombros inexistentes o menos marcados (PERIAGO et al., 2006). Ambas especies comparten muchos rasgos morfológicos. Su cuerpo está cubierto por pequeñas espinas que se dirigen hacia atrás, cuya función es mantener la posición del parásito en el interior de los conductos biliares, así como erosionar su epitelio y el de los vasos sanguíneos. Poseen una ventosa oral en el extremo anterior, otra ventral, a la altura de lo que se podría llamar hombros. El sistema digestivo comprende la boca, situada en la ventosa oral, la prefaringe, la faringe, el esófago y los ciegos intestinales. Los ciegos intestinales se bifurcan a poca distancia de la ventosa oral, formando ramas primarias y secundarias que se extienden

hasta la parte posterior del cuerpo. El poro genital se abre anteriormente a la ventosa ventral (QUIROZ, 2000; URQUHART & ARMOUR, 2001). En *F. gigantica* los ciegos están más ramificados que en *F. hepatica* (PERIAGO et al., 2006). El ovario ramificado está localizado en el lado derecho, posteriormente al acetábulo. A partir del ovario se origina un oviducto estrecho. Tras el ootipo se forma un útero tubuloso y ondulado. Las glándulas vitelógenas están formadas por células arracimadas en forma de folículos, situadas en los márgenes laterales del cuerpo, cuya función es la de producir sustancias de reserva. Presenta dos testículos ramificados, uno anterior y el otro posterior, que ocupan una considerable porción del cuerpo del parásito. A partir de ellos, se originan dos conductos eferentes que se dirigen hacia la parte anterior y se unen para formar el conducto deferente en la base de la bolsa del cirro, a nivel de la ventosa ventral. La primera parte de este conducto es ancho y forma la vesícula seminal. Posteriormente sufre un estrechamiento y se rodea de glándulas prostáticas, terminando en el órgano copulador, o cirro, bien desarrollado (OLSEN, 1977). El sistema excretor está formado por solenocitos o células en flama que están conectados a túbulos que liberan los productos de desecho del parásito a través de un poro ubicado en la porción terminal del cuerpo (FAIRWEATHER et al., 1999). Los huevos de las especies de *Fasciola* son operculados, ovalados, amarillentos y no embrionados en el momento de la puesta. En el extremo opuesto al opérculo puede identificarse, ocasionalmente, un área irregular, si bien este carácter tiene variabilidad poblacional (VALERO et al., 2009a). Los huevos de *F. hepatica* suelen medir entre 130 y 150 µm de largo por 63/90 µm de ancho, mientras que los de *F. gigantica* son un poco mayores en longitud y anchura, aunque morfológicamente indistinguibles de los de *F. hepatica*, estando relacionadas sus dimensiones con la especie del hospedador definitivo que aloje al parásito (VALERO et al., 2001b, 2002, 2009a).

1.2. Ciclo biológico

El ciclo de *F. hepatica* se describió por primera vez por THOMAS, (1883). El ciclo biológico (Fig. 1.1) del parásito presenta dos estadios de multiplicación, uno sexuado en el hospedador definitivo vertebrado, y el otro asexuado en el hospedador intermediario invertebrado.

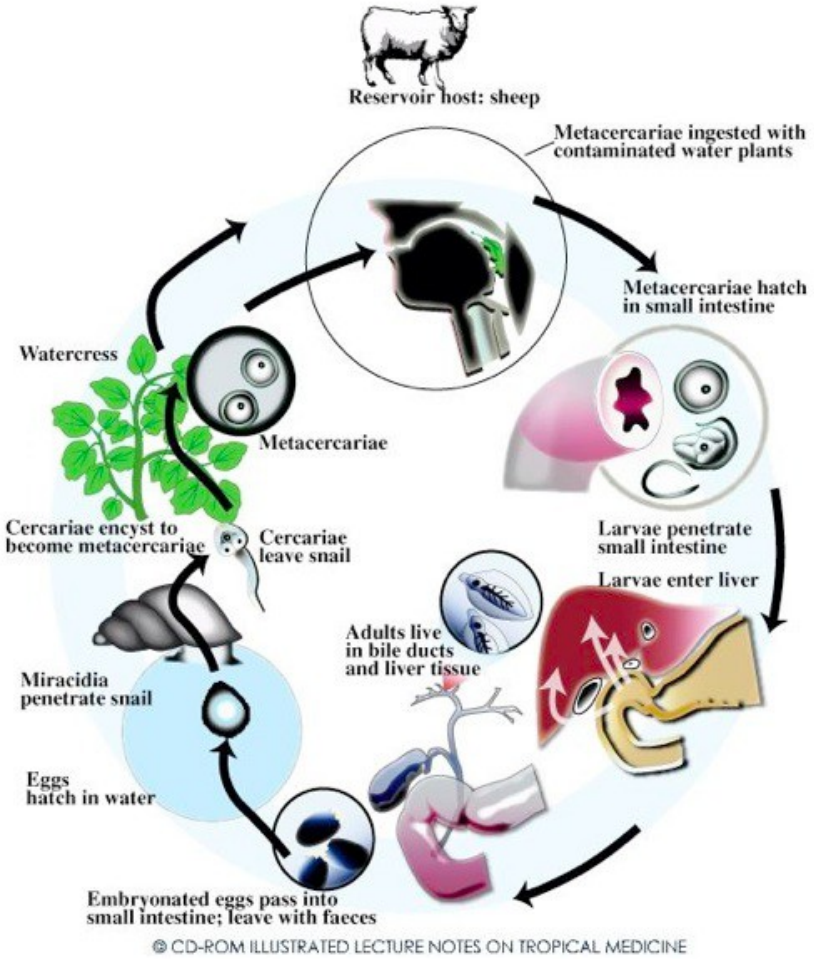

FIGURA 1.1: Ciclo biológico de *Fasciola hepatica*. Imagen tomada del CD-ROM Tropical Medicine. (PETERS & PASVOL, 2006).

El ciclo biológico de ambas especies es similar y dura 14–23 semanas (MAS-COMA et al., 2014c). Los vectores de *F. hepatica* y *F. gigantica* son los caracoles gasterópodos de agua dulce de la familia Lymnaeidae. Las diferentes especies de lymnaeidos que transmiten a los dos fasciólidos muestran una especificidad marcada y diferente. Hay especies de lymnaeidae que no pueden transmitir fasciólidos, otras especies de lymnaeidos que sólo transmiten *F. hepatica*, otras especies que transmiten *F. gigantica* y muy pocas que son capaces de transmitir a las dos especie fasciólidos. *Fasciola hepatica* principalmente es transmitida por una especie de pequeño tamaño que pertenece al grupo *Galba/Fossaria* (BARGUES et al. 2007,

2011a), incluyendo *Galba truncatula* como vector principal, único en Europa, pero que también se presenta en África, Asia y Sudamérica. Otra especie de este grupo actúa como vector en las Américas *Lymnaea tomentosa* es el transmisor en Australia. *Fasciola gigantica* es transmitida por especies del género *Radix*, principalmente *R. natalensis* en África y variedades de *R. auricularia* y *R. viridis* en Asia. En Europa, sin embargo, las especies de *Radix* no muestran ninguna importancia epidemiológica, dado que sólo está presente *F. hepatica* (BARGUES et al. 2001). *Pseudosuccinea* es un género monoespecífico que incluye a la especie *P. columella*, que ha colonizado todos los continentes, y que parece ser capaz de transmitir a ambas especies de *Fasciola* (BARGUES et al. 2011c). La presencia de los lymnaeidos vectores no sólo define la distribución geográfica de la fascioliasis, sino también puede explicar tanto la distribución de infección humana dentro de un país, como recientemente se ha observado en Venezuela (BARGUES et al. 2011b) y Chile (ARTIGAS et al. 2011), como, dentro de una zona endémica, que la transmisión sea estacional o permanente (MAS-COMA et al. 1999). De forma similar a otras enfermedades parasitarias humanas, los focos de transmisión están distribuidos de forma "parcheada" dentro de una zona endémica. Como hospedadores definitivos, además del humano, pueden actuar una gran variedad de mamíferos. Los trematodos adultos parasitan los canales biliares y la vesícula biliar del hospedador definitivo. Como hospedadores reservorios actúan especies de ganado doméstico, incluyendo especies representantes de las familias Bovidae, Equidae, y Camelidae. Los mamíferos silvestres también pueden contraer la enfermedad, actuando también como reservorios. Por ejemplo se han citado especies de reservorios silvestres pertenecientes a Cervidae, Marsupialia y Lagomorpha (BORAY, 1969). Así, cabe destacar principalmente como hospedadores a ovejas, cabras, bovinos y muchos otros animales herbívoros domésticos y silvestres, incluyendo caballos, asnos, mulas y también camélidos del Viejo y el Nuevo Mundo. Búfalos, ciervos, la oveja salvaje, el cerdo salvaje, varios marsupiales, el conejo, la liebre y la nutria son también hospedadores susceptibles. Los cerdos domésticos también puede ser parasitados, pero este hospedador por lo general muestra una resistencia natural más alta contra la duela del hígado (MAS-COMA & BARGUES, 1997). El ganado ovino y el ganado bovino son las especies más afectados por *F. hepatica*, mientras que el búfalo parece ser el hospedador más importante para *F. gigantica*. Entre los hospedadores silvestres definitivos en Europa, *F. hepatica* parece estar menos

adaptada al corzo (*Capreolus capreolus*) cuando se compara con otros ciervos (el ciervo rojo y el gamo), la nutria (*Myocastor coypus*) actúa como un reservorio importante en Francia y quizás también en áreas de Sudamérica, de donde procede. El humano es un hospedador susceptible a la infestación por ambas especies de *Fasciola* (MAS-COMA et al. 2009a). El ciclo consta de 7 fases:

- desarrollo del adulto en el hospedador definitivo;
- eliminación de los huevos desde el hospedador definitivo;
- desarrollo de los huevos en el ambiente;
- formación del miracidio y búsqueda del hospedador intermediario en el agua;
- desarrollo de los estadios larvarios en los caracoles;
- salida de las cercarias de los caracoles y su enquistamiento;
- e ingestión de las metacercarias por el hospedador definitivo.

Empezando por el parásito adulto en los canalículos biliares hepáticos del hospedador definitivo, un parásito adulto pone entre 8,000 y 25,000 huevos al día. A pesar de que el trematodo adulto es hermafrodita, se cree que la fecundación cruzada entre dos parásitos adultos es la forma más común de reproducción sexuada (CHEN & MOTT, 1990). Los huevos no embrionados pasan a través del hígado hasta las heces. Las condiciones anaeróbicas encontradas en el bolo fecal impiden cualquier desarrollo y, por lo tanto, sólo los huevos libres en agua van a embrionar y eclosionar. ROWCLIFFE & OLLERENSHAW (1960) indican que los huevos pueden mantener su viabilidad en el interior de las heces por un periodo variable entre unas semanas a varios meses, dependiendo de la humedad. Respecto a su desarrollo, éste está condicionado por la temperatura ambiental. Así, se requiere un periodo de 6 meses para su completo desarrollo cuando la temperatura es de 10 °C, sólo son necesarios 8 días cuando la temperatura es de 30 °C, y por encima de 30 °C, la mortalidad de los huevos se incrementa. Temperaturas más bajas a 10 °C inhiben su desarrollo. Sin embargo, se ha demostrado que pueden sobrevivir a condiciones invernales desfavorables, eclosionando en la siguiente primavera, pero con una alta mortalidad. La intensidad de la luz, la tensión de oxigeno, así como el

pH del medio pueden influir significativamente en su desarrollo. Cuando los huevos se activan por la luz, los miracidios alteran la permeabilidad de la membrana del huevo, resultando un aumento de la presión interna, con la posterior apertura del opérculo (WILSON, 1969). Después de la eclosión, el miracidio tiene una expectativa de vida de aproximadamente 24 horas, y en este intervalo de tiempo debe encontrar al hospedador intermediario. Su longevidad esta influenciada por la temperatura medioambiental. Así, la vida media de un miracidio disminuye desde 36 horas a 6 °C hasta 6 horas a 25 °C, mientras que a 10 °C es de aproximadamente 24 horas (ROWCLIFFE & OLLERENSHAW, 1960). Como el miracidio tiene energía para sólo 24 horas, la penetración en el caracol ocurre tan pronto lo encuentre. En cuanto entre en contacto con él, tras la penetración, pierde los cilios y se transforma en esporocisto. Tras la formación de redias madres e hijas, se producen múltiples cercarias, entre la 5 a 7 semana post-infección (HOPE CAWDERY et al., 1978). El proceso de reproducción asexuada dentro del caracol permite a un único miracidio producir de 10 a 700 metacercarias. La longevidad de la metacercaria en el medio externo es dependiente de la edad (VALERO & MAS-COMA, 2000). Se ha citado que en condiciones ideales, las metarcercarias permanecen viables durante 5 meses en heno seco (HASEEB et al., 2002), de 6 a 7 meses en periodos invernales (ROWCLIFFE & OLLERENSHAW, 1960), o durante 2 o 3 meses con bajas temperaturas y escasa humedad (ENIGK & HILDEBRANDT, 1964). De modo general, las metacercarias pueden mantenerse viables en el medio ambiente durante largos periodos, si las condiciones de humedad son superiores al 70 %. Una vez ingerida la metacercaria por el hospedador definitivo, la metacercaria, se va a desenquistar y tornarse activa en dos pasos, primero en el rumen/estómago por la temperatura y la concentración de dióxido de carbono, seguidamente, al pasar el duodeno, la presencia de las enzimas y sales biliares van a estimular el desquistamiento. Una vez libre del quiste, la forma joven del parásito va a penetrar en la mucosa intestinal, entrando en la cavidad peritoneal, en general a las 24 horas, migrando hasta llegar al hígado del hospedador definitivo. Después de pasar la cápsula de Glisson, empieza su periodo de migración a través del parénquima hepático. Va a crecer y alimentarse en el parénquima y después penetrará en los canalículos biliares, para completar su desarrollo a adulto (ANDREWS, 1999). El periodo prepatente, o sea, el tiempo transcurrido entre la infección hasta que se puedan detectar huevos en heces, es, en ratones de 6 semanas, en ovino de 8 a 10

semanas, y en bovinos entre 12 y 15 semanas. Una vez adultos, los trematodos pueden vivir por un periodo variado de tiempo, entre 9 a 12 meses en vacunos, hasta 11 años en ovejas y 9 a 13 años en el hombre (MAS-COMA et al., 2014c).

1.3. Heterogeneidad epidemiológica de la fascioliasis humana

Un análisis global de la distribución geográfica de casos humanos muestra que la esperada correlación entre la fascioliasis animal y humana sólo aparece en un nivel básico. Así, altas prevalencias en humanos no necesariamente se relacionan con áreas en donde la fascioliasis es un gran problema veterinario. En la actualidad se conoce que la fascioliasis humana es un problema de salud en países Andinos (Bolivia, Perú, Chile, Ecuador), el Caribe (Cuba), África del Norte (Egipto), Oriente Próximo (Irán y países vecinos), Sudeste Asiático (Vietnam) y Europa Occidental (Portugal, Francia y España) (ESTEBAN et al., 1998; MAS-COMA et al., 2005, 2009a). En áreas de hiperendemia humana se presenta con mayor prevalencia y con mayores cargas parasitarias en niños (con un pico en la categoría de edad entre los 9-11 años), si bien los sujetos adultos también están parasitados. Los sujetos adultos pueden mantener a los parásitos adquiridos cuando eran jóvenes o bien pueden re-infectarse debido al alto riesgo de infección. Vale la pena mencionar el efecto de género en la fascioliasis humana. Las prevalencias y/o intensidades en áreas humanas hiperendémicas parecen ser considerablemente más altas en mujeres. En países Andinos, las mujeres emiten un mayor número de huevos que los varones (ESTEBAN et al., 1999, 2002).En Egipto, la prevalencia en mujeres es considerablemente más alta que en varones (ESTEBAN et al., 2003). Después de muchos años de estudio sobre diferentes áreas que presentan la parasitación humana por fasciólidos por todo el mundo, la clasificación de las situaciones epidemiológicas propuestas por MAS-COMA et al. (1999) todavía es totalmente válida y útil. Esta clasificación incluye las siguientes situaciones: (1) casos autóctonos, aislados, no constantes; (2) casos importados; situaciones endémicas incluyendo (3) hipoendemia, (4) mesoendemia y (5) hiperendemia; y también situaciones epidémicas que comprenden (6) epidemias en zonas no endémicas humanas pero endémicas de animales y (7) epidemias en zonas endémicas humanas. La fascioliasis humana

presenta un amplio espectro de patrones de transmisión y modelos epidemiológicos, incluyendo desde áreas hipoendémicas hasta áreas hiperendémicas. Esta variedad se relaciona con una gran diversidad de entornos, incluyendo diferentes situaciones de endemia/epidemia; demografías humanas diferentes, razas, dietas, hábitos, tradiciones y religiones; diferentes especies de reservorios mamíferos domésticos y silvestres; diferentes especies de lymnaéidos transmisoras; localizaciones tanto en el hemisferio norte como en el hemisferio sur; altitudes oscilando entre 27 m por debajo del nivel del mar hasta 4,200 m; precipitaciones anuales escasas o pronunciadas; potencial de evapotranspiración medio anual bajo o alto; y estaciones con ausencia de período seco o con ausencia de periodo de lluvias. Estas áreas incluyen desde altiplanos a valles, de islas a continentes, de sistemas de irrigación naturales a artificiales, de lagos a lagunas, de grandes ríos a pequeñas corrientes y de cuerpos de agua permanentes a temporales (MAS-COMA et al., 2003). MAS-COMA (2005) propuso una clasificación del modelo de transmisión, la cual ha sido actualizada progresivamente para ofrecer una línea de partida de futuras investigaciones (MAS-COMA et al., 2009a). Hasta la actualidad, se han distinguido los siguientes patrones: (1) un modelo de alta altitud en países Andinos incluyendo el modelo altiplánico y el modelo de valle (VALERO et al., 2012c), (2) un modelo caribeño insular, (3) un modelo relacionado con tierras bajas Afro mediterráneas, (4) un modelo relacionado con los alrededores del mar Caspio y (5) un modelo relacionado con tierras bajas del sudeste asiático. MAS-COMA et al. (2009a) ha propuesto el término de "superposición zonal" para designar a zonas endémicas incluyendo, tanto tierras de altitud con transmisión de *F. hepatica* como tierras bajas vecinas con transmisión de *F. gigantica*. Se incluyen en este tipo de patrón epidemiológico de "superposición zonal" zonas endémicas de países africanos como Etiopía (YILMA & MALONE, 1998) o el sur de Tanzania (WALKER et al., 2008), y también en países asiáticos como la parte norte de la provincia de Punjab en Pakistán (KENDALL, 1954; KENDALL & PARFITT, 1959; AFSHAN et al., 2013, 2014) o la provincia de Guilan en Irán (ASHRAFI et al., 2006a, b, 2007). Desde un punto de vista epidemiológico, estas áreas de fascioliasis lamentablemente no han sido evaluadas suficientemente para distinguir si todas ellas presentan o no un modelo de transmisión y una situación epidemiológica uniforme. Desde un punto de vista de salud pública, el problema de infección humana sólo ha sido destacado en la provincia iraní de Guilan, mientras que en las otras áreas indicadas hasta la fecha

los casos humanos no tienen un gran impacto. Concretamente, no se han descrito ninguna infección humana en zonas de altitud o zonas bajas de Tanzania, se han citado tasas de infección humanas relativamente bajas en Etiopía (BAYU et al., 2005; FENTIE et al., 2013), y un área relativamente restringida con prevalencias en humanos en Pakistán (QURESHI et al., 2005; QURESHI & TANVEER, 2009; AFSHAN et al., 2014).

1.4. Estacionalidad e impacto del cambio climático y cambio global

Los factores climáticos son decisivos en la transmisión de la fascioliasis, principalmente la temperatura, la precipitación y/a la evapotranspiración potencial (MAS-COMA et al., 2009b). Principalmente la variación de la precipitación y la temperatura da lugar a la diferente estacionalidad de la fascioliasis. En Europa, la transmisión de la enfermedad es típicamente bi-estacional, debido a los períodos de actividad del lymnaeido vector en primavera y otoño. En el Altiplano boliviano, sin embargo, la transmisión ocurre a lo largo del año, ya que las poblaciones del lymnaeido vector están siempre presentes debido a que habitan cuerpos de agua permanentes en vez de temporales, ante las altas tasas evapotranspiración de las áreas geográficas de la alta altitud (MAS-COMA et al., 1999). En otras áreas, la transmisión aparece mono-estacional, debido a la existencia de sólo un período intra-anual con disponibilidad de agua. El cambio climático solapado con las modificaciones antropogénicas y ambientales se incluyen en el amplio término de "cambio global" (MAS-COMA et al., 2009b). Así, la irrigación artificial de los campos de cultivo parece ser suficiente para tener la transmisión de la fascioliasis en el Altiplano peruano (ESTEBAN et al., 2002). En la provincia de Punjab, en Pakistán, la transmisión incluye la bi-estacionalidad, con un pico relacionado con la precipitación natural y otro pico relacionado con la irrigación artificial (AFSHAN et al., 2014). Punjab es la primera zona endémica donde la aparición de infección humana se ha correlacionada con un aumento significativo de riesgo de transmisión de la fascioliasis debido al impacto del cambio climático a lo largo de un período de 20 años (AFSHAN et al., 2014).

1.5. Diagnóstico

Dado al incremento en el número de casos de fascioliasis humana por todo el mundo, es fundamental la caracterización del agente etiológico implicado en la enfermedad. Para el diagnóstico diferencial de *F. hepatica*, *F. gigantica*, híbridos y formas intermedias, los métodos clínicos, patológicos, coprológicos o inmunológicos no son útiles. A pesar de la importancia de poder distinguir entre infecciones por una u otra especie de fasciólido, debido a sus diferentes características de transmisión, epidemiología, patología y control, no hay, lamentablemente, ninguna prueba de coproantígeno directa, ni prueba indirecta inmunológica disponible para su diagnóstico diferencial (VALERO et al., 2009a, b, 2012a, b; MAS-COMA et al., 2014b). De hecho, las herramientas diagnósticas disponibles actualmente son sólo útiles para diferenciar a la fascioliasis de otras enfermedades. Hasta ahora, la diferenciación específica sólo puede ser realizada por estudios fenotípicos de parásitos adultos mediante herramientas morfométricas (PERIAGO et al., 2006; VALERO et al., 2009a, 2012c; ASHRAFI et al., 2006a, 2015; ASHRAFI et al., 2013) o estudios genotípicos por herramientas moleculares (MAS-COMA et al., 2009a). Esto es un problema en las áreas geográficas en las que se superponen las dos especies de fasciólidos, dada la importancia de este diagnóstico diferencial de las formas de fasciólidos implicadas. En el caso de que existan formas intermedias, las medidas del huevo pueden superponerse. Clásicamente, la distinción entre las especies de *Fasciola* se ha hecho en base a su morfología. Sin embargo, en general se ha aceptado que la diferenciación específica de estos trematodos hepáticos no se puede conseguir únicamente por examen morfológico (PERIAGO et al., 2008), siendo necesario un análisis genético detallado (ITAGAKI & TSUTSUMI, 1998; MARCILIA et al., 2002). Además, la hibridación entre diferentes genotipos de *Fasciola* puede dar lugar a la generación de nuevas formas (MAS-COMA et al., 2001). A pesar del desarrollo reciente de muchas pruebas moleculares, la secuenciación del ADN todavía permanece como el único método apropiado para tanto el haplotipaje de la dos especies "puras" de fasciólidos, como para la detección de su hibridación o de formas intermedias. Para tal objetivo, las secuencias completas de los dos espaciadores rDNA ITS-1 e ITS-2 conjuntamente con la secuencia completa de los genes mtDNA cox1 y nad1 hasta la fecha han demostrado ser los marcadores

de elección (MAS-COMA et al., 2009a). Ya se ha publicado la nomenclatura para estos cuatro marcadores (MAS-COMA et al., 2009a). No obstante, las diferencias en solo 5 posiciones para el ITS-1 y otras 5 posiciones en el ITS-2, diferencias típicas de organismos que pueden hibridar, como de hecho ocurre entre estas dos especies de fasciólidos (MAS-COMA et al., 2009a), hace que frecuentemente se utilicen criterios morfológicos para la identificación del agente etiológico.

1.6. Importancia económica de la fascioliasis

La fascioliasis afecta un gran número de animales sobre todo los animales rumiantes de importancia en ganadería (ovejas, vacas, búfalos y cabras). Infecciones agudas con cargas elevadas pueden causar la muerte, sobre todo en el ganado ovino y caprino. También pueden causar alteración de la fertilidad, producción de leche (ganado) y lana (ovejas), así como reducción de la capacidad de trabajo de estos animales. Todos estos déficits colocan a la fascioliasis como una de las enfermedades zoonóticas con un mayor impacto económico global (CWIKLINSKI et al., 2015). La fascioliasis es la enfermedad parasitaria más importante del ganado y una enfermedad de importancia económica en rumiantes en Europa, América, Australia y Nueva Zelanda. El triclabendazol es el fármaco de elección utilizado en programas de control, pero el alto costo del tratamiento impide el uso generalizado por los ganaderos en los países en vías de desarrollo. Por otra parte, la resistencia al triclabendazol ha sido citada en ovinos afectados con *F. hepatica* (OVEREND & BOWEN, 1995), lo que sugiere que la selección de parásitos resistentes a la larga pueden comprometer el uso de este fármaco. Además, a pesar de que la quimioterapia se ha utilizado durante más de dos décadas con cierta eficacia en la reducción de las tasas de morbilidad, ésta quimioterapia es sólo una medida provisional, dejando sin cambios las tasas de transmisión, debido a la continua re-infección en áreas endémicas (SPITHILL et al., 1999; MEEUSEN & PIEDRAFITA 2003). Un estudio realizado por SCHWEIZER et al. (2005) en Suiza sugiere que la prevalencia de infección por *F. hepatica* en el ganado se encontraba por encima del 16 %, representando una pérdida económica media de cerca de 52 millones de Euros, con un rango de entre 22 y 92 millones por año. En el Reino Unido, la prevalencia de la infección en las distintas categorías de ganado

vacuno oscila entre el 45 y 84 % en Irlanda, y las pérdidas anuales se estiman en 60 millones de Euros (TENDLER & SIMPSON, 2008). Para calcular el costo potencial de las pérdidas se requiere el conocimiento de la prevalencia y la intensidad de la infección y la habilidad de utilizar la información meteorológica para predecir las prácticas de manejo zootécnico (QUIROZ, 1995).

1.7. Relevancia e hipótesis del estudio

La diferenciación morfológica específica entre *F. hepatica* y *F. gigantica* en áreas dónde ambas especies se superponen es una tarea a veces muy complicada por distintas razones. En primer lugar, los trabajos previamente realizados no han seguido un método estandarizado de medición morfométrica y por lo tanto los resultados no son comparables. Además, estos estudios no han considerado el crecimiento alométrico que albergan los adultos. También hay que tener en consideración que la especie de hospedador definitivo puede influir en el fenotipo, tanto en la fase adulta de *F. hepatica* como en la de huevo, principalmente debido al tamaño del conducto biliar (VALERO et al. 2001a, b, 2002, 2009a). Sin embargo, la infectividad metacercariana no parece diferenciarse en aislados obtenidos a partir de especies hospedadoras diferentes (VALERO & MAS-COMA, 2000). Las clásicas medidas morfométricas de los ejemplares adultos y huevos de ambas especies en las revisiones clásicas (YAMAGUTI, 1958; WATANABE, 1962; BORAY, 1969, BORCHET, 1981; BOCH & SUPPERER, 1982; URQUHART et al., 1987; MAS-COMA & BARGUES, 1997; MAS-COMA et al., 2000, MAS-COMA, 2004a), demuestran un solapamiento entre ambas especies. Por lo tanto, no se puede disponer de datos concluyentes en muchas ocasiones porque el material parasitario utilizado en las diferentes revisiones generalmente resulta ser una mezcla de diferentes especies hospedadoras. Dado que hay una superposición en la distribución geográfica de *F. hepatica* y *F. gigantica* en áreas tropicales de África y Asia (MAS-COMA et al., 2009a), existen en muchas ocasiones problemas de identificación especifica en estas zonas tanto a nivel humano como animal (MAS-COMA et al., 2009a; ICHIKAWA et al., 2011). En el caso concreto de Asia, el solapamiento en la distribución de *F. hepatica* y *F. gigantica* ha conducido a una controversia que concierne la identidad taxonómica de la especie *Fasciola* encontrada en los países del Extremo Oriente,

en el cual unos se parecen a *F. hepatica*, mientras que los otros se parecen a *F. gigantica*, con formas intermedias también presentes y fenómenos de gametogénesis anormal, diploidia, triploidia y mixoploidia, partenogénesis e hibridación entre genotipos diferentes (MAS-COMA et al., 2009a). Finalmente, ASHRAFI et al. (2006b) describió la presencia de formas intermedias en oriente medio, concretamente en Irán. Ante esta situación morfométrica y genética, las cuestiones radican en que si los fasciólidos presentes en otras áreas de solapamiento fuera del sudeste asiático o Iran son también formas intermedias o híbridas. Estudios multidisciplinares, sin embargo, han demostrado que *F. hepatica* y *F. gigantica* deben ser consideradas como especies válidas, a pesar de su capacidad de hibridación y dar lugar a formas intermedias en las áreas donde se superponen (MAS-COMA et al., 2009a). La meta de este estudio es tratar de contestar estas preguntas desde el punto de vista morfométrico. Se ha realizado el fenotipaje mediante un sistema de análisis de imágenes digitales por ordenador teniendo en cuenta: (i) el uso de medidas estandarizadas, (ii) el crecimiento alométrico, (iii) la influencia del hospedador definitivo, y (vi) la distribución geográfica del hospedador intermediario, dada la especificidad del lymnaeido. Para poder realizar un estudio morfométrico de ambas especies y poder identificar los fasciólidos presentes en las diferentes áreas de endemia, sólo se han utilizado parásitos adultos procedentes de hospedadores naturalmente infectados. Se han utilizado como representantes de *F. hepatica* ejemplares de la zona mediterránea de España y Francia, (MAS-COMA et al., 2003), donde sólo está presente esta especie. Ejemplares de Burkina Faso se han utilizados como representantes de *F. gigantica*, ya que *Radix natalensis* es la única especie de lymnaeido presente es este país y porque *F. gigantica* fue originalmente descrita en una jirafa del África sub-sahariana, encontrada en un zoológico ambulante en Inglaterra (COBBOLD, 1855).

El jabalí (*Sus scrofa* Linnaeus, 1758) es uno de los mamíferos terrestres más ampliamente distribuidos (LUCCINI et al., 2005). El área de distribución originaria incluye el norte de áfrica, Europa, sur de Rusia, China, Oriente Medio, India, Sri Lanka e Indonesia (WILSON & REEDER, 1993). El jabalí es un animal con diferentes tipos de dietas, las cuales incluyen frutas, anfibios, reptiles, setas, aves y sus huevos, pequeños roedores, carroña y larvas de insectos (SOLAYMANI-MOHAMMADI et al., 2003; MOWLAVI et al., 2006). Debido a sus hábitos alimentarios, el jabalí pueden desempeñar un papel significativo en la circulación y el

mantenimiento de ciertos patógenos (ANTOLOVA et al., 2006; MOWLAVI et al., 2006), o sea, puede actuar como reservorio de muchas enfermedades infecciosas de animales domésticos importantes, tales como la clásica peste porcina, la brucelosis y la triquinosis. En el caso de los humanos, actúa de reservorio de enfermedades como la hepatitis E, la tuberculosis, la leptospirosis y la triquinosis (GIBBS, 1997). Las especies de *Fasciola* son capaces de parasitar una amplia gama de animales rumiantes domésticos y silvestres, y el hombre (MAS-COMA et al., 2014c). Todos estos animales actúan como hospedadores definitivos de *Fasciola* sp. Esta gran cantidad de hospedadores sitúa a los fasciólidos entre los trematodos digéridos capaces de parasitar a una mayor gama de hospedadores definitivos, difiriendo de otros trematodos, tales como *Schistosoma mansoni*, patógeno humano, que tiene una gama de hospedadores definitivos mucho más restringida (CWIKLINSKI et al., 2015). Dado que la fascioliasis tiene un carácter zonótico, es importante evaluar la capacidad de los animales de actuar como reservorios silvestres, ya que son condición esencial de la persistencia de la zoonosis en una determinada área geográfica (HUDSON et al., 2002). A pesar de que el jabalí es un animal silvestre, en las últimas décadas, distintos factores, como cambios del hábitat humano a áreas suburbanas y un mayor uso de las tierras para la agricultura y la deforestación (GIBBS, 1997) han favorecido el acercamiento de estos animales al hombre. GORTÁZAR et al. (2003) cita la caza humana del jabalí con el objetivo de mejorar la cosecha y creación de granjas semi-extensivas. Además, POPIOLEK et at. (2010) cita, como otro factor que contribuye al acercamiento jabalí-hombre, el aumento de la población de jabalí, con una tendencia creciente constante desde 1960. Existen pocos trabajos científicos publicados sobre la presencia de *F. hepatica* en el jabalí. Así, cabe destacar la presencia de *F. hepatica* en el jabalí de la Península Ibérica (CORDERO DEL CAMPILLO et al., 1994; DE SOUSA et al., 2004), con un porcentaje de 60,9 % o en el oeste de Escocia (THOMPSON et al., 2009). A pesar de la baja cantidad de estudios que apuntan al jabalí como reservorio de *F. hepatica*, se sabe que el hábito de compartir los mismos pastos y las mismas fuentes de agua que el ganado, favorece la diseminación de la fascioliasis (KUKIELKA et al., 2013). Concretamente, el jabalí se alimenta de especies herbáceas localizadas en los cursos de agua habitados por caracoles de la familia Lymnaeidae, hospedadores intermediarios de *F. hepatica*. El jabalí con fascioliasis

puede defecar en estos cursos de agua, los huevos del parásito emitidos por las heces, en un ambiente propicio de luz, temperatura y humedad, completa el ciclo de la *Fasciola*. El ganado al beber agua en estos sitios pueden ingerir metacercarias, forma infestante del parasito. Finalmente se ha abordado la cuestión de que si los adultos de *F. hepatica* son capaces de alcanzar un desarrollo normal en el jabalí europeo, en comparación con los hospedadores considerados como normales, como son el ganado ovino y bovino.

1.8. Objetivos

El objetivo general de la presente investigación es la caracterización fenotípica de faciólidos adultos de zonas endémicas con características epidemiológicas heterogéneas. En este sentido hemos focalizado el estudio en áreas con modelos de transmisión diferentes, incluyendo áreas en donde ambas especies de fasciólidos coexisten y áreas en donde únicamente existe *F.hepatica*.
En el primer caso, el estudio se ha dirigido a los siguientes objetivos específicos:

- a) caracterizar los individuos de fasciólidos adultos de Pakistán y compararlos con los patrones morfométricos específicos de cada especie, utilizando como previa base, patrones específicos de *F.hepatica* y *F. gigantica* de poblaciones alopátricas (*F.hepatica* de España, y *F. gigantica* de Burkina Faso).
- b) caracterizar los individuos de fasciólidos adultos de Bangladesh y compararlos con los patrones morfométricos específicos de cada especie, utilizando como previa base, patrones específicos de *F.hepatica* y *F. gigantica* en poblaciones alopátricas (*F.hepatica* de España y Francia y *F. gigantica* de Burkina Faso).
- c) evaluar la distribución geográfica (analizando longitud, latitud y altitud) de *F.hepatica* y *F. gigantica* en la provincia de Guilan y establecer una metodología, utilizando herramientas morfométricas, que pueda ser usada para evaluar otras áreas de fascioliasis similares;

- d) analizar la influencia ejercida por la especie hospedadora definitiva sobre los rasgos métricos de los adultos de *F. gigantica* utilizando herramientas morfométricas

En el segundo caso, el estudio se ha dirigido al siguiente objetivo específico:

- e) caracterizar los individuos de *F.hepatica* en el jabalí en España y su comparación con individuos de *F.hepatica* procedentes de especies consideradas como reservorios principales de la enfermedad (ovinos y bovinos) procedentes de la misma área geográfica.

Para abordar estos objetivos, la presente Tesis Doctoral ha sido desglosada en cinco capítulos. Así, tras el presente primer capítulo introductorio, en el segundo de los capítulos incluimos los materiales estudiados procedentes de zonas asiáticas, europeas y africanas. Dentro del mismo capítulo se incluye las técnicas utilizadas, tanto referentes a las técnicas mastozoológicas, como a las técnicas helmintológicas generales, técnicas de imagen computerizadas (CIAS) para el estudio morfométrico y finalmente los análisis estadísticos empleados. En el tercer y cuarto capítulo se aborda el fenotipaje de fasciólidos de países asiáticos, incluyendo Pakistán, Bangladesh e Irán. En cada país se incluye después de los resultados, la pertinente discusión de los mismos. En el quinto capítulo se aborda el fenotipaje de fasciólidos obtenidos en el jabalí europeo, focalizando el estudio en el desarrollo uterino del parasito y centrando la discusión sobre el posible papel de reservorio de esta especie hospedadora silvestre. El sexto y último capítulo comprende las conclusiones alcanzadas.

Capítulo 2

Material y Métodos

Material and Methods

2.1. Material

2.1.1. Zonas de Estudio

2.1.1.1. España

España presenta una superficie territorial de 504 645 km^2. Comparte fronteras terrestres con Francia y con Andorra al norte, con Portugal al oeste y con el territorio británico de Gibraltar al sur. Su territorio está organizado en diecisiete comunidades autónomas, comprende también los archipiélagos de Canarias y Baleares, otras islas menores y las ciudades de Ceuta y Melilla, situadas en el norte del continente africano. El PIB per capita de España, en 2014, fue de 32,360 dólares, por lo que se encuentra en el puesto 32 de 196 países. Su economía está basada principalmente en el turismo, siendo el tercer país más visitado del mundo. La agricultura en España es un sector estratégico de gran importancia social, territorial, medioambiental y económica. La mitad de la superficie de España se destina a actividades agrícolas o ganaderas (el 33 % del territorio corresponde a tierras de cultivo y el 16 % a prados y pastos) y el sector agroalimentario es uno de los más pujantes de la economía española (La Moncloa, Govierno de España, 2015).

El material parasitario estudiado en la presente Tesis Doctoral procede de dos zonas geográficas diferentes, el área mediterránea, en donde se obtuvo material del matadero de Mercavalencia, de la zona de los alrededores de la Albufera de Valencia, en la Comunidad Valenciana y el área atlántica, en donde se obtuvo material de Galicia (véase Fig. 2.1), región localizada en el noroeste de España y que cubre un área de 29 574 km^2 (latitud 42°23‘60N, longitud 7°4’W). En Galicia el área de tierra dedicada a la agricultura comprende 874,379 ha y hay actualmente 548,642 vacas en 36,919 granjas y 279,942 pequeños rumiantes en 25,288 rebaños (Consellería do Medio Rural e do Mar, 2015). Los prados ocupan aproximadamente el 60.0 % de la tierra útil agrícola, y la ganadería por lo general pasta a lo largo

FIGURA 2.1: Mapa de España, situando Galicia.
www. wikipedia. com.

del año. El tipo de ganadería y las características climáticas de la región (clima marítimo) favorecen la transmisión de helmintiasis a través del pasto. Según datos oficiales, se cazaron 27,000 jabalíes a lo largo del período de estudio (de 2007 hasta 2010).

2.1.1.2. Francia

La mediterránea isla de Córcega presenta una superficie de 8 721 km^2, siendo su longitud máxima de 183 km desde el cabo de Córcega hasta el estrecho de Bonifacio. Se sitúa a 12 km de Cerdeña y a 82 km de Italia. Está caracterizada por la insularidad y su pronunciado relieve (altitud media superior a 568 m). El material parasitario estudiado en la presente Tesis Doctoral procede del matadero de Portovechio, en Córcega del Sur (véase Fig. 2.2).

FIGURA 2.2: Ubicación geográfica de la isla de Córcega, Francia. www. wikipedia. com.

2.1.1.3. Burkina Faso

Burkina Faso es un país de África Occidental, con un área de 274 00 km^2, que limita al noroeste con Malí, al noreste con Níger, al sur con Costa de Marfil, Ghana, Togo y Benín. La altitud media es de 400 metros y la diferencia entre el punto más elevado y el más bajo es inferior a 600 metros. En general, Burkina Faso es un país plano. El material parasitario estudiado en la presente Tesis Doctoral procede del matadero de Bobo Dioulaso, la capital del País (véase Fig. 2.3).

FIGURA 2.3: Mapa de Burkina Faso.
www. wikipedia. com.

2.1.1.4. Pakistán

Pakistán, incluye cuatro provincias, Punjab, Sindh, Baluchistán y la Provincia Fronteriza Noroccidental. El país tiene fronteras con la India por el este, con Afganistán por el oeste y el norte, con Irán por el suroeste y con China en su extremo noreste. La superficie de Pakistán es de 796 095 km^2. El PIB es de 167 millones de dólares estadounidenses, lo que le hace la 48° economía más grande del mundo, siendo considerada como la segunda economía más grande en el sur de Asia. Pakistán es un país agrícola. Según datos de la FAO (2014), la agricultura proporciona alimentos e ingresos para 80 % de la población, es el sector más influyente responsable de sacar a las familias más pobres del hambre, de la desnutrición y de la dependencia de ayuda externa. Además, la agricultura tiene una influencia directa sobre si los hogares rurales pueden mantenerse, ofreciendo servicios médicos y manutención de escuelas. Los principales cultivos son de algodón, arroz, caña de

azúcar, maíz y trigo. Sólo estos cultivos representan cerca del 90 % del sector agrario (GUÍA PAÍS PAKISTÁN, 2006). El tipo y método empleado para el cultivo de ciertos alimentos pueden propiciar el ambiente ideal para el desarrollo de ciertas parasitosis. Cabe recordar que los hospedadores intermediarios de fasciólidos son caracoles gasterópodos de agua dulce de la familia Lymnaeidae que necesitan de colecciones de agua dulce adecuadas para su supervivencia. Este ambiente está presente en el cultivo de arroz, donde el método de irrigación es por inundación y estos campos a menudo, permanecen inundados durante toda la temporada de cultivo. La presencia del ambiente ideal para el desarrollo del hospedador intermediario de especies de fasciólidos, añadido a presencia del ganado que representa su principal hospedador definitivo, propicia el ambiente ideal para la incidencia de la fascioliasis animal en Pakistán. No obstante, no sólo el cultivo de arroz propicia el desarrollo del parásito en Pakistán, el país es conocido por su excelente red de canales de riego y tierras agrícolas ricas, con tres grandes ríos. Así, los canales y zanjas cubren alrededor de 1 609 344 kilómetro (ALVI & SHARIF, 1995). Estos cursos de agua y canales agravarían el riesgo de fascioliasis en el país. Según SPITHILL et al. (1999) el uso de estanques para el consumo de agua, hábitat primario del hospedador intermediario de *F. gigantica*, es un riesgo para los animales en el pastoreo. Este fenómeno se ve apoyado por la mayor tasa de infección en animales que utilizan estanques como fuente de agua, seguido por los que utilizan ríos y fuentes (KHAN et al., 2009). Para la población que vive de la agricultura, los animales son una parte fundamental, al ser responsables de arar la tierra y el transporte, además del aporte, en términos de productos, de carne, leche, cueros, etc. La ganadería representa el 46,8 % del valor añadido del sector agrario y el 10,8 % del PNB. Entre 30 y 35 millones de personas viven de la cría de ganado o éste representa una parte fundamental de sus ingresos (GUÍA PAÍS PAKISTÁN, 2006). La parasitosis implica perdidas en la productividad de estos animales y prejuicios no sólo en las familias que dependen del ganado, sino también en el sector agrícola. En Pakistán la fascioliasis es endémica en varios distritos (CHAUDHRY & NIAZ, 1984; MASUD & MAJID, 1984; MAQBOOL et al., 2002; KHAN et al., 2009). Los primeros casos de fascioliasis humana se han citado en las áreas rurales del distrito de Lahore, provincia de Punjab (QURESHI et al., 2005; QURESHI, 2008; QURESHI & TANVEER, 2009). En Punjab, se ha citado una prevalencia del 14,71 % de casos humanos (MAQBOOL et al., 2002), con mayor presencia de

F. gigantica que de *F. hepatica* (KHAN et al., 2009). Además, dentro los bovinos, los más afectados son los búfalos. Según LIGDA (1998) el factor más importante que contribuye la alta presencia de la fascioliasis en el búfalo, es su hábito de vivir en zonas pantanosas, zonas donde están los hospedadores intermediarios del parásito. El área de estudio comprende tierras de regadío, con un sistema de canales de agua bien establecido de la cuenca del río Indo, comúnmente denominada como Punjab y localizada en la zona central de Pakistán. Los búfalos analizados procedían de diferentes distritos de la zona central de Punjab, concretamente de los distritos de Lahore, Faisalabad, Okara, Sahiwal y Jhang (véase Fig. 2.4).

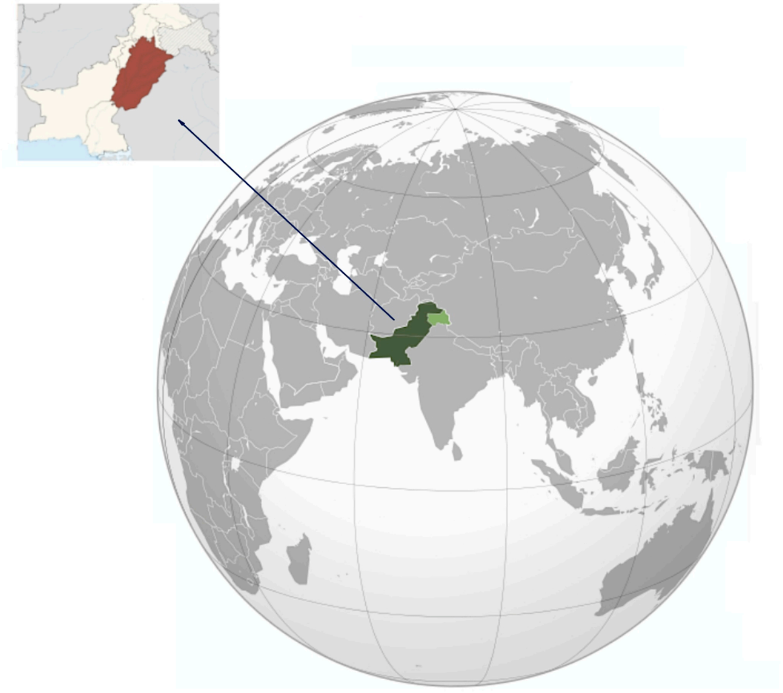

FIGURA 2.4: Mapa de Pakistán, situando la región de Punjab.
www. wikipedia. com.

2.1.1.5. Bangladesh

Bangladesh se encuentra en el sur de Asia y está rodeada casi en su totalidad por la India, a excepción de una pequeña frontera en el sureste con Birmania (Myanmar) y una línea costera a lo largo de la bahía de Bengala (véase Fig. 2.5).

FIGURA 2.5: Mapa de Bangladesh.
www. wikipedia. com.

La superficie de Bangladesh es de 147 570 km^2. Es un país pobre, siendo uno de los países más densamente poblado del mundo y eminentemente rural. Según datos de la CIA WORLD FACTBOOK, el PIB de Bangladesh del año 2012 estuvo distribuido en 17,7 % para agricultura, 28,5 % para industria y 53,9 % para servicios. La participación en el PIB de los subsectores individuales de la agricultura son aproximadamente el 13 % para cultivos, el 3 % para el ganado, 5 % para la pesca y el 2 % para el sector forestal. Bovinos, caprinos y suínos representan el sustento de muchas familias no sólo por la obtención de leche y carne, sino también para la ejecución de los servicios agrícolas. Los datos de CHOWDHURY et al. (2003) apuntan esta realidad, de los 20 millones de cabras en Bangladesh, 10,4 millones de cabras están criadas en sistema de patio trasero por agricultores rurales, especialmente las mujeres pobres o los niños. Estos animales no sólo sirven de sustento para la población más pobre, sino representan una parte importante de la economía rural del país. Existen alrededor de 38,1 millones de pequeños rumiantes (cabras y ovejas) en Bangladesh FAO (2005), representando una cantidad

considerable de divisas por exportación de pieles y otros subproductos (KAMARUDDIN, 2003). Según dados de la FAO (2007), la agricultura en Bangladesh se está transformando gradualmente del sistema de producción de subsistencia a una agricultura comercial. La importancia de los cultivos comerciales tradicionales (yute, caña de azúcar, tabaco, etc.) ha disminuido con el tiempo. Por necesidad, los agricultores están recurriendo a los cultivos de alimentos tales como el arroz, el trigo, frutas y verduras para la producción comercial y para ingresos en efectivo. Análogamente a Pakistán, Bangladesh cultiva el arroz y tiene este cultivo como el cultivo más dominante del país. Como hemos mencionado previamente, las irrigaciones de los arrozales y los estanques aumenta la incidencia de fascioliasis en animales. Bangladesh es uno de los países asiáticos donde la fascioliasis es la enfermedad parasitaria más frecuente en bovinos, búfalos, cabras y ovejas (NOORUDDIN & EL ISLAM, 1996; HOSSAIN et al., 2011). Una encuesta anterior reveló que 71,55 % de los bovinos, el 14,6 % de las ovejas y las cabras y el 100 % de los búfalos se ven afectados por la fascioliasis (BHUIYAN, 1970). Un informe más reciente (BAS-USDA, 2012) mostró que el 10,42 % de ganado, 9 % cabras y 36,61 % de los búfalos están infectados con *Fasciola* sp. El área de estudio de la presente Tesis Doctoral comprende 9 lugares de 8 distritos de cuatro zonas agro-ecologicas diferentes.

2.1.1.6. Irán

Irán limita al norte con Armenia, Azerbaiján, el mar Caspio y Turkmenistán, al este con Afganistán y Pakistán, al oeste con Turquía e Irak y al sur con el golfo Pérsico y el mar de Omán. La superficie de Irán es de 1 648 000 km^2, presentan como ciudades principales Teherán, Tabriz, Meshed, Ispahán, Shiraz, Abadán, Ahwaz y Kermanshah. El PIB per cápita es de 6,359 Dólares USA. Los principales productos de exportación son el petróleo, frutas, tapetes y acero. La economía de Irán está principalmente marcada por la dependencia de las exportaciones de petróleo y gas. A parte, también está presente la agricultura, industria y servicios. El sector agrícola está basado en el cultivo de cereales como el trigo, el arroz, caña de azúcar, árboles frutales, frutos secos y algodón. La produccion ganadera se centra en la producción lana, leche y otros productos lácteos (CIA WORLD

FACTBOOK, 2014). En Asia, el Oriente Próximo aparece como un importante foco de fascioliasis humana. En Irán, la fascioliasis está presente en muchas provincias, incluyendo el Kurdistán, Zanjan, Kermanshah, Mazandaran, Teherán, Azerbaiyán, Gilan, Fars y Juzestán (AMOR et al., 2011). La fascioliasis animal está presente en la mayor parte del país y su prevalencia alcanza hasta el 50 % en algunas áreas (MOGHADDAM et al., 2004a; ROKNI, 2008; MOSHFE et al., 2003). Entre los animales infectados están el ganado vacuno, ovino, búfalos y cabras. La fascioliasis causada por *F. hepatica*, *F. gigantica* y formas intermedias está presente en la provincia Guilan, con una situación epidemiológica complicada, y donde se ha descrito las tasas más altas de infección humana en Iran. El primér brote implicó alrededor de 10,000 personas en 1987 y el segundo brote de alrededor de 5,000 personas, diez años después. La mayoría de los casos humanos en Guilan se detectaron en personas que vivían en las tierras bajas de los distritos de Bandar-Anzali y de Rasht, en una zona situada a 23 m por debajo del nivel del mar. Estos son los brotes de fascioliasis humana que han implicado un mayor número de pacientes hasta el momento. El material de Iran analizado en el presente trabajo procede de Guilan (véase Fig. 2.6), situada a lo largo de las orillas occidentales de Mar Caspio, incluyendo tierras bajas con un llano costero y colinas en donde se presentan los casos humanos de fascioliasis y áreas montañosas (tierras altas). Guilan tiene un clima húmedo subtropical con la precipitación más elevada del país, sobre todo entre septiembre y diciembre. La humedad es muy alta debido al carácter pantanoso de los llanos costeros y puede alcanzar el 90 % en el verano, con temperaturas por encima de 26 °C. La línea de la costa es más fría. Gran parte de la provincia es montañosa, verde y arbolada. El llano costero a lo largo de Mar Caspio se usa principalmente para campos de arroz, con altitudes que se extienden entre 27 m por debajo del nivel de mar y 300 m por encima de nivel de mar, y en donde el 60 % del terreno incluye pantanos, con numerosos canales de irrigación y el cultivo del arroz como la actividad agrícola principal (ASHRSAFI & MAS-COMA, 2014).

FIGURA 2.6: Mapa de Irán, situando la región de Gilan.
www. wikipedia. com.

2.1.2. Material Parasitológico

Todos los ejemplares de fasciólidos utilizados para el estudio morfométrico fueron obtenidos de los conductos biliares de animales naturalmente infectados. Los huevos se recolectaron de los úteros de los Trematodos adultos grávidos, aplicando un poco de presión en el acetábulo para forzar la salida de los huevos maduros a través del poro genital. A continuación se detalla el material analizado, indicando la localización geográfica y especie hospedadora.

Material de España: 81 trematodos adultos de 7 individuos de ganado vacuno (*B. taurus*) del Matadero de Mercavalencia (Valencia); 35 trematodos adultos de 11 individuos de jabalí (*Sus scrofa*) de Galicia; 88 trematodos adultos procedentes de 7 ovinos (*Ovis aries*) de Galicia y 133 trematodos adultos procedentes de 7 individuos de ganado vacuno (*B. taurus*) de Galicia (véase Fig. 2.1).

Material de Francia: 84 ejemplares de 6 individuos de ganado vacuno (*B. taurus*) del Matadero de Portovechio (Córcega, Francia).

Material de Burkina Faso: 81 ejemplares de 4 cebús *(Bos indicus)* del Matadero de Bobo-Dioulasso (Burkina Faso).

Material de Pakistán: 81 ejemplares de 19 búfalos *(Bubalus bubalis)* de diferentes distritos de la zona central de Punjab (véase Fig. 2.4).

Material de Bangladesh: 102 ejemplares de 51 búfalos *(B. bubalis)* y 51 cebús *(B. indicus)* (véase Fig. 2.5).

Material de Irán: se escogieron parásitos adultos de mataderos localizados en regiones con una mayor diversidad, zonas montañosas, de tierras bajas y colinas. Se estudiaron un total de 69 hígados incluyendo 4713 adultos (*Fasciola*) procedentes de cuatro especies hospedadoras: vacuno (*B. taurus*), búfalos *(B. bubalis)*, ovinos *(O. aries)* y cabras *(Capra aegagrus hircus)*. De estos fasciólidos se seleccionaron los de mejor estado, un total de 1361 parásitos adultos (véase Fig. 2.6). De estos se analizaron:

- *Fasciola hepatica*, 153 procedentes del ganado bovino, 8 de búfalo, 130 de ovinos y 301 de cabras (localizadas principalmente en zonas montañosas y colinas);
- *F. gigantica*, 625 de ganado vacuno, 123 de búfalos y 21 de ovinos.

2.2. Métodos y Técnicas

2.2.1. Técnicas Mastozoológicas

Estas técnicas van a proporcionar información sobre el tratamiento de los hospedadores definitivos utilizados para poder recuperar los diferentes estadios (adultos y huevos) de los Digénidos.

2.2.1.1. Estudio del hígado y canales biliares

Dado que en *F. hepatica* y *F. gigantica* el microhábitat de parasitación específico del adulto es el hígado, se realizó un minucioso estudio de este órgano. Los hígados infectados procedentes de los distintos mataderos fueron trasladados hasta el laboratorio en fresco y sin congelación para procurar mantener los fasciólidos vivos. La colección de los especimenes fue realizada a través de una necropsia parasitaria del hígado. El propósito de esta necropsia es colectar los fasciólidos de la cara visceral hepática, la vesícula biliar, los conductos biliares mayores y finalmente los menores que se encuentran en la parte más profunda del parénquima hepático. Los canales biliares aumentan mucho su diámetro en la fase de estado, siendo fácil detectar si van a existir adultos de *F. hepatica* o *F. gigantica* en ellos. Al abrir el canal biliar con unas tijeras, los Digénidos adultos salen vivos, procediéndose a su recolección y fijación. Una vez extraídos los diferentes especimenes, se colocaron en agua para permitir que el fasciólido libere restos del jugo biliar (PANOVA, 2002; PERIAGO, 2008).

2.2.2. Técnicas Helmintológicas

Las técnicas helmintológicas se utilizan para la preparación y estudio de los Digénidos adultos, para su posterior estudio morfométrico y morfoanatómico.

2.2.2.1. Técnicas Microscópicas Generales

Estas técnicas incluyen los procesos realizados desde la recogida de los vermes hasta su estudio en el microscopio, es decir, la fijación de los Trematodos, su conservación, coloración, diferenciación, deshidratación y montaje.

2.2.2.1.1 Fijación

Para que la visualización de la anatomía interna del Trematodo sea óptima, el verme se debe fijar in vivo y ser sometido a una ligera presión. El proceso de fijación se detalla a continuación. En ejemplares pequeños se coloca el Trematodo vivo con un pincel sobre un portaobjetos en una gota de agua. Se añade una gota de líquido de Bouin en la cara inferior de un cubreobjetos y se deja caer sobre el Trematodo, cuidando que éste no se encuentre ladeado, en cuyo caso se enderezará con ayuda de una aguja enmangada. El verme debe permanecer entre porta y cubreobjetos unos 20 minutos. Luego se levanta y se traslada el Trematodo con un pincel a una placa Petri con fijador de Bouin, en el que permanecerá unos 30 minutos (MARCOS, 1993).

En ambos casos, transcurrido el tiempo establecido, se llevan a alcohol de 70º, cambiando éste diariamente hasta que el verme pierda la coloración amarilla dada por el fijador; en nuestro caso oscila entre 30 a 90 días dependiendo del tamaño del Trematodo. El líquido de Bouin se considera uno de los mejores fijadores topográficos. Su composición por cada 100 ml es la siguiente:

Solución acuosa de ácido pícrico
75 ml Formol (solución comercial al 40 %)
25 ml Ácido acético 5 ml

Se prepara de la siguiente manera: en un matraz, se pone un litro de agua y se calienta hasta que esté templada. Se coloca el matraz sobre un agitador y se le va añadiendo poco a poco ácido pícrico hasta conseguir una solución saturada del mismo, es decir, hasta que el ácido pícrico no se disuelva en el agua. Una vez conseguido se deja reposar 24 horas (solución madre). Para utilizar el fijador se añade a pequeñas fracciones de la mezcla la cantidad correspondiente de ácido

acético (5 partes de ácido acético por cada 100 partes de solución) (PANOVA, 2002). El líquido de Bouin fija de forma homogénea y penetra rápidamente. La retracción en el momento de la fijación es más débil que con otras buenas mezclas fijadoras (BARGUES, 1986).

2.2.2.1.2 Conservación

La conservación se realiza en viales con alcohol de 70°, en el cual los Digénidos pueden permanecer hasta el momento de su tinción (PANOVA, 2002).

2.2.2.1.3 Coloración

Las tinciones utilizadas dan lugar a preparaciones permanentes, imprescindibles en el estudio llevado a cabo. El colorante utilizado ha sido el Carmín Borácico de Grenacher, cuya fórmula es la siguiente:

Solución acuosa de bórax al 4 % (8 gramos de bórax en 200 cc de agua destilada)
Carmín (casa Merck)

Se detalla a continuación el protocolo de preparación del carmín Borácico de Grenacher. En un matraz redondo se pone la solución acuosa anterior y se le añaden 5 gramos de carmín. Se calienta en un recipiente de reflujo, en el cual tenemos un cazo con tierra y el matraz esférico, con un refrigerador por el que entra y sale agua. Se deja hervir suavemente durante 30 minutos y se le añaden 200 cc de alcohol de 70°. Se deja reposar 24 horas y se filtra (PANOVA, 2002). Los Digénidos obtenidos deben permanecer en el colorante un tiempo determinado que depende de su grosor y su capacidad de tomar el colorante. Los adultos de *F. hepatica* y *F. gigantica* deben teñirse de forma suficiente pero no excesiva para facilitar su posterior diferenciación. Si los ejemplares se tiñen en exceso deben estar más tiempo en el líquido diferenciador, lo que puede alterar el inicial de la tinción e incluso los tejidos del verme. En líneas generales diremos que los adultos de *F. hepatica* de mayor grosor y los adultos de *F. gigantica* se han teñido durante 24 horas en la mezcla de carmín Borácico de Grenacher. La tinción óptima se controla periódicamente bajo la lupa binocular.

2.2.2.1.4 Diferenciación

Una vez que el Trematodo ha sido teñido efectuamos la diferenciación en alcohol clorhídrico. Con un pincel bien seco extraemos los vermes del colorante y los colocamos, de uno en uno, en una placa Petri añadiendo, gota a gota, ácido clorhídrico comercial (35 %). El Trematodo va alcanzando una tonalidad rosada con luz superior de la lupa, debiéndose observar con la inferior todas las estructuras del parásito por transparencia. La duración de esta operación depende del tamaño y grosor del Trematodo (PANOVA, 2002).

2.2.2.1.5 Deshidratación

Tras la diferenciación colocamos una serie de placas Petri con distintos alcoholes en los que, con ayuda de un pincel, iremos pasando sucesivamente los parásitos. En la primera placa, con etanol de 70°, el Digénido debe estar 10 minutos. Después lo llevamos a otra placa con alcohol de 96°, 15 minutos. De ésta pasa a alcohol de 100° 15 minutos más, otros 15 minutos en alcohol butílico y finalmente 15 minutos en xilol, terminando con este paso la cadena de deshidratación.

2.2.2.1.6 Montaje

El montaje se realiza con un portaobjetos, un cubreobjetos y bálsamo de Canadá, resina comercial que solidifica rápidamente y proporciona preparaciones de larga vida. Sin embargo, para poderla emplear es necesario realizar una previa deshidratación del material, ya que no es soluble en agua. Para realizar el montaje se coloca sobre el portaobjetos una gota de bálsamo de Canadá y sobre ella el Trematodo en posición ventral, es decir, con la ventosa o acetábulo ventral de cara al cubreobjetos. Con un pincel mojado en xilol eliminamos las posibles burbujas que se hayan podido formar en el bálsamo antes de colocar el cubreobjetos sobre él. Si una vez montado, el parásito no presenta suficiente bálsamo, o si éste se retrae, se puede añadir más pincelando con xilol los bordes entre porta y cubre, añadiendo una gota de bálsamo de Canadá, que entra en la preparación por capilaridad (MARCOS, 1993). Por último, se introducen las preparaciones en la estufa a 20

°C de 4 a 24 horas, hasta que estén secas. Si el bálsamo se retrae por el calor, se repite la operación anteriormente descrita de pincelado con xilol y bálsamo por capilaridad (PANOVA, 2002).

2.2.2.2. Estudio Morfométrico CIAS

Para la obtención de imágenes y para el cálculo de las diferentes medidas morfométricas con un analizador de imágenes, tanto unidimensionales como bidimensionales y para la obtención de imágenes se han utilizado los siguientes programas:

- Image-Pro Plus, versión 5.1 para Windows (Media Cybernetics Inc., Silver Spring, USA): con dicho programa comercializado se han creado macros para el cálculo de las dimensiones en imágenes digitalizadas mediante cámara digital Nikon Coolpix 5400 y Leica V-LUX 1 (para medidas de adultos) y mediante un microscopio Nikon modelo SE equipado con un revolver de 4 objetivos (4x, 10x, 40x, 100x y 2 oculares de 10x), conectado con videocámara de color 3CCD (Sony DXC-930P) (para medidas de ventosas y faringes).
- Microsoft Excel: programa comercializado para análisis y cálculos de datos. Los datos obtenidos a través de Image-Pro®Plus se exportaban a los archivos de dicho programa para poder operar después con ellos.

La estandarización de la metodología para la realización de las medidas es fundamental en cualquier estudio morfométrico (VALERO & MAS-COMA, 1985; VALERO, 1986; VALERO et al., 1987; VALERO et al., 1996, 2005; PERIAGO et al., 2006, 2008). Los trabajos de VALERO et al. (1996, 2005) y PERIAGO et al. (2006, 2008) proponen la metodología Computer Image Analysis System (CIAS) para el estudio morfométrico de los adultos de *F. hepatica* y *F. gigantica*. Se han realizado mediciones de adultos de fasciólidos en material fijado y montado en preparaciones permanentes. Los parámetros morfométricos analizados en el huevo de los fasciólidos son los siguientes:
Características biométricas lineales: EL = Longitud del huevo; EW = anchura del huevo; EP = perímetro del huevo; ER = circularidad del huevo (ER=EP2/4πEW).

Superficies: EA = área del huevo.
Relaciones: EL/EW = relación entre longitud del huevo y anchura del huevo.

Los parámetros morfométricos analizados en el fasciólido adulto son los siguientes (véase Fig. 2.7):
Características biométricas lineares: BL= longitud corporal; BW= anchura corporal; BWOv= anchura corporal a nivel del ovario; BP= perímetro corporal; BR= circularidad corporal (BR=BP2/4πBW); CL= longitud del cono; CW= anchura de cono; OSMax= diámetro máximo de la ventosa oral; OSmin= diámetro mínimo de la ventosa oral; VSMax= diámetro máximo de la ventosa ventral; VSmin= diámetro mínimo de la ventosa ventral; A-VS= distancia entre el extremo anterior del cuerpo y la ventosa ventral; OS-VS= distancia entre la ventosa oral y ventosa ventral; VS-Vit= distancia entre la ventosa ventral hasta el final de las glándulas vitelógenas; Vit-P= distancia entre las glándulas vitelógenas hasta la parte posterior corporal; VS-P= distancia entre la ventosa ventral y la parte posterior corporal; PhL= longitud de la faringe; PhW: anchura de la faringe; TL= longitud testicular; TW= anchura testicular; TP= perímetro testicular; TR= circularidad testicular (TR=TP2/4πTW). Superficies: BA= superficie corporal; OSA= superficie de la ventosa oral; VSA= superficie de la ventosa ventral; PhA= superficie de la faringe; TA= superficie testicular. Relaciones: BL/BW= relación entre longitud corporal y anchura corporal; BWOv/CW= relación entre la anchura corporal a nivel de ovario y distancia entre la ventosa ventral y el final corporal; OS/VS= relación entre área de la ventosa oral y área de la ventosa ventral; BL/VS-P= relación entre la longitud corporal y la es el objeto más grande son los valores (PERIAGO et al., 2008).
La medida de la circularidad se utilizó para cuantificar su forma. Esta medida sirve para conocer como de circular es un objeto. Un objeto circular tendrá una circularidad de 1.0, cuanto más regular.

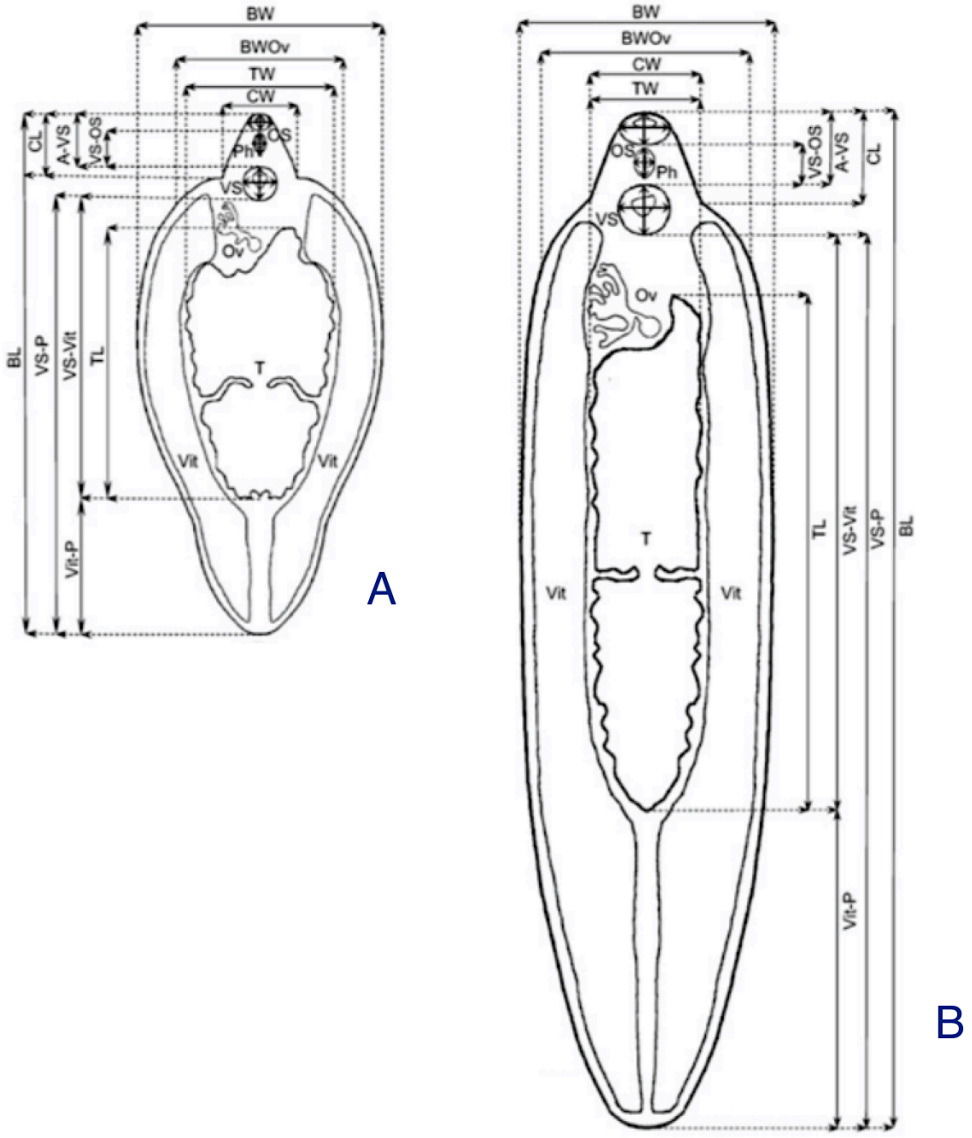

FIGURA 2.7: Medidas estandarizadas aplicadas a espécimes adultos grávidos de *F. hepatica*-like (A) y *F. gigantica*-like (B) (PERIAGO et al., 2006).

2.2.2.3. Análisis Estadístico

Los fasciólidos adultos sufren un proceso marcado de desarrollo en el hospedador definitivo. Los cambios de los diferentes parámetros biométricos de los Trematodos

a diferentes tiempos han sido analizados previamente en *F. hepatica* (VALERO et al., 1998, 2001a, b, 2005, 2011). Las correspondientes curvas de crecimiento obtenidas son logísticas, lo cuál implica que el desarrollo morfométrico de *F. hepatica* no es ilimitado, sino "damped" y no puede exceder de ciertos valores máximos característicos ym (=valor máximo alcanzado por una variable biométrica). Este modelo describe la variación de los valores biométricos del adulto de *F. hepatica* a lo largo del tiempo, desde la migración de parásito por el parénquima hepático a la localización del adulto en el interior del conducto biliar. La entrada en el conducto biliar induce a la maduración y la producción de huevos. El modelo logístico que representa el crecimiento del cuerpo y el desarrollo se caracteriza por dos fases (VALERO et al., 1998, 2005). La parte exponencial de crecimiento logístico corresponde al desarrollo de cuerpo durante la migración en la cavidad abdominal y el parénquima hepático, y la fase saturada corresponde al desarrollo, maduración sexual y producción de huevos en el conducto biliar. Por consiguiente, para evitar la influencia de la edad y el crecimiento sobre la comparación morfométrica, la variación morfológica la hemos cuantificado a través de la morfometría geométrica (ROHLF & MARCUS, 1993), una técnica que ofrece una estimación del tamaño y las diferentes tasas de crecimiento se integran en una única variable (ROHLF & MARCUS, 1993).

2.2.2.3.1 Análisis de los Componentes principales (ACP)

El análisis de los componentes principales (ACP) permite explorar la variación individual y reconocer eventualmente los agrupamientos particulares sobre un "mapa factorial", que generalmente se trata de un plot de los dos primeros componentes principales (BOOKSTEIN, 1989; ROHLF & MARCUS, 1993; KLINGERBERG, 1996; DUJARDIN et al., 2002; DUJARDIN & LE PONT, 2004). Este análisis no toma en cuenta la pertenencia de cada individuo a grupos distintos. Es un análisis ciego y frecuentemente puede detectar estos grupos a partir de los valores individuales (DUJARDIN et al., 2002). En resumen, la función del análisis en componentes principales es doble. Por una parte, permite reconocer una estructuración en particular de los individuos, correspondiente o no a su clasificación inicial, así que puede ser utilizado como instrumento de clasificación. Por otra parte, el análisis en componentes principales presenta el interés adicional de proporcionar una variable

de tamaño, en general el primer componente principal (CPI), variable que será utilizada después para eliminar el efecto tamaño de las poblaciones en comparación (DUJARDIN et al., 2002). Así, el análisis en componentes principales brinda una variable de tamaño (CPI), que es la combinación lineal que representa la varianza máxima. Además, geométricamente CPI se corresponde con la dirección del eje más largo a través de la nube de puntos. Las subsiguientes variables (CPII, CPIII, etc.), matemáticamente independientes, alcanzan la varianza máxima, siendo un eje ortogonal al resto de los componentes (KLINGERBERG, 1996). Además, esta clase de análisis permite separar la influencia del tamaño de la influencia de la forma sobre la estructura observada (DUJARDIN et al., 2002). El análisis se llevó a cabo usando el software BAC v.2 (DUJARDIN, 2002), utilizando varios módulos CLIC package (by J.P. Dujardin, http://momedujardin.wordpress.com). Para realizar de manera óptima estos dos objetivos del análisis en componentes principales, los datos iniciales deben transformarse en logaritmos naturales. Esta transformación presenta numerosas ventajas estadísticas (normalización de las distribuciones, igualación de las varianzas, etc.) y permite comparar las diferencias relativas más que las absolutas (DUJARDIN et al., 2002). Se han utilizado las siguientes medidas no redundantes (una medida no incluye a la otra) en el estudio de los adultos: BL, BW, BP, OS max, OS min, VS max, VS min, A-VS, VS-Vit, Vit-P, PhL, PhW and TL, donde al menos se ha tenido en cuenta una dimensión de los caracteres morfológicos más importantes.

2.2.2.3.2 Correlaciones

Para el calculó de la correlación entre dos variables se utilizó la correlación de Pearson. Se han considerado resultados estadísticamente significativos cuando $P<0.05$.

Capítulo 3

Fenotipaje de fasciólidos adultos en Pakistán y Bangladesh

Phenotyping of adult fasciolids in Pakistan and Bangladesh

3.1. Phenotyping of adult fasciolids in Pakistan

Pakistan is one of the Asian countries where both fasciolid species overlap, and fascioliasis is highly prevalent in livestock, including cattle, buffaloes, sheep and goats (KHAN et al., 2010), and the two fasciolid species are frequently reported (MAQBOOL et al., 1994, 2002; SIDDIQI & SHAH, 1984; CHAUDHRY & NIAZ, 1984; MASUD & MAJID, 1984; KHAN et al., 2009). In the Punjab province of Pakistan, fascioliasis is of special concern in bovines. Fascioliasis has been found to be endemic in various districts, with prevalences ranging from 10.48 to 40.31 % (CHAUDHRY & NIAZ, 1984; MASUD & MAJID, 1984; MAQBOOL et al., 2002; KHAN et al., 2009), and results indicate the presence of *F. gigantica* to be more widespread than *F. hepatica* in five Punjabi districts (KHAN et al., 2009).
Furthermore, human cases have also been described in rural areas of Lahore, Central Punjab, Pakistan, thus suggesting fascioliasis to be an even greater public heath problem than previously estimated (QURESHI et al., 2005).

Faced with the present situation, it is evident that further studies about the epidemiology and transmission of the disease to humans and animals are required to obtain the baseline on which to establish appropriate control measures in that endemic area of Pakistan. This study represents a step further in this endeavour, by analysing the morphometric characteristics of fasciolid adults infecting the buffalo in Pakistan, one of the main livestock species present. Unfortunately, appropriate phenotypic analyses to assess the presence and phenotypic features of the fasciolids have never been carried out in Pakistán. Studies were performed on the basis of standardised measurements known to be useful for the differentiation of both fasciolid species (PERIAGO et al., 2006). Therefore, a computer image analysis system (CIAS) method (VALERO et al., 2005) was applied to flukes collected from buffaloes. Since it is the first time that such a study has been performed in Pakistan, the results are compared to pure fasciolid populations, namely (i) *F. hepatica* from the European Mediterranean area and (ii) *F. gigantica* from Burkina Faso, i.e. geographical areas where both species do not co-exist, according

to previously obtained results (PERIAGO et al., 2006).The definitive animal host species is known to pronouncedly influence the phenotype of both adult stage and egg of the liver fluke, mainly due to the different size of the liver duct microhabitat (VALERO et al., 2001a, 2002, 2009a). For this reason, only parasites obtained from bovines were used in this study.

3.1.1. Material and Methods

3.1.1.1. Material

Only parasites obtained from bovines were used in this study.The Material used is detailed in Section 2.1.2 in the Material & Methods. The study area is described in 2.1.1.4 Section

3.1.1.2. Morphometrics

An accurate morphometric study was conducted to phenotypically discriminate between *F. hepatica* and *F. gigantica*. Only adult flukes found in naturally infected bovines were used for the phenotypic comparison. All measurements of adult worms and eggs were made according to a previously described standardised methodology (VALERO et al., 2005, 2009a; PERIAGO et al., 2006, 2008). After egg collection from the uteri of flukes, standardised measurements were taken using a microscope and images captured by a digital camera (Nikon Coolpix), which were then analysed by image analysis software (ImagePro plus version 5.0 for Windows, Media Cybernetics, Silver Spring, USA) (see 2.2.2.8 Section). For adult fasciolids, the standardised measurements described in 2.2.2.8 Section were taken (Fig. 2.7). Since the morphometric maximum values are characteristic for each population, they are considered the comparative base of this study (Table 3.1).

3.1.1.3. Data analysis

Morphological variation is quantified by geometrical morphometrics (ROHLF & MARCUS, 1993). The principal component analysis is used to summarize most

of the variations in a multivariate dataset in few dimensions (DUJARDIN & LE PONT, 2004) and is described in 2.3 Section. The following non-redundant measurements (one measurement is not included in another) used for fasciolid adults were BL, BW, BP, OS max, OS min, VS max, VS min, A-VS, VS-Vit, Vit-P, PhL, PhW and TL, where at least one dimension was measured among the most important morphological characters. The remaining variables were all significantly correlated with the first principal component (PC1), contributing 72 % to overall variations. PC1 could therefore be accepted as a general indicator of size (BOOKSTEIN, 1989), so that the resulting factor maps (Fig. 3.1) clearly illustrate global size differences in the populations analysed.

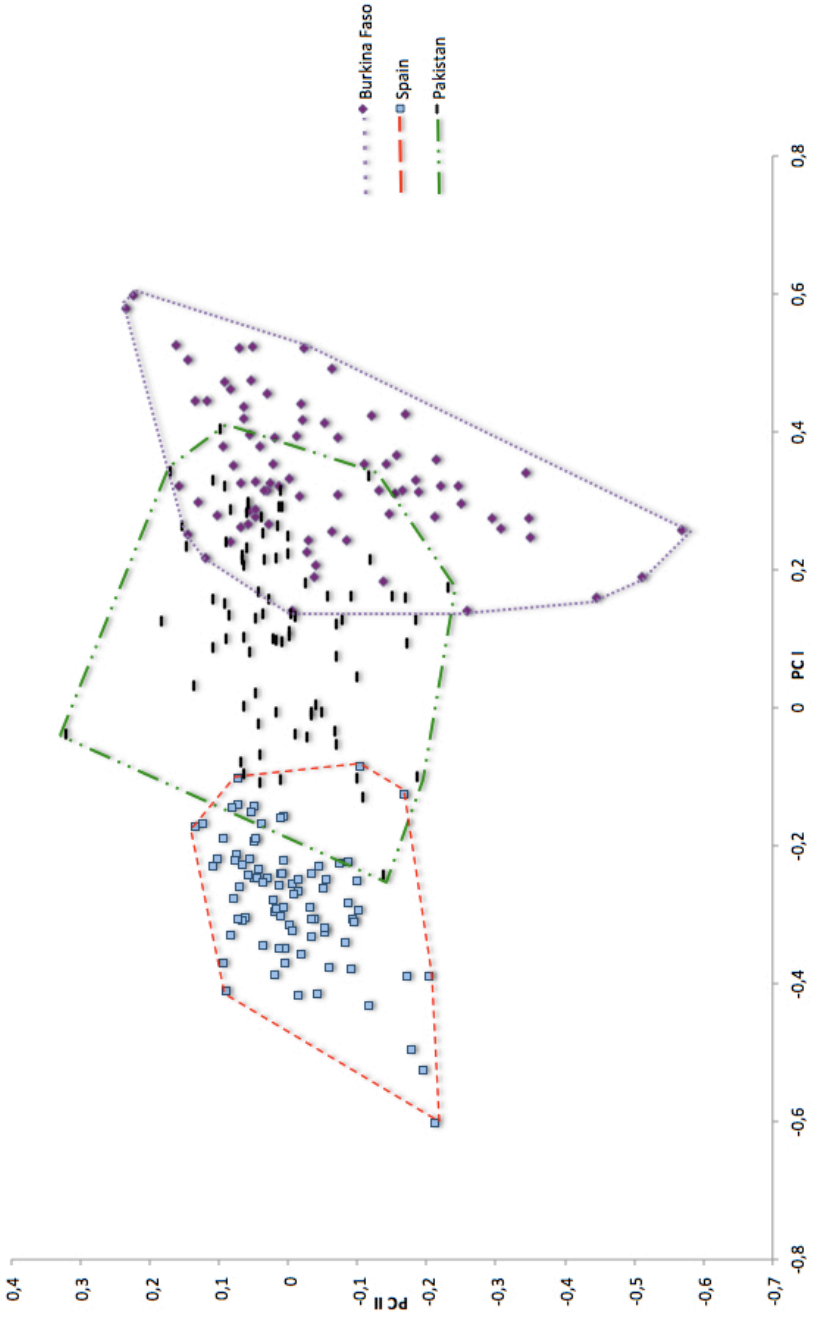

FIGURA 3.1: Principal component analysis of adult fasciolids from natural infections in bovines of Pakistan (dashed and double dotted line) compared with *F. hepatica* from Valencia, Spain (dashed line) and Corsica, France (dashed-dotted line) and *F. gigantica* from Burkina Faso (dotted line) (PERIAGO et al., 2006). Samples are projected on to the first (PCI, 70 %) and second (PCII, 13 %) principal components.

Adult measurements	Pakistan	Burkina Faso	Spain
	Fasciola sp. n=81	*F. gigantica* n=81	*F. hepatica* n=84
Body length (BL)	29.09 ± 5.23 17.43 - 41.34	39.72 ± 0.58 28.82-52.30	17.41 ± 0.23 11.64-22.93
Body width (BW)	9.64 ± 1.29 6.37 - 12.45	8.45 ± 0.14 6.03-11.84	10.02 ± 0.17 6.41-13.88
Body width ovary level (BWOv)	7.83 ± 1.07 5.48 - 10.49	7.59 ± 0.13 5.33-11.55	7.90 ± 0.14 4.53-11.67
Body perimeter (BP)	70.84 ± 11.40 43.42 - 96.87	85.25 ± 1.21 63.19-113.71	43.05 ± 0.55 28.58-54.40
Body roundness (BR)	1.91 ± 0.21 1.53 - 2.65	2.47 ± 0.05 1.71 -3.65	1.23 ± 0.01 1.06-1.58
Cone length (CL)	2.16 ± 0.26 1.59 - 2.68	2.67 ± 0.04 2.10-3.36	1.09-2.92 2.02 ± 0.04
Cone width (CW)	3.52 ± 0.37 2.59 - 4.37	3.74 ± 0.06 2.23-5.08	3.20 ± 0.04 2.35-4.21
Maximum diameter of oral sucker (OS max)	1.00 ± 0.12 0.72 - 1.78	0.83 ± 0.02 0.52-1.17	0.85 ± 0.01 0.57-1.03
Minimum diameter of oral sucker (OS max)	0.32 - 1.62 0.66 ± 0.16	0.62 ± 0.02 0.21 -0.95	0.61 ± 0.01 0.44-0.77
Maximum diameter of ventral sucker (VS max)	1.55 ± 0.13 1.08 - 1.88	1.50 ± 0.02 0.87-1.92	1.13 ± 0.01 0.92-1.49
Minimum diameter of ventral sucker (VS max)	1.41 ± 0.14 0.61 - 1.66	1.38 ± 0.02 0.80-1.83	1.12 ± 0.01 0.86-1.35
Distance between anterior end of body and VS (A-VS)	1.94 ± 0.29 0.97 - 2.48	2.36 ± 0.03 1.46-3.01	2.05 ± 0.04 1.12-2.92
Distance between suckers (OS-VS)	1.29 ± 0.28 0.16 - 1.91	1.71 ± 0.03 1.15-2.22	1.42 ± 0.04 0.57-2.4.1
Distance between Vs and union of vitelline gland (VS-Vit)	18.67 ± 3.59 10.71 - 26.60	22.68 ± 0.45 12.26-34.11	9.60 ± 0.17 6.57-14.31
Distance between Vit and posterior end of body (Vit-P)	8.83 ± 2.26 1.38 - 15.32	13.45 ± 0.32 8.97-21.43	4.73 ± 0.10 2.63-7.57
Distance between VS and posterior end of body (VS-P)	27.50 ± 5.30 15.88 - 39.57	36.39 ± 0.59 26.28-50.09	14.40 ± 0.22 9.51 -19.94
Pharynx length (PL)	0.93 ± 0.11 0.63 - 1.28	0.78 ± 0.02 0.46-1.06	0.70 ± 0.01 0.52-1.00
Pharynx width (PW)	0.56 ± 0.09 0.34 - 0.75	0.42 ± 0.01 0.23-0.68	0.44 ± 0.01 0.26-0.83
Testicular space length (TL)	13.79 ± 3.14 7.80 - 22.26	18.76 ± 0.38 12.76-29.38	7.91 ± 0.15 5.45-11.68
Testicular space width (TW)	5.68 ± 0.91 3.71 - 7.64	5.51 ± 0.12 3.24-8.66	6.61 ± 0.12 4.18-8.93
Testicular space perimeter (TP)	37.29 ± 7.43 22.48 - 59.63	44.62 ± 0.87 25.97-68.06	25.32 ± 0.42 17.10-34.34
Body area (BA)	212.47 ± 54.59 87.33 - 332.11	249.38 ± 7.48 162.58-482.91	123.83 ± 3.28 54.90-197.40
Oral sucker area (OSA)	0.52 ± 0.11 0.28 - 0.84	0.46 ± 0.03 0.10-1.11	0.41 ± 0.01 0.25-0.56
Ventral sucker area (VSA)	1.75 ± 0.26 1.03 - 2.46	1.86 ± 0.08 0.56-3.52	0.99 ± 0.02 0.67-1.57
Pharynx area (PA)	0.40 ± 0.08 0.21 - 0.64	0.33 ± 0.02 0.12-0.64	0.31 ± 0.01 0.16-0.57
Testicular space area (TA)	62.10 ± 19.65 22.41 - 123.46	80.75 ± 3.44 41.05-187.50	40.45 ± 1.24 17.85-70.62
Ratio between BL and BW (BL/BW)	3.04 ± 0.53 2.02 - 4.85	4.70 ± 0.08 3.40-6.77	1.74 ± 0.03 1.29-2.77
Ratio between BWOv and CW (BWOv/CW)	2.24 ± 0.29 1.66 - 2.88	2.03 ± 0.05 1.34-3.63	2.47 ± 0.04 1.52-3.46
Ratio between Suckers area OSA/VSA	0.30 ± 0.05 0.17 - 0.44	0.25 ± 0.01 0.08-0.40	0.41 ± 0.01 0.24-0.60
Ratio between BL ad VS-P (BL/VS-P)	1.06 ± 0.02 1.01 - 1.12	1.09 ± 0.004 0.95-1.20,	1.21 ± 0.004 1.14-1.31

TABLA 3.1: Comparative morphometrics in mm (mean +/- SD and ranges) of 81 adult liver flukes from buffaloes of Pakistan. n. number of adult liver flukes measured; lineal biometric characters in mm. areas in mm^2.

3.1.2. Results

Fasciolid populations from Pakistan were grouped according to the maximum and minimum values of given differentiating morphological measurements previously proposed for *F. hepatica*, *F. gigantica* or *Fasciola* sp. (= intermediate forms) (PERIAGO et al., 2006): BR: 1.06–1.58 in *F. hepatica*; 1.71–3.65 in *F. gigantica*; BL/BW: 1.29–2.80 in *F. hepatica*; 3.40–6.78 in *F. gigantica* and VS-P: 8.86–25.08 mm in *F. hepatica*; 26.28–50.09 mm in *F. gigantica*. The specimens from Pakistan were first grouped according to BR and BL/ BW, and, secondarily, according to VS-P. Using these criteria, adult specimens from Pakistan were grouped into *F. hepatica*-like (3.7 %), *F. gigantica*-like (22.2 %) or *Fasciola* sp.-like (74.1 %).

Secondly, integration of morphological data from all adult specimens from Pakistan (without the above-mentioned previous classification) through Principal Component Analysis was made. Adults from Spain and Burkina Faso were included in the same analysis as *F. hepatica* and *F. gigantica* standard populations, respectively. Fasciolid variables all significantly correlated with PC1. The resulting factor maps (Fig. 3.1) clearly illustrate global size differences in the populations analysed, where each group is represented by its perimeter. Two independent zones can be distinguished: one zone is made up of samples from Spain, while the other zone consists only of samples from Burkina Faso. These zones overlap with the samples from Pakistan. The multivariate analysis used to measure the changes in size of fasciolid adults from Pakistan and compared with the above-mentioned standard populations for each pure fasciolid species showed that the size of most fasciolids from Pakistan is situated between *F. hepatica* and *F. gigantica* standard populations. These results confirm that intermediate forms of fasciolids exist in Central Punjab. Nevertheless, it is worth mentioning that the samples from Pakistan overlap with *F. hepatica* and *F. gigantica* standard populations, but in the absence of specimens of standard *F. hepatica* and *F. gigantica* from Pakistan it is also possible that the forms identified as *F. hepatica*-like and *F. gigantica*-like could be the extreme values of the morphometric distribution of the *Fasciola* sp. - like specimens.

3.1.3. Discussion

In Pakistan, fascioliasis in humans from Lahore, Central Punjab, was diagnosed in the chronic stage of the disease, i.e. through the coprological detection of fasciolid eggs (QURESHI et al., 2005). Liver fluke disease has recently proved to be highly pathogenic when chronic, and not only in the acute phase as previously thought. This aspect is of great importance in human endemic areas of developing countries where the majority of infected subjects, mainly children, are detected in the chronic stage (VALERO et al., 2003, 2006a, 2008). Additionally, appropriate surveys in these human endemic areas have shown that liver fluke infected patients are usually concomitantly infected by other parasites, related to an immune-suppression caused by *Fasciola* infection in chronic fascioliasis (GIRONES et al., 2007).

This pathological scenario of great concern may be aggravated when considering that results of recent studies indicate that the occurrence of *F. gigantica* is more widespread than that of *F. hepatica* in five Punjabi districts (KHAN et al., 2009), similarly to northern Iran (ASHRAFI et al., 2004). The considerably larger size of *F. gigantica* adult worms when compared to *F. hepatica* and consequently its higher pathogenicity must be taken into account in Pakistan. Moreover, fascioliasis is a trematode disease highly susceptible to climatic factors (FUENTES et al., 1999, 2001), which explains the impact of climate change on transmission of this disease (MAS-COMA et al., 2008). Pakistan's latitudinal situation is prone to be affected by climate change, which has indeed already been detected, showing a rise in the mean temperature in coastal areas, a 10-15 % decrease of precipitation in the coastal belt, the presence of hyper-arid plains, and an increase in summer and winter precipitation in the northern part of the country (FAROOQ & KAHN, 2004; CRUZ et al., 2007). When considering all the aforementioned facts, it becomes evident that assessing the present situation of *F. hepatica* and *F. gigantica* is a priority for Pakistan.

The present phenotypic study on fasciolids infecting buffaloes from Central Punjab demonstrates the presence of intermediate forms (*Fasciola* sp.) (Table 3.1, Fig. 3.1).

	Pakistan	Spain	Burkina Faso
Measurements	*Fasciola sp.* n=345	*F. hepatica* n=113	*F. gigantica* n=142
Length, EL	150.75±14.73	129.80±0.83	156.80±1.07
	115.07-186.68	107.30-152.70	129.61-204.51
Width, EW	93.1±5.99	69.59±0.60	89.45±0.75
	79.12-114.09	52.44-89.11	61.63-112.56
Perimeter, EP	411.16±30.46	319.29±1.70	390.14±2.26
	323.21-481.64	270.45-360.07	335.52-471.84
Roundness, ER	1.24±0.06	1.17±0.01	1.09±0.01
	1.00-1.57	1.05-1.33	1.00-1.34
Area, EA	10925.41±1332.61	6983.80±75.9	11144.09±124.34
	8223.94-14045.6	5137.25-9183.46	7846.34-15890.70
Length/Width ratio	1.19-2.14	1.46-2.54	1.77±0.02
	1.63±0.19	1.88±0.02	1.32-2.64

TABLA 3.2: Comparative morphometrics in μm (mean +/- SD and ranges) of mature eggs of fasciolids from Pakistan with data previously published by PERIAGO et al. (2006) for standard populations from Spain and Burkina Faso; n=number of eggs measured; Length (EL), Width (EW), Perimeter (EP) in μm and Area (EA) in μm^2.

The size measurements of eggs of fasciolid intermediate forms in the current study, of 115.07-186.68/79.12-114.09 µm (Table 3.2), show intermediate values when compared to the standard fasciolid populations of both species previously described (PERIAGO et al., 2006) and which molecularly proved to be genetically pure *F.*

hepatica and pure *F. gigantica* (MAS-COMA et al., 2009a). Fasciolid egg size ranges depend upon livestock species and geographical areas, respectively, as previously described by applying the CIAS methodology (VALERO et al., 2009a). Egg length minimum values obtained in the present study approach minimum values found in *F. hepatica* from Bolivian cattle, *F. hepatica* from Egyptian cattle and those from Georgia. Egg length maximum values approach maxima obtained in *F. gigantica* from Egyptian cattle and *F. gigantica* from Vietnamese cattle, but are smaller than the maxima described in those from Burkina Faso. The results obtained from fasciolids encountered in the Central Punjab area agree with results from previous studies carried out in other Asian countries, where a large egg measurement overlap was detected (WATANABE, 1962; AKAHANE et al., 1970; SAHBA et al., 1972; KIMURA et al., 1984; SRIMUZIPO et al., 2000).

3.2. Phenotyping of adult fasciolids in Bangladesh

Bangladesh is one of the Asian countries where fascioliasis is the most prevalent parasitic disease in cattle, buffaloes, goats and sheep (NOORUDDIN & ISLAM, 1996; HOSSAIN et al., 2011). An earlier survey revealed that 71.55 % of cattle, 14.6 % of sheep and goats and 100 % of buffaloes are affected by the liver-fluke (BHUIYAN, 1970). A more recent report (BAS-USDA, 2012) showed that 10.42 % cattle, 9 % goats and 36.61 % of buffaloes are infected with *Fasciola* among snail borne trematode infections in Bangladesh. Recent post-mortem examinations revealed a *Fasciola* infection rate of 35.27 % irrespective of geographical areas and host animal species (unpublished data, 2013). Unfortunately, appropriate phenotypic analyses to assess the presence and phenotypic features of the fasciolids have never been carried out in Bangladesh. The present study represents the first attempt within that endeavour. Bovines, as the most widespread livestock species in the country, have been the hosts selected. An approach by means of a computer image analysis system (CIAS) (VALERO et al., 2005, 2012c) was applied for the morphometric characterisation of the liver flukes.

3.2.1. Material and Methods

3.2.1.1. Material

Only parasites obtained from bovines were used in this study.The Material used is detailed in section 2.1.2 in the Material & Methods. The study area is described in 2.1.1 Section.

3.2.1.2. Morphometrics

An accurate morphometric study was conducted to phenotypically discriminate between *F. hepatica* and *F. gigantica*. All measurements of adult worms and eggs were made according to a previously described standardised methodology (VALERO et al., 2005, 2009a; PERIAGO et al., 2006, 2008). After egg collection from the uteri of flukes, standardised measurements were taken using a microscope and

images captured by a digital camera (Nikon Coolpix), which were then analysed by image analysis software (ImagePro plus version 5.0 for Windows, Media Cybernetics, Silver Spring, USA) (see 2.2.2.2. section). For adult fasciolids, the standardised measurements described in 2.2.2.8 Section section were taken (Fig. 2.7). Since the morphometric maximum values are characteristic for each population, they are considered the comparative base of this study (Table 3.3).

3.2.1.3. Data analysis

Morphological variation is quantified by geometrical morphometrics (ROHLF & MARCUS, 1993). The principal component analysis is used to summarize most of the variations in a multivariate dataset in few dimensions (DUJARDIN & LE PONT, 2004) and is described in 2.3 Section. The following non-redundant measurements (one measurement is not included in another) used for fasciolid adults were BL, BW, BP, OS max, OS min, VS max, VS min, A-VS, VS-Vit, Vit-P, PhL, PhW and TL, where at least one dimension was measured among the most important morphological characters. The remaining variables were all significantly correlated with the first principal component (PC1), contributing 72 % to overall variations. PC1 could therefore be accepted as a general indicator of size (BOOKSTEIN, 1989), so that the resulting factor maps (Table 3.3) clearly illustrate global size differences in the populations analysed. For adult fasciolids, the following standardised measurements were taken (Fig. 2.7). Since the morphometric maximum values are characteristic for each population, they are considered the comparative base of this study.

Adult measurements	Bangladesh	Spain	Corsica	Burkina Faso
	F. sp. n=102	*F. hepatica* n=84	*F. hepatica* n=86	*F. gigantica* n=81
Body length, BL	30.29±0.77 20.19-53.58	11.64–22.93 17.41±0.23	12.22–29.00 20.45±0.37	28.82–52.30 39.72±0.58
Body width, BW	10.32±0.17 6.98-15.39	6.41–13.88 10.02±0.17	4.88–14.07 10.71±0.18	6.03–11.84 8.45±0.14
BW at ovary level, BWOv	9.03±0.19 5.10-12.90	4.53–11.67 7.90±0.14	4.46–11.46 8.06±0.15	5.33–11.55 7.59±0.13
Body perimeter, BP	81.23±1.71 57.22-130.96	28.58–54.40 43.05±0.55	30.21–66.43 49.21±0.81	63.19–113.71 85.25±1.21
Body roundness, BR	1.69±0.02 1.39-2.25	1.06–1.58 1.23±0.01	1.10–1.55 1.25±0.01	1.71–3.65 2.47±0.05
Cone length, CL	2.46±0.05 1.90-3.30	1.09–2.92 2.02±0.04	1.58–3.04 2.21±0.03	2.10–3.36 2.67±0.04
Cone width, CW	3.24±0.09 2.00-5.00	2.35–4.21 3.20±0.04	2.30–4.01 3.08±0.04	2.23–5.08 3.74±0.06
OS maximum diameter, OSmax	0.72±0.01 0.50-1.00	0.57–1.03 0.85±0.01	0.60–0.99 0.84±0.01	0.52–1.17 0.83±0.02
OS minimum diameter, OSmin	0.47±0.02 0.20-0.90	0.44–0.77 0.61±0.01	0.40–0.83 0.65±0.01	0.21–0.95 0.62±0.02
VS maximum diameter, VSmax	1.12±0.03 0.85-1.85	0.92–1.49 1.13±0.01	0.69–1.38 1.10±0.01	0.87–1.92 1.50±0.02
VS minimum diameter, VSmin	0.98±0.02 0.79-1.80	0.86–1.35 1.12±0.01	0.83–1.26 1.08±0.01	0.80–1.83 1.38±0.02
Distance between anterior end of body and VS, A-VS	2.50±0.03 2.00-3.00	1.12–2.92 2.05±0.04	1.35–3.04 2.48±0.04	1.46–3.01 2.36±0.03
Distance between suckers, OS-VS	1.82±0.08 0.70-4.30	0.57–2.41 1.42±0.04	0.89–2.32 1.82±0.03	1.15–2.22 1.71±0.03
Distance between VS and union of vitelline glands, VS-Vit	18.36±0.53 9.35-36.04	6.57–14.31 9.60±0.17	5.97–18.22 11.64±0.26	12.26–34.11 22.68±0.45

TABLA 3.3: Comparative morphometric data of adult liver flukes from bovines of Bangladesh with previously published data of standard populations from Spain, France and Burkina Faso described by PERIAGO et al. (2006). n. number of adult liver flukes measured; lineal biometric characters in mm. areas in mm^2.

Distance between Vit and posterior end of body, Vit-P	9.42±0.32 5.20-17.02	2.63–7.57 4.73±0.10	2.90–8.99 5.17±0.15	8.97–21.43 13.45±0.32
Distance between VS and P, VS-P	27.78±0.77 17.71-50.82	9.51–19.94 14.40±0.22	8.86–25.08 16.90±0.36	26.28–50.09 36.39±0.59
Pharynx length, PhL	0.74±0.03 0.27-1.57	0.52–1.00 0.70±0.01	0.10–0.97 0.76±0.01	0.46–1.06 0.78±0.02
Pharynx width, PhW	0.60±0.02 0.26-1.14	0.26–0.83 0.44±0.01	0.29–0.63 0.41±0.01	0.23–0.68 0.42±0.01
Testicular space length, TL	17.17±0.38 12.00-29.00	5.45–11.68 7.91±0.15	5.38–15.99 9.85±0.23	12.76–29.38 18.76±0.38
Testicular space width, TW	5.27±0.14 3.00-8.00	4.18–8.93 6.61±0.12	3.17–10.11 7.39±0.14	3.24–8.66 5.51±0.12
Testicular space perimeter, TP	44.88±1.02 30.00-74.80	17.10–34.34 25.32±0.42	15.75–40.29 29.85±0.56	25.97–68.06 44.62±0.87
Body area, BA	318.41±11.20 164.11-637.60	54.90–197.40 123.83±3.28	46.80–261.71 153.61±4.70	162.58– 482.91 249.38±7.48
Oral sucker area, OSA	0.36±0.02 0.10-0.90	0.25–0.56 0.41±0.01	0.22–0.59 0.43±0.01	0.10–1.11 0.46±0.03
Ventral sucker area, VSA	1.16±0.06 0.68-3.33	0.67–1.57 0.99±0.02	0.45–1.30 0.93±0.02	0.56–3.52 1.86±0.08
Pharynx area, PhA	0.50±0.03 0.08-1.79	0.16–0.57 0.31±0.01	0.04–0.54 0.31±0.01	0.12–0.64 0.33±0.02
Testicular space area, TA	95.20±4.65 36.00-243.60	17.85–70.62 40.45±1.24	13.77–100.47 56.30±1.96	41.05–187.50 80.75±3.44
BL/BW ratio	2.96±0.07 1.83-4.87	1.29–2.77 1.74±0.03	1.33–2.80 1.91±0.03	3.40–6.77 4.70±0.08
BWOv/CW ratio	2.89±0.06 1.97-4.08	1.52–3.46 2.47±0.04	1.47–3.86 2.62±0.05	1.34–3.63 2.03±0.05
Sucker ratio, OSA/VSA	0.30±0.01 0.15-0.48	0.24–0.60 0.41±0.01	0.21–0.99 0.46±0.01	0.08–0.40 0.25±0.01
BL/VS-P ratio	1.10±0.003 1.05-1.15	1.14–1.31 1.21±0.004	0.95–1.38 1.21±0.01	0.95–1.20 1.09±0.004

TABLA 3.3: Comparative morphometric data of adult liver flukes from bovines of Bangladesh with previously published data of standard populations from Spain, France and Burkina Faso described by PERIAGO et al. (2006). n. number of adult liver flukes measured; lineal biometric characters in mm. areas in mm^2.

3.2.2. Results

Fasciolid populations from cattle from Bangladesh were grouped according to the maximum and minimum values of given differentiating morphological measurements previously proposed for *F. hepatica*, *F. gigantica* or *Fasciola* sp. (= intermediate forms) (PERIAGO et al., 2006): BR: 1.06–1.58 in *F. hepatica*, 1.71–3.65 in *F. gigantica*; BL/BW: 1.29–2.80 in *F. hepatica*, 3.40–6.78 in *F. gigantica*; and VS–P: 8.86–25.08 mm in *F. hepatica*, 26.28–50.09 mm in *F. gigantica*. The specimens from Bangladesh were first grouped according to BR and BL/BW, and, secondarily, according to VS–P. Using these criteria, adult specimens from cattle were grouped into *F. hepatica*-like (11.76 %), *F. gigantica*-like (47.06 %) or *Fasciola* sp.-like (41.18 %). Similarly, adult specimens from buffaloes were grouped into *F. hepatica*-like (5.88 %), *F. gigantica*-like (39.22 %) or *Fasciola* sp.-like (54.90 %). The resulting factor maps clearly illustrate global size differences in the cattle and buffalo populations analysed, each group being represented by its perimeter (Fig. 3.3). Two independent zones can be distinguished: one zone is made up of samples from Spain and Corsica, while the other zone consists only of samples from Burkina Faso.

For Bangladesh the Figure 3.3 indicates that 24 % of specimens fit the *F. gigantica*-morph and that 65 % are intermediate forms, white 11 % fit the *F. hepatica*-morph.

For Pakistan Figure 3.3 indicates that 40 % of specimens fit the *F. gigantica*-morph and that 42 % are intermediate forms, white 18 % fit the *F. hepatica*-morph.

Principal component analysis of adult fasciolids from natural infections in bovines of Bangladesh (dashed line) compared with *F. hepatica* from Valencia, Spain (dotted line) and Corsica, France (dashed-dotted line) and *F. gigantica* from Burkina Faso (solid line) (PERIAGO et al., 2006). Samples are projected on to the first (PCI, 58 %) and second (PCII, 29 %) principal components. These zones overlap with the samples from Bangladesh. The multivariate analysis used to measure the changes in size of fasciolid adults from Bangladesh and compared with the above mentioned standard populations for each pure fasciolid species showed that the

size of most fasciolids from Bangladesh is situated between *F. hepatica* and *F. gigantica* standard populations.

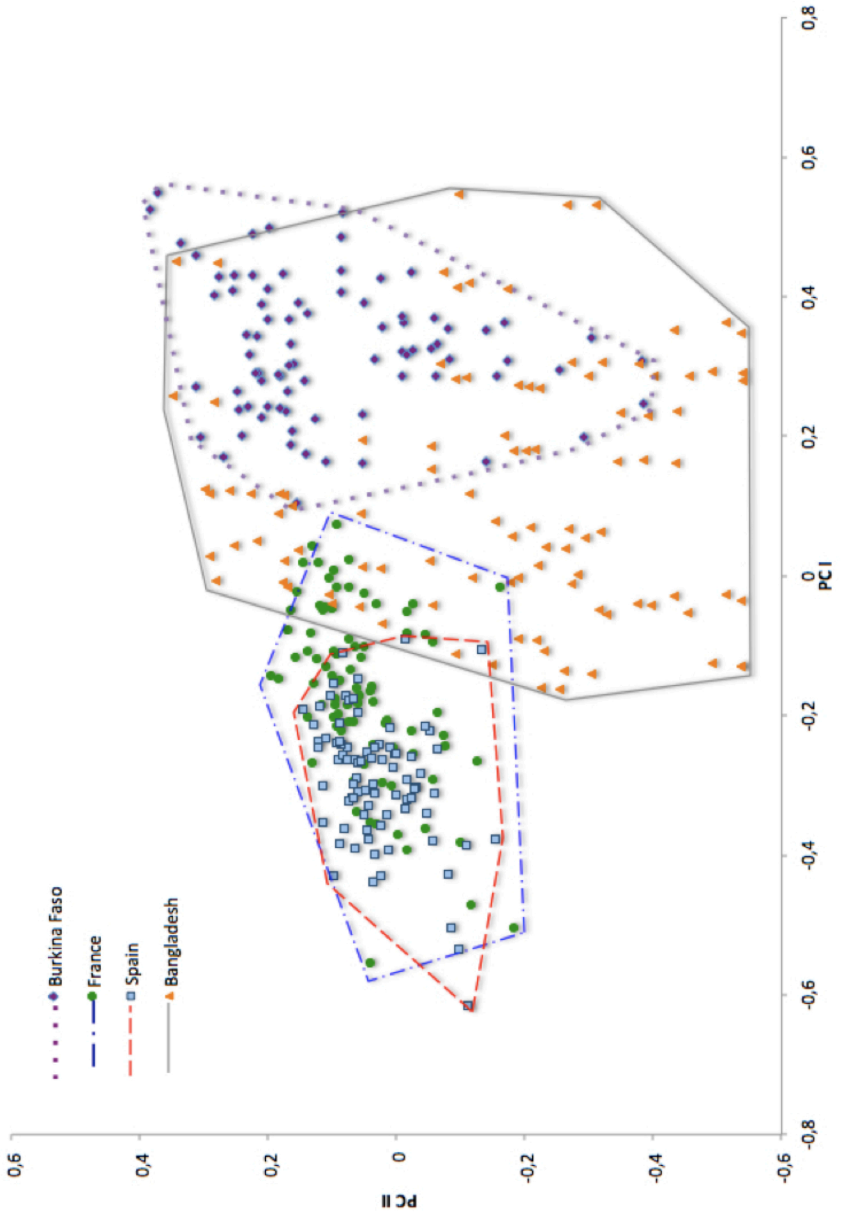

FIGURA 3.2: Principal component analysis of adult fasciolids from natural infections in bovines of Bangladesh (solid line) compared with *F. hepatica* from Valencia, Spain (dashed line) and Corsica, France (dashed-dotted line) and *F. gigantica* from Burkina Faso (dotted line) (PERIAGO et al., 2006). Samples are projected on to the first (PCI, 58 %) and second (PCII, 29 %) principal components.

These results demonstrate that intermediate forms of fasciolids exist in Bangladesh. Nevertheless, it is worth mentioning that the samples from Bangladesh overlap with *F. hepatica* and *F. gigantica* standard populations, but in the absence of specimens of standard *F. hepatica* and *F. gigantica* from Bangladesh it is also possible that the forms identified as *F. hepatica*-like and *F. gigantica*-like could be the extreme values of the morphometric distribution of the *Fasciola* sp.-like specimens.

3.2.3. Discussion

In the material from Bangladesh, the CIAS detection of the three phenotypic groups of *F. hepatica*-like forms (cattle: 11.76 %; buffalo: 5.88 %), *F. gigantica*-like forms (cattle: 47.06 %; buffalo: 39.22 %) or *Fasciola* sp.-like forms (cattle: 41.18 %; buffalo: 54.90 %) represent highly interesting results and pose several question marks. The first aspect to be highlighted is the geographical and physiographical similarities between Bangladesh and the lowlands of Pakistan, where human fascioliasis has been reported recently (QURESHI et al., 2005; QURESHI & TANVEER, 2009) and where human infection by *Fasciola* has more recently proved to be related to climate and global changes (AFSHAN et al., 2014). Bangladesh presents abundant water bodies, irrigation fields and canals, making it a suitable habitat for the lymnaeid snail vectors. Geographical locations, agroecological and agrobiological conditions, and climate change with the particular trend of increasing temperatures are enhancing the abundance, seasonality, reproduction and distribution of different species of snail borne trematodes throughout the country. Bangladesh is globally recognised as the most vulnerable country to climate change, e.g. in Bangladesh the average temperature has registered an upward trend of about 1°C in May and 0.5°C in November along the 14 year period from 1985 to 1998 (IPCC, 2007). According to general analyses, climate change is bound to have a severe impact on the transmission of this disease in Bangladesh (MAS-COMA et al., 2008, 2009b). Thus, similarity results suggest that a risk for human infection in Bangladesh should be considered. The percentages of the three forms found in the bovines studied, with similar proportions in both cattle and buffaloes, and with a patent domination of *F. gigantica*-like forms and *Fasciola* sp.-like forms closer to *F. gigantica* than to *F. hepatica*, agree with the known evolutionary historical

scenario throughout southern Asia (MAS-COMA et al., 2009a). The detection of *F. hepatica*-like forms poses the question mark about the lymnaeid vectors which may be needed for their transmission in Bangladesh. *Fasciola hepatica* is mainly transmitted by small size lymnaeid species of the *Galba/Fossaria* group (BARGUES et al., 2007, 2011a), including *Galba truncatula* as the main vector and the only one in Europe, but also present in Asia, Africa and South America. *Fasciola gigantica* is transmitted by lymnaeids of larger size of the *Radix* group (BARGUES & MAS-COMA, 2005). The presence of lymnaeid vectors defines not only the distribution of fascioliasis, but may also explain the distribution of human infection within a country, as has been recently observed in different countries (ARTIGAS et al., 2011; BARGUES et al., 2011b,c), and within an endemic area, as well as its seasonality or permanent transmission (BARGUES et al., 2012). In southern Asia G. truncatula is restricted to the highlands in countries such as Afghanistan and Pakistan (KENDALL, 1954,1965) and its absence as well as the lack of appropriate *Galba/Fossaria* lymnaeid species for the development of *F. hepatica* in India and eastward up to South-east Asia is well known (MAS-COMA et al., 2009a). In Bangladesh, G. truncatula has never been found, nor does the country have temperatures below 15°C (except for about 10 days a year), which indicates a scenario not appropriate for the transmission of *F. hepatica*. Consequently, it may be perhaps better to consider that the forms identified as *F. hepatica*-like could indeed correspond to the extreme values of the morphometric distribution of the *Fasciola* sp.-like specimens.

	Burkina Faso	Spain	Pakistan	France	Bangladesh
Burkina Faso	0.00				
Spain	6.23	0.00			
Pakistan	3.36	4.19	0.00		
France	6.15	1.53	4.87	0.00	
Bangladesh	4.56	3.72	4.53	2.90	0.00

TABLA 3.4: Mahalanobis distances between liver-flukes from Burkina Faso, France, Spain, Bangladesh and Pakistan.

3.3. Adult fasciolids from Pakistan vs Bangladesh

3.3.1. Results

The PC analysis of fasciolid populations from bovines from Pakistan, Bangladesh, Spain, France and Burkina Faso are indicated in (Fig. 3.3). The resulting factor maps clearly illustrate global size differences in the cattle and buffalo populations analysed, each group being represented by its perimeter. The results shows an overlap of the perimeters from Pakistan and Bangladesh populations, indicating a strong phenotypic homogeneity. Mahalanobis distances showed maximum distances when Burkina Faso, France, Spain, Bangladesh and Pakistan were compared in (Table 3.4).

3.3.2. Discussion

Previous studies showed the presence of morphologically intermediate forms in geographically sympatric areas of *F. hepatica* and *F. gigantica* in Africa and Asia. In Africa, the presence of intermediate forms has been morphometrically evidenced by means of the CIAS methodology in Egypt (PERIAGO et al., 2008). In Asia, a varied spectrum of morphological forms of fasciolids has been described in several countries, such as India (VARMA, 1953), Japan (WATANABE & IWATA, 1954; WATANABE, 1962; OSHIMA et al., 1968a, b; TERASAKI et al., 2000), Korea (CHU & KIM, 1967), the Philippines (KIMURA et al., 1984), Thailand (SRIMUZIPO et al., 2000), Iran (ASHRAFI et al., 2006b), Vietnam (ITAGAKI et al., 2009) and China (PENG et al., 2009) based on traditional microscopic measurements. In Iran, however, the presence of intermediate forms was verified by the same methodology as the one applied in this study (PERIAGO et al., 2008). In relation to the distribution of fasciolids in Asia, historical, archeological, biogeographic, climatic and lymnaeid faunal data indicate that two different main routes from the Fertile Crescent and eastwards separated by the large Himalayan chain need to be considered (MAS-COMA et al., 2009a). One northward from the Himalaya includes the spread of mainly *F. hepatica* and another route southward from the Himalaya concerns the spread of mainly *F. gigantica* through the

southern Asian region. This southward spread appears to have been linked to the higher temperatures and presence of the appropriate *Radix* vector species in the lowlands of Afghanistan, Pakistan, India and eastward up to South East Asia. This spread should have been facilitated by the extensive trade between the two primary centres of India and the Fertile Crescent during the 4000-1000 BC period and the later, very intense and long distance commercial exchanges between those southern Asian countries and Near East countries, for instance, through the southern routes of the Silk Road, which was active over 15 centuries, from around 138 years BC until the 15th century. Camels, taurine and zebu cattle were mainly used for the transportation of goods and merchandise, while dromedaries were later incorporated into the most southern routes of the Silk Road through Afghanistan, Pakistan and India because of their better adaptation to warmer climates (MAS-COMA et al., 2009a).

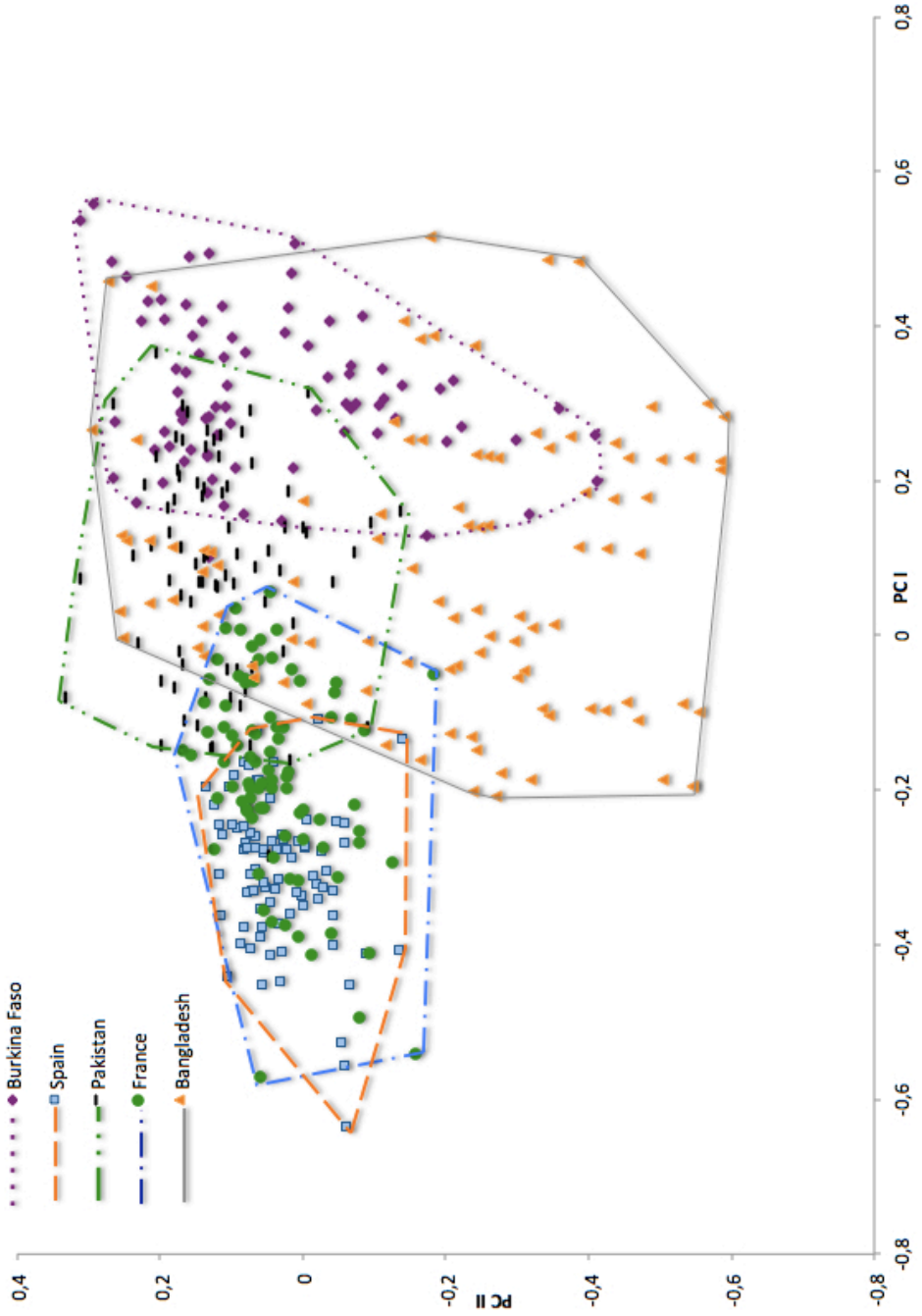

FIGURA 3.3: Principal component analysis of adult fasciolids from natural infections in bovines of Pakistan (dashed and double dotted line) and Bangladesh (solid line) compared with *F. hepatica* from Valencia, Spain (dashed line), Corsica, France (dashed-dotted line) and *F. gigantica* from Burkina Faso (dotted line) (PERIAGO et al., 2006). Samples are projected on to the first (PCI, 54 %) and second (PCII, 29 %) principal components.

Capítulo 4

Fenotipaje de fasciólidos adultos en Irán

Phenotyping of fasciolids in Iran

4.1. Relationships between zonal overlap and phenotypic traits

The Iranian province of Guilan is, on the contrary, of particular interest due to the wide human fascioliasis endemic area it includes and mainly to the two very large human epidemics which occurred in the past (MASSOUD, 1990; WHO, 1995; ASSMAR et al., 1991; FORGHANPARAST et al., 1993; FORGHANPARAST & ASHRAFI, 2001; MOGHADDAM et al., 2004a; TALAIE et al., 2004; ASHRAFI et al., 2004, 2006a,b, 2007). The first outbreak involved an estimate of around 10,000 people in 1987 and the second of around 5000 people occurred ten years later. The majority of human cases in Guilan were detected in people who lived in the lowlands of Bandar-Anzali and Rasht districts, in an area located at 23 m b.s.l. These are the human fascioliasis outbreaks known to have involved a higher number of patients so far. Descriptions of human fascioliasis out-breaks have so far only included a relatively small number of related patients, such as members of a family in an animal endemic area in Argentina (MERA & SIERRA et al., 2011), or relatively reduced in the number of patients (usually fewer than 100) in human endemic areas usually after a particularly rainy year, such as in France or Cuba (ESTEBAN et al., 1998). The causes of the two large outbreaks in Guilan still remain to be elucidated, but the different aspects evoked to be potentially linked to them do unfortunately not explain them. The implication of local traditional foods (ASHRAFI et al., 2006b) would explain the outbreaks affecting humans but not livestock (no epidemic situation in animals was reported during these two periods), although this does not appear to be the origin as indeed these local foods have always been consumed in Guilan. Increased precipitation, noted to be the cause of these outbreaks (SALAHI-MOGHADDAM et al., 2011; SALAHI- MOGHADDAM & ARFAA, 2013), did neither explain them as indeed the previous rainy periods did not appear to be exceptional (no flooding in the area), parallel animal outbreaks were not reported, and similar human outbreaks were never reported before nor have they afterwards in the Guilan area. Multidisciplinary research is warranted to clarify the transmission pattern and epidemiological situation in the

human fascioliasis endemic area of the Iranian province of Guilan, as the needed baseline to elucidate what really happened in those two large human outbreaks, to forecast similar epidemics in the future and establish appropriate control measures, not only in Guilan but also in other zonal overlap endemic areas in other countries. The present study aims to assess the geographical distribution of *F. hepatica* and *F. gigantica* in the Guilan province in depth. The specific differentiation of fasciolids has been made by means of phenotypic characterisation of adult flukes (VALERO et al., 2005) infecting the different livestock species throughout the province and including both lowland and highland areas. This is the first time that such an exhaustive study is made in a zonal overlap endemic area and is therefore expected to establish a methodology which may be used to evaluate other similar fascioliasis areas adequately. Moreover, it is also the first time that morphometric tools are used to analyse the decisive influence exercised by different host species on the metric traits of *F. gigantica* adults.

4.1.1. Materials and Methods

Only parasites obtained from bovines were used in this study. The Material used is detailed in Section 2.1.2 in the Material & Methods. The study area is described in 2.1.1.6 Section.

4.1.2. Morphometrics

An accurate morphometric study was conducted to phenotypically discriminate between *F. hepatica* and *F. gigantica*. All measurements of adult worms and eggs were made according to a previously described standardised methodology (VALERO et al., 2005, 2009a; PERIAGO et al., 2006, 2008). After egg collection from the uteri of flukes, standardised measurements were taken using a microscope and images captured by a digital camera (Nikon Coolpix), which were then analysed by image analysis software (ImagePro plus version 5.0 for Windows, Media Cybernetics, Silver Spring, USA) (see 2.2.2.8 Section). For adult fasciolids, the standardised measurements described in 2.2.2.8 Section were taken (Fig. 2.7).

Since the morphometric maximum values are characteristic for each population, they are considered the comparative base of this study.

4.1.3. Data analysis

Morphological variation is quantified by geometrical morphometrics (ROHLF & MARCUS, 1993). The principal component analysis is used to summarize most of the variations in a multivariate dataset in few dimensions (DUJARDIN & LE PONT, 2004) and is described in 2.3 Section. The following non-redundant measurements (one measurement is not included in another) used for fasciolid adults were BL, BW, BP, OS max, OS min, VS max, VS min, A-VS, VS-Vit, Vit-P, PhL, PhW and TL, where at least one dimension was measured among the most important morphological characters. The remaining variables were all significantly correlated with the first principal component (PC1), contributing 72 % to overall variations. PC1 could therefore be accepted as a general indicator of size (BOOKSTEIN, 1989), so that the resulting factor maps, clearly illustrate global size differences in the populations analysed. Ovoposition is the inflection point of logistic growth, marking the end of the exponential period and the beginning of the "saturated" period, i.e. the beginning of egg shedding to the external environment constitutes the biological factor that marks the inflection point (VALERO et al., 2005, 2006b), which implies that immature (= without eggs in the uterus) and small individuals correspond to recent infections. Consequently, the simultaneous presence of juvenile and fully developed adult worms was used as the determining criterion to assign the geographical area in which parasite transmission may mainly occur.

4.1.4. Results

4.1.5. Analysis of altitudinal relationships

Integration of morphological data from all adult specimens from Guilan through Principal Component Analysis was made. The fasciolid variables all significantly correlated with PC1. The resulting factor maps clearly illustrate global size differences in the populations analysed, where each group is represented by its perimeter. Two independent zones can be distinguished: one zone corresponds to the vast predominance of *F. hepatica*-like specimens (independently of the host species and its breeding location), while the other zone corresponds to the vast predominance of *F. gigantica*-like (independently of the host species and its breeding location). These zones overlap in a few specimens. These results suggest that intermediate forms of fasciolids exist in our sample analysis. Nevertheless it is worth mentioning that, in the absence of specimens of standard *F. hepatica* and *F. gigantica* from Iran, it is also possible that the forms identified as *F. hepatica*-like and *F. gigantica*-like could be the extreme values of the morphometric distribution of the *Fasciola* sp.- like specimens (Fig. 4.1). *Fasciola hepatica*-like flukes were present in cattle, buffaloes, sheep and goats from -27 m up to 1,821 m altitude, while *F. gigantica*-like flukes were present in cattle, buffaloes and sheep from -27 m up to 1,691 m altitude (Fig. 4.1). Of the 69 livers analysed, 22 were co-infected with both fasciolids: 17 from cattle, three from buffaloes, one from sheep and one from goats.

For an in-depth analysis of the zonal distribution, *F. hepatica*-like and *F. gigantica*-like specimens detected in the 69 livers analysed were divided into four groups according to altitude:

- 27 m b.s.l. to 0 m (-27-0 m) with 99 F.hepatica-like and 1,282 *F. gigantica*-like,
- 1-99 m a.s.l. (1-99 m) with 958 F.hepatica-like and 690 *F. gigantica*-like,
- 100-999 m a.s.l. (100-999 m) with 880 F.hepatica-like and 368 *F. gigantica*-like,
- 1,000-2,000 m a.s.l. (1,000-2,000 m) with 163 F.hepatica-like and 3 *F. gigantica*-like.

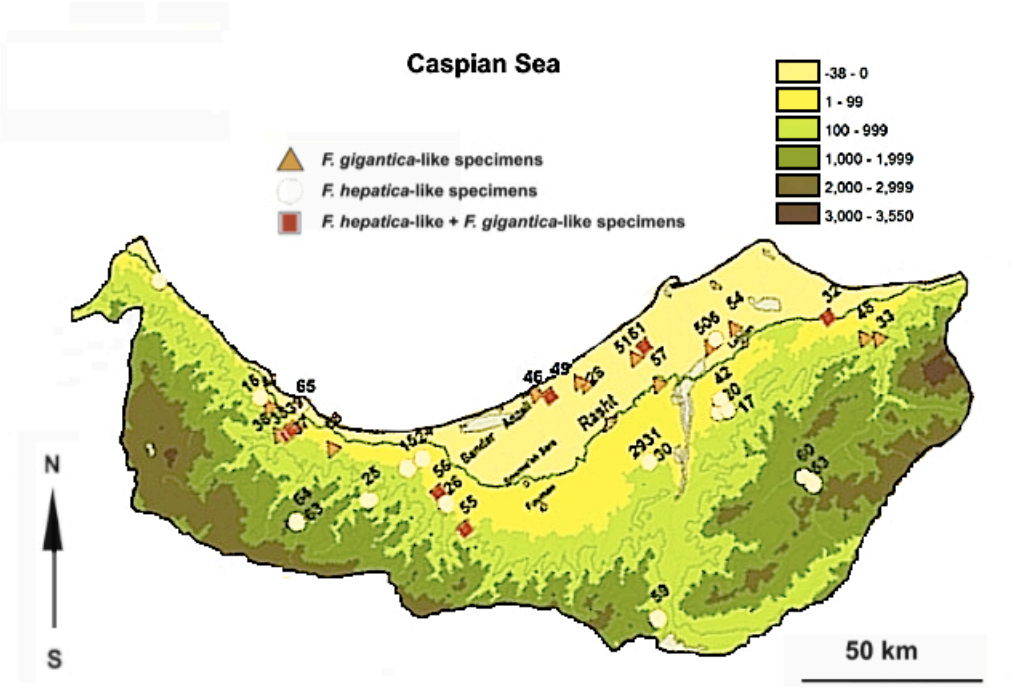

FIGURA 4.1: Map of Guilan, Iran

Sample sizes of the different groups analysed in each species, the year of sample collection, definitive host species, the breeding site of the definitive host (with latitude, longitude and altitude), and the slaughterhouse where sample collection took place (with latitude, longitude and altitude) are indicated in (Table 4.1). The distribution of the average % of *F. hepatica*-like and *F. gigantica*-like specimens detected in each liver versus average of altitude in each group (m) is shown in (Fig. 4.2).

Relationship between altitude and the average of, *Fasciola hepatica*-like and *F. gigantica*-like specimens.

The presence of *F. gigantica*-like specimens mainly in locations below sea level (11.23 % F.h., 88.77 % F.g.), the presence of both species with similar intensity at 1-99 m (56.95 % F.h., 43.05 % F.g.), and the presence of *F. hepatica*-like specimens mainly from 100 m onwards (100-999 m: 71.69 % F.h., 28.31 % F.g.), as well as the almost disappearance of *F. gigantica*-like specimens at the highest altitudes (1,000-2,000 m: 97.48 % F.h., 2.52 % F.g.), are noteworthy. The bivariant correlation

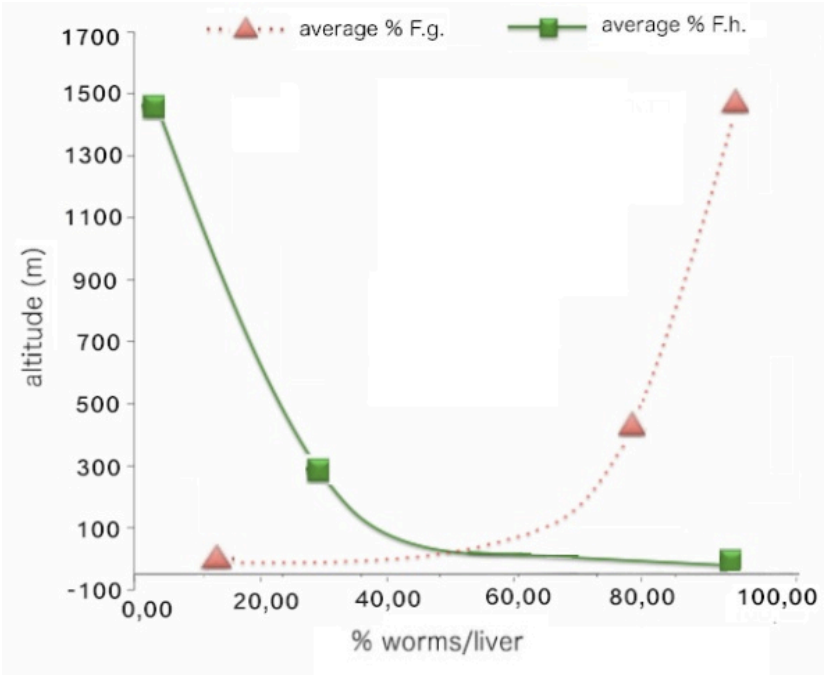

FIGURA 4.2: Relationship between altitude and the average of % *Fasciola hepatica*-like and *F. gigantica*-like specimens.

between altitude vs % F.h., Ln(% F.h.), % F.g. and Ln(% F.g.) was calculated. A significant positive correlation was obtained between altitude and % F.h. (r = 0.339, P< 0.001) and Ln(% F.h.) (r = 0.300, P< 0.05). A significant negative correlation was obtained between altitude and % F.g. (r = -0.339, P< 0.001) and Ln(% F.g.) (r = -0.279, P< 0.001).

					Livestock breeding site				Slaughterhouse					
Host	Age	Sex	Year[a]	Code[b]	Locality	Latitude	Longitude	Altitude	Locality	Latitude	Longitude	Altitude	*F.h. (*)*	*F.g.(*)*
Buffalo	3	F	2003	65	Talesh	37°49'55'' N	48°57'14"E	-11	Talesh	37°48'00'' N	48°54'54"E	45	17 (8)	73 (39)
Cattle	3	F	2002	2	Khomam	37°23'53'' N	49°35'56"E	-23	Anzali	37°28'23'' N	49°27'14"E	-23	-	17 6(29)
Cattle	♂	♂	2002	4	Lavandavil	38°18'50'' N	48°51'22"E	-10	Astara	38°28'26'' N	48°51'54"E	-25	-	157 (17)
Cattle	7	F	2002	6	Astaneh	37°15'54'' N	49°56'46"E	-8	Syahkal	37°08'02'' N	49°53'17"E	61	61 (9)	-
Cattle	4	F	2003	28	Hassanrood	37°24'58'' N	49°35'13"E	-24	Khoshkebijar	37°21'50'' N	49°47'06"E	-12	-	100 (23)
Cattle	5	F	2003	32	Amlash	37°07'01'' N	50°13'19"E	-2	Lahijan	37°12'14'' N	50°00'07"E	5	4	269 (75)
Cattle	2	F	2003	43	Khomam	37°24'18'' N	49°40'8"E	-19	Anzali	37°28'23'' N	49°27'14"E	-23	-	105
Cattle	25	F	2003	46	Anzali	37°27'47'' N	49°28'30"E	-25	Anzali	37°28'23'' N	49°27'14"E	-23	-	150 (29)
Cattle	3	M	2003	49	Khomam	37°25'52'' N	49°30'7"E	-27	Khoshkebijar	37°21'50'' N	49°47'06"E	-12	3 (3)	21 (15)
Cattle	2	F	2003	50	Astaneh	37°15'54'' N	49°55'19"E	-14	Lahijan	37°12'14'' N	50°00'07"E	5	2	13 (10)
Cattle	6	F	2003	51	Khoshkebijar	37°22'05'' N	49°46'55"E	-13	Lahijan	37°12'14'' N	50°00'07"E	5	10 (3)	110 (81)
Cattle	4	F	2003	54	Lahijan	37°15'40'' N	50°00'11"E	-15	Lahijan	37°12'14'' N	50°00'07"E	5	1	49 (31)
Cattle	5	F	2003	57	Khoshkebijar	37°16'37'' N	49°45'11"E	0	Lahijan	37°12'14'' N	50°00'07"E	5	1	44 (32)
Cattle	3	F	2003	61	Khoshkebijar	37°22'05'' N	49°44'42"E	-14	Khoshkebijar	37°21'50'' N	49°47'06"E	-12	-	15 (8)

27 m below sea level to

TABLA 4.1: Sample sizes of the *Fasciola hepatica*-like (F.h.) and *F. gigantica*-like (F.g.) groups analysed in each definitive host species (including sex and age in years), the year of sample collection, the breeding site of the definitive host (with latitude, longitude and altitude), and the slaughterhouse where sample collection took place (with latitude, longitude and altitude) in Guilan province, Iran. (*) = In parenthesis the number of parasites included in the morphometrical analysis. F = female; M = male. a = Year of sample collection. b = Number code used to identify the samples.

Livestock breeding site										Slaughterhouse								
	5	F	2002	10	Rezvanshahr	37°32'31" N	49°05'10" E	94	Rezvanshahr	37°33'04" N	49°08'53"E	4	14	4				
Buffalo	6	F	2002	11	Rezvanshahr	37°33'04" N	49°08'46" E	4	Rezvanshahr	37°33'04" N	49°08'53"E	4	2	17				
Buffalo	3	M	2003	45	Talesh	37°53'20" N	48°53'10" E	48	Talesh	37°48'00" N	48°54'54"E	45	-	176 (84)				
Cattle	3	M	2002	5	Syahkal	37°09'47" N	49°49'8"E	22	Syahkal	37°08'02" N	49°53'17"E	61	49	-				
Cattle	¿	¿	2002	14	Amlash	37°05'38" N	50°10'19" E	45	Roodsar	37°08'35" N	50°16'05"E	-22	-	27				
Cattle	3	M	2003	22	Astara	38°18'58" N	48°50'53" E	2	Masal	37°22'23" N	49°09'07"E	36	84 (50)	-				
Cattle	6	F	2003	37	Talesh	37°48'00" N	48°52'48" E	87	Talesh	37°48'00" N	48°54'54"E	45	5 (4)	10 (6)				
Cattle	4	F	2003	38	Talesh	37°48'04" N	48°54'47" E	40	Talesh	37°48'00" N	48°54'54"E	45	-	45 (18)				
Cattle	¿	¿	2003	39	Talesh	37°47'56" N	48°54'0"E	55	Talesh	37°48'00" N	48°54'54"E	45	7 (4)	53 (37)				
Cattle	4	F	2003	41	Masal	37°25'52" N	49°07'8"E	53	Masal	37°22'23" N	49°09'07"E	36	-	23				
Cattle	10	F	2003	47	Roodsar	37°02'13" N	50°19'23" E	36	Roodsar	37°08'35" N	50°16'05"E	-22	2	6				
Cattle	4	F	2003	56	Masal	37°25'30" N	49°06'50" E	66	Masal	37°22'23" N	49°09'07"E	36	45 (40)	40 (20)				
Cattle	2	M	2003	62	Talesh	37°47'46" N	48°53'38" E	68	Khoshkebijar	37°21'50" N	49°47'06"E	-12	5					
Cattle	6	F	2003	68	Asalem	37°42'00" N	48°57'22" E	46	Anzali	37°28'23" N	49°27'14"E	-23	-	130 (44)				
Cattle	4	F	2004	69	Masal	37°24'50" N	49°08'20" E	39	Anzali	37°28'23" N	49°27'14"E	-23	1	29				
Goat	1	M	2003	42	Syahkal	37°08'13" N	49°51'32" E	61	Syahkal	37°08'02" N	49°53'17"E	61	53 (41)	-				
Sheep	¿	¿	2002	7	Rahimabad	37°02'10" N	50°19'16" E	36	Roodsar	37°08'35" N	50°16'05"E	-22	21	-				
Sheep	4	F	1905	21	Rezvanshahr	37°34'52" N	49°04'44" E	26	Rezvanshahr	37°33'04" N	49°08'53"E	4	2	-				
Sheep	4	F	1905	23	Masal	37°23'13" N	49°08'49" E	29	Masal	37°22'23" N	49°09'07"E	36	2	-				
Sheep	4	F	2003	21	Rezvanshahr	37°34'52" N	49°04'44" E	26	Rezvanshahr	37°33'04" N	49°08'53"E	4	2	-				
Sheep	4	F	2003	23	Masal	37°23'13" N	49°08'49" E	29	Masal	37°22'23" N	49°09'07"E	36	2	-				
Sheep	3	F	2003	24	Rezvanshahr	37°31'23" N	49°07'48" E	47	Rezvanshahr	37°33'04" N	49°08'53"E	4	8 (8)	-				
Sheep	3	F	2003	26	Masal	37°23'38" N	49°06'40" E	59	Masal	37°22'23" N	49°09'07"E	36	21 (5)	-				
Sheep	1	F	2003	27	Masal	37°23'56" N	49°08'42" E	30	Masal	37°22'23" N	49°09'07"E	36	74	-				
Sheep	3	F	1905	66	Rahimabad	37°02'20" N	50°20'17" E	34	Rahimabad	37°03'11" N	50°19'16"E	26	9	400				
Sheep			1905	67	Masal	37°24'29" N	49°08'31" E	35	Masal	37°22'23" N	49°09'07"E	36	550	-				

1-99 m above sea level

TABLA 4.1: Sample sizes of the *Fasciola hepatica*-like (F.h.) and *F. gigantica*-like (F.g.) groups analysed in each definitive host species (including sex and age in years), the year of sample collection, the breeding site of the definitive host (with latitude, longitude and altitude), and the slaughterhouse where sample collection took place (with latitude, longitude and altitude) in Guilan province, Iran. (*) = In parenthesis the number of parasites included in the morphometrical analysis. F = female; M = male. a = Year of sample collection. b = Number code used to identify the samples.

Livestock breeding site										Slaughterhouse						
Cattle	3	F	2002	8	Talesh	37°49'01" N	48°51'54" E	286	Talesh	37°48'00" N	48°54'54"E	45	12			
Cattle	6	F	2002	9	Talesh	37°49'44" N	48°51'54" E	186	Talesh	37°48'00" N	48°54'54"E	45	2			
Cattle	4	♂	2003	33	Rahimabad	36°59'49" N	50°17'46" E	109	Rahimabad	37°03'11" N	50°19'16"E	26	-			
Cattle	2	F	2003	36	Talesh	37°49'01" N	48°51'47" E	286	Talesh	37°48'00" N	48°54'54"E	45	-			
Cattle	8	F	2003	48	Roodsar	37°01'19" N	50°15'58" E	504	Roodsar	37°08'35" N	50°16'05"E	-22	3			
Cattle	4	F	2003	52	Syahkal	37°07'37" N	49°51'40" E	104	Syahkal	37°08'02" N	49°53'17"E	61	5			
Cattle	5	F	2003	55	Masal	37°18'32" N	49°06'50" E	305	Masal	37°22'23" N	49°09'07"E	36	40 (40)	36 (26)		
Goat	4	F	2002	12	Rezvanshahr	37°33'07" N	49°01'1"E	381	Rezvanshahr	37°33'04" N	49°08'53"E	4	17	1		
Goat	4	F	2002	15	Rezvanshahr	37°31'48" N	49°05'10" E	243	Rezvanshahr	37°33'04" N	49°08'53"E	4	162 (93)	-		
Goat	3	F	2002	16	Talesh	37°54'58" N	48°52'59" E	119	Talesh	37°48'00" N	48°54'54"E	45	20 (20)	-		
Goat	2	M	2002	18	Fooman	37°06'58" N	49°13'55" E	523	Fooman	37°13'05" N	49°16'19"E	61	1	-		
Goat	3	F	2003	29	Rasht	37°08'02" N	49°36'47" E	126	Sangar	37°09'04" N	49°40'59"E	39	146 (65)	-		
Goat	3	F	2003	30	Rasht	37°08'02" N	49°36'47" E	126	Sangar	37°09'04" N	49°40'59"E	39	99 (46)	-		
Goat	♂	♂	2003	31	Rasht	37°08'02" N	49°36'47" E	126	Sangar	37°09'04" N	49°40'59"E	39	81 (28)	-		
Goat	3	M	2003	40	Fooman	37°12'00" N	49°06'50" E	931	Fooman	37°13'05" N	49°16'19"E	61	25	-		
Sheep	2	F	2002	17	Syahkal	37°06'11" N	49°51'14" E	155	Syahkal	37°08'02" N	49°53'17"E	61	57 (26)	-		
Sheep	3	F	2002	19	Fooman	37°06'58" N	49°13'55" E	523	Fooman	37°13'05" N	49°16'19"E	61	2	-		
Sheep	2	F	2003	25	Rezvanshahr	37°31'44" N	48°57'4"E	666	Rezvanshahr	37°33'04" N	49°08'53"E	4	48 (28)	-		
Sheep	1	F	1905	35	Talesh	37°49'01" N	48°51'47" E	286	Talesh	37°48'00" N	48°54'54"E	45	31	-		
Sheep	4	F	1905	44	Talesh	37°49'05" N	48°53'31" E	118	Talesh	37°48'00" N	48°54'54"E	45	10	-		
Sheep	1	F	2003	35	Talesh	37°49'01" N	48°51'47" E	286	Talesh	37°48'00" N	48°54'54"E	45	31	-		
Sheep	4	F	2003	44	Talesh	37°49'05" N	48°53'31" E	118	Talesh	37°48'00" N	48°54'54"E	45	10	-		
Sheep	1	F	2003	58	Roodbar	36°48'25" N	49°22'59" E	743	Roodbar	36°47'10" N	49°56'02"E	1922	-	5		
Sheep	1	F	2003	59	Roodbar	36°48'25" N	49°22'59" E	743	Roodbar	36°47'10" N	49°56'02"E	1922	-	40 (21)		

100-999 m above sea level

TABLA 4.1: Sample sizes of the *Fasciola hepatica*-like (F.h.) and *F. gigantica*-like (F.g.) groups analysed in each definitive host species (including sex and age in years), the year of sample collection, the breeding site of the definitive host (with latitude, longitude and altitude), and the slaughterhouse where sample collection took place (with latitude, longitude and altitude) in Guilan province, Iran. (*) = In parenthesis the number of parasites included in the morphometrical analysis. F = female; M = male. a = Year of sample collection. b = Number code used to identify the samples.

Livestock breeding site										Slaughterhouse					
Goat	3	F	2002	1	Kermanshah	34°17'56" N	47°03'14" E	1437	Anzali	37°28'23" N	49°27'14"E	-23	26	-
Goat	1	M	2003	60	Deylaman	36°50'35" N	49°54'58" E	1691	Deilaman	36°51'40" N	49°56'02"E	1348	15 (8)	2
Sheep	♂	F	2002	3	Astara	38°14'35" N	48°17'56" E	1351	Astara	38°14'35" N	48°17'56"E	1351	47	-
Sheep	6	F	2002	13	Hamadan	34°47'46" N	48°30'22" E	1821	Roodsar	37°08'35" N	50°16'05"E	-22	16	-
Sheep	♂	♂	2003	53	Deylaman	36°49'37" N	49°55'37" E	1445	Syahkal	37°08'02" N	49°53'17"E	61	16 (12)	1
Sheep	5	F	2003	63	Matash	37°36'14' N	48°46'19" E	1446	Talesh	37°48'00' N	48°54'54"E	45	25 (10)	-
Sheep	4	F	2003	64	Matash	37°36'14' N	48°46'19" E	1446	Talesh	37°48'00' N	48°54'54"E	45	18 (7)	-

1,000-2,000 m above sea level

TABLA 4.1: Sample sizes of the *Fasciola hepatica*-like (F.h.) and *F. gigantica*-like (F.g.) groups analysed in each definitive host species (including sex and age in years), the year of sample collection, the breeding site of the definitive host (with latitude, longitude and altitude), and the slaughterhouse where sample collection took place (with latitude, longitude and altitude) in Guilan province, Iran. (*) = In parenthesis the number of parasites included in the morphometrical analysis. F = female; M = male. a = Year of sample collection. b = Number code used to identify the samples.

4.1.6. Morphometric assessment

For the morphological analysis, the fasciolid populations from Guilan were also grouped according to shape into *F. hepatica*-like and *F. gigantica*-like, host species (F.h. from cattle, buffaloes, sheep and goats; F.g. from cattle, buffaloes and sheep) and geographical altitude (-27-0 m; 1-99 m; 100-999 m; 1,000-2,000 m) into 16 groups (Table 4.1):

F. hepatica-like specimens from: cattle: F.h. cattle -27-0 m, F.h. cattle 1-99 m; F.h. cattle 100-999 m, buffaloes: F.h. buffaloes -27-0 m, sheep: F.h. sheep 1-99 m; F.h. sheep 100-999 m; F.h. sheep 1,000-2,000 m, goats: F.h. goat -27-0 m; F.h. goat 100-999 m; F.h. goat 1,000-2,000 m.

F. gigantica-like specimens from: cattle: F.g. cattle -27-0 m, F.g. cattle 1-99 m; F.g. cattle 100-999 m; F.g. cattle 1,000-2,000 m, buffaloes: F.g. buffaloes -27-0 m, F.g. buffaloes 1-99 m, sheep: F.g. sheep 100-999 m. The size of liver fluke adults was studied by multivariate analyses. A scatterplot of the first two principal components (PC) is shown in (Fig. 4.3). The first common principal component (PCI) of the 16 populations analysed can be interpreted as a measure of overall size, all significantly correlated with PCI, thus contributing 69.0 % to overall variation. The comparison of *F. hepatica*-like vs *F. gigantica*-like specimens showed the presence of intermediate forms in all livestock species analysed (cattle, buffalo, sheep and goat). The scatterplot can be divided into two parts related to smaller (juveniles and ingravid specimens) and larger specimens (fully developed and gravid specimens) in both *F. hepatica*-like and *F. gigantica*-like populations. The results show that *F. hepatica* from mountains, hills and lowland populations in cattle, buffalo, sheep and goat share their higher values, but smaller specimens are present mainly in populations with positive altitudinal values (mainly 1-99 m), independently of the host species. All this indicates that, in lowlands, transmission of *F. hepatica*-like specimens takes place in zones above sea level. Furthermore, *F. hepatica*-like specimens are the main morph detected in highlands (mainly above 100 m).

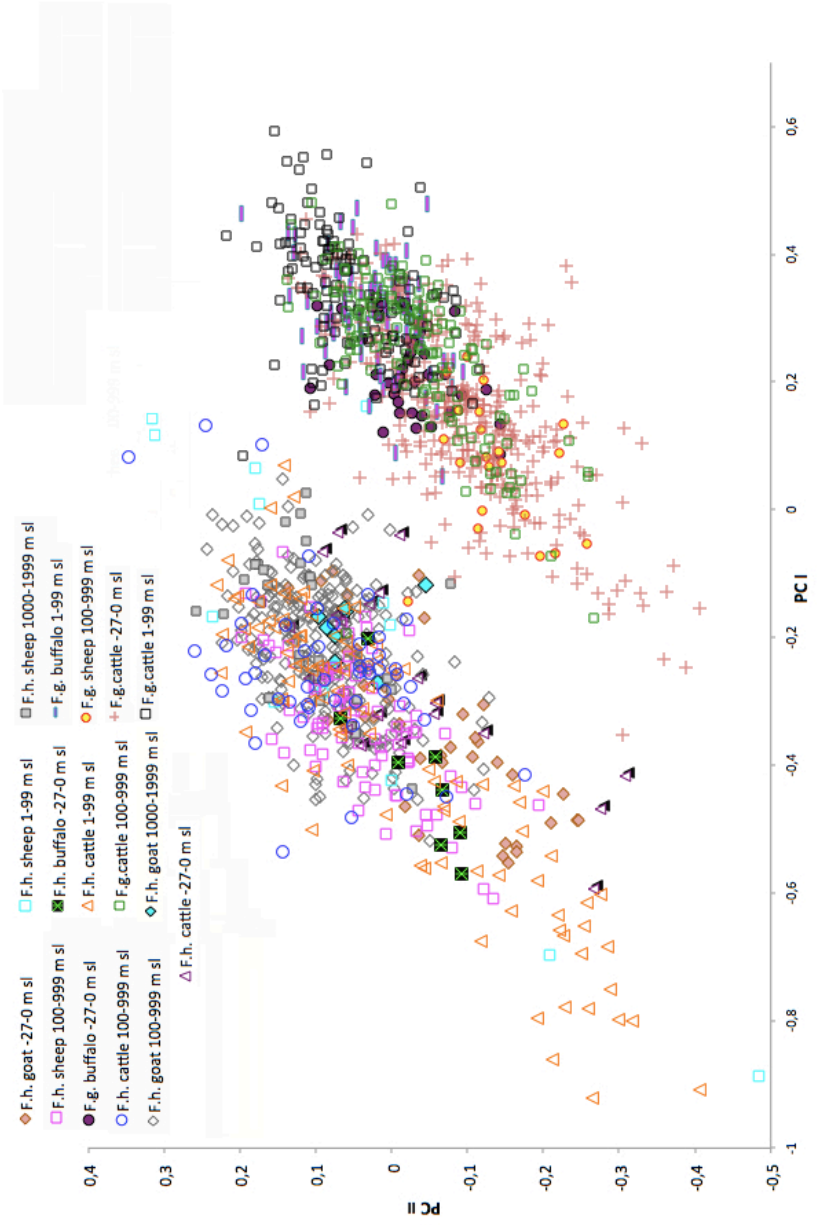

FIGURA 4.3: Altitude influence on fasciolid distribution: Factor map corresponding to worms of *Fasciola* from naturally infected livestock from Guilan province according to altitude range groupings: *Fasciola hepatica*-like specimens from cattle (F.h. cattle -27 - 0 m, F.h. cattle 1-99 m; F.h. cattle 100-999), buffaloes (F.h. buffaloes -27 - 0 m), from sheep (F.h. sheep 1-99 m; F.h. sheep 100-999; F.h. sheep 1,000-2,000 m), and goats (F.h. goat -27 - 0 m; F.h. goat 100-999 m; F.h. goat 1,000-2,000 m); *F. gigantica*-like specimens from sheep (F.g. sheep 100-999 m), cattle (F.g. cattle -27 - 0 m, F.g. cattle 1-99 m; F.g. cattle 100-999; F.g. cattle 1,000-2,000 m), and buffaloes (F.g. buffaloes -27 - 0 m, F.g. buffaloes 1-99 m). Samples are projected onto the first (PCI, 69 %) and second (PCII, 11 %) principal components.

Altitude influence on fasciolid distribution. Factor map corresponding to worms of *Fasciola* from naturally infected livestock from Guilan province according to altitude range groupings: *Fasciola hepatica*-like specimens from cattle (F.h. cattle -27 - 0 m, F.h. cattle 1-99 m; F.h. cattle 100-999), buffaloes (F.h. buffaloes -27 - 0 m), from sheep (F.h. sheep 1-99 m; F.h. sheep 100-999; F.h. sheep 1,000-2,000 m), and goats (F.h. goat -27 - 0 m; F.h. goat 100-999 m; F.h. goat 1,000-2,000 m); *F. gigantica*-like specimens from sheep (F.g. sheep 100-999 m), cattle (F.g. cattle -27 - 0 m, F.g. cattle 1-99 m; F.g. cattle 100-999; F.g. cattle 1,000-2,000 m), and buffaloes (F.g. buffaloes -27 - 0 m, F.g. buffaloes 1-99 m). Samples are projected onto the first (PCI, 69 %) and second (PCII, 11 %) principal components.

The results show that *F. gigantica*-like populations in cattle, buffaloes and sheep share higher values, but smaller specimens are present mainly in lowland populations located below sea level, independently of the host species (cattle, buffalo). *F. gigantica*-like flukes from lowland cattle presented a larger worm variability. Summing up, the results obtained indicate that there are four different fascioliasis transmission areas in Guilan (Fig. 4.4):

a) lowland coastal areas neighbouring the Caspian Sea shore, below sea level, where basically *F. gigantica*-like specimens are found;

b) a coastal plain with an altitude between 1-99 m where both species co-exist;

c) areas with altitude values of 100-999 m where mainly *F. hepatica*-like specimens are found;

d) highland mountainous areas where basically *F. hepatica*-like specimens are found.

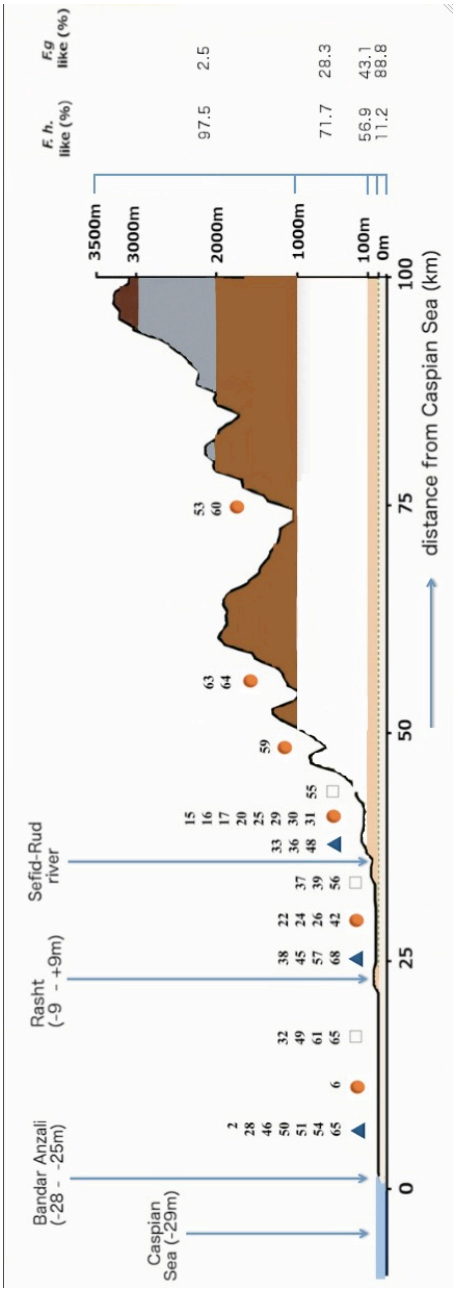

FIGURA 4.4: Relationship between the topographic profile of Guilan province (section through Bandar Anzali and Rasht cities) and the altitudinal presence of *Fasciola hepatica*-like and *F. gigantica*-like specimens. To assess the topographic profile ArcGis software, using an irregular triangle network (TIN) and a raster from SRTM30 dataset- CGIAR-SRTM data aggregated to seconds, produced by NASA Shuttle Radar Topographic Mission (SRTM). Circle = *Fasciola hepatica*-like specimens; triangle = *Fasciola gigantica*-like specimens; square = coexistence of *Fasciola hepatica*-like and *Fasciola gigantica*-like specimens in the same liver.

The study of the influence of the host species on the liver fluke was also carried out by PCI analysis and size-out analysis. Regarding size, variables of fasciolid adults from domestic animals (cattle, buffalo, sheep and goat) all significantly correlated with PCI, thus contributing 69 % to overall variation. The resulting factor maps (Fig. 4.6) clearly illustrate global size differences in *F. gigantica*-like populations analysed vs bovines (cattle and buffaloes) and sheep. The results show that the liver fluke size of *F. gigantica*-like populations from sheep is smaller than that from cattle and buffaloes; these differences are not as marked. However, when *F. hepatica*-like specimens are compared, i.e. specimens from cattle are slightly larger than those from sheep, which, in turn, are slightly larger than specimens from goats and buffaloes. The *F. gigantica*-like population from buffaloes presented a similar size than that found in cattle populations. Regarding shape, PCII versus PCIII analysis illustrates global shape differences in *F. gigantica*-like populations analysed vs bovines (cattle and buffaloes) and sheep. Mahalanobis distances showed maximum distances when *F. hepatica* and *F. gigantica* were compared. When comparing *F. gigantica*-like from sheep vs *F. gigantica*-like specimens from bovines (cattle and buffaloes, respectively), distances detected were larger than in the inter-bovine comparison of *F. gigantica*-like specimens (cattle vs buffaloes). Likewise, the maximum distance (3.51) was obtained when *F. hepatica*-like specimens from sheep were compared with *F. hepatica*-like specimens from buffaloes (Fig. 4.6).

Regarding shape, PCII versus PCIII analysis illustrates global shape differences in *F. gigantica*-like populations analysed vs bovines (cattle and buffaloes) and sheep. Mahalanobis distances showed maximum distances when *F. hepatica*-and *F. gigantica*-were compared (Table 4.2). When comparing *F. gigantica*-like from sheep vs *F. gigantica*-like specimens from bovines (cattle and buffaloes, respectively), distances detected were larger than in the inter-bovine comparison of *F. gigantica*-like specimens (cattle vs buffaloes). Likewise, the maximum distance (3.51) was obtained when *F. hepatica*-like specimens from sheep were compared with *F. hepatica*-like specimens from buffaloes (Fig. 4.5).

	F.h. goat -27-0m	F.h. goat 100-999m	F.h. goat 1000-1999m	F.h. sheep 1-99m	F.h. sheep 100-999m	F.h. sheep 1000-1999m	F.g. sheep 100-999m	F.h. buffalo -27-0m	F.g. buffalo -27-0m	F.g. buffalo 1-99m	F.h. cattle -27-0m	F.h. cattle 1-99m	F.h. cattle 100-999m	F.g. cattle -27-0m	F.g. cattle 1-99m	F.g. cattle 100-999m
F.h. goat -27-0m	0.0															
F.h. goat 100-999m	1.57	0.00														
F.h. goat 1000-1999m	2.93	2.34	0.00													
F.h. sheep 1-99m	2.57	1.80	3.24	0.00												
F.h. sheep 100-999m	2.93	1.99	2.27	2.24	0.00											
F.h. sheep 1000-1999m	2.39	1.91	2.29	2.14	1.15	0.00										
F.g. sheep 100-999m	6.84	7.18	6.09	7.12	7.67	7.37	0.00									
F.h. buffalo -27-0m	2.72	2.70	3.12	3.51	2.61	2.68	7.31	0.00								
F.g. buffalo -27-0m	6.32	6.73	6.01	6.51	7.31	6.88	1.60	6.85	0.00							
F.g buffalo 1-99m	6.60	6.96	6.38	6.54	7.56	7.17	1.98	7.42	1.39	0.00						
F.h. cattle -27-0m	1.71	1.79	2.36	2.46	2.47	2.02	6.24	1.85	5.64	6.11	0.00					
F.h. cattle 1-99m	1.75	1.59	2.49	2.60	1.86	1.70	7.31	1.49	6.86	7.31	1.49	0.00				
F.h. cattle 100-999m	2.04	1.22	2.06	2.40	1.36	1.18	7.60	2.46	7.20	7.52	1.95	1.32	0.00			
F.g. cattle -27-0m	6.70	7.24	6.86	6.89	7.98	7.52	2.42	7.43	1.27	1.46	6.21	7.41	7.83	0.00		
F.g. cattle 1-99m	6.52	6.96	6.49	6.43	7.59	7.09	2.67	7.61	1.92	1.20	6.18	7.31	7.51	1.75	0.00	
F.g. cattle 100-999m	6.57	7.18	6.66	6.84	7.87	7.33	2.34	7.59	1.58	1.36	6.25	7.40	7.70	1.28	1.14	0.00

TABLA 4.2: Mahalanobis distances between the 16 fluke adult groups distinguished in Guilan province, Iran, according to parasite species (Fh = *F. hepatica*-like; Fg = *F. gigantica*-like), host species (*F. h.* from cattle, buffaloes, sheep and goats;*F. g.* from cattle, buffaloes and sheep) and geographical altitude (ranges: -27- 0 m; 1-99 m; 100-999 m; 1,000-2,000 m).

Host influence on fasciolid size: Factor map showing principal component analysis of adult fasciolids from natural infections in livestock in Guilan province, Iran. F.h. = *Fasciola hepatica*-like specimens from cattle, buffaloes, sheep and goats; F.g. = *Fasciola gigantica*-like specimens from cattle, buffaloes and sheep. Samples are projected on to the first (PCI) and second (PCII) principal components. Each group is represented by its perimeter.

4.1.7. Discussion

4.1.7.1. Morphometric modelling of overlap areas

Given that fascioliasis is able to follow different transmission patterns and gives rise to different epidemiological situations (MAS-COMA, 2005; MAS-COMA et al., 2009a), the characterisation of local scenarios and patterns of human and animal infection must always be considered the starting point before implementing any measure of infection control. For this purpose, the first step is always to ascertain the causal agent(s) present and its (their) distribution in the endemic area, bearing in mind the different transmission, epidemiological, pathological and control characteristics depending on the fasciolid species involved. In overlap areas, specific adscription of parasite adults and eggs remains difficult (MAS-COMA et al., 2014a). The only tools available for fasciolid discrimination are based on molecular biology techniques based on DNA sequencing (MAS-COMA et al., 2009a) or mathematical assessment of morphometry considering fluke development, allometric growth and host-induced variation (VALERO et al., 2005, 2009a). The application of DNA sequencing techniques is costly and special equipment is required. Therefore, morphology has traditionally been the most commonly used criterion for systematic studies on fasciolids and its application is less costly. Yet, an accurate morphological discrimination between fasciolid species remains difficult due to the many variations in their metric traits (BERGEON & LAURENT, 1970) and also because of the fact that intermediate fasciolid forms of adults usually appear in overlap areas (PERIAGO et al., 2008). In Asia, especially Japan, Taiwan, the Philippines and Korea, a wide range of morphological types has been detected using traditional microscopic measurements (WATANABE, 1962; OSHIMA et al., 1968a; AKAHANE et al., 1970; KIMURA et al., 1984; SRIMUZIPO et al., 2000;

TERASAKI et al., 2000). Concretely, in Iran, intermediate forms of *Fasciola* have been found in Mazandaran (MOGHADDAM et al., 2004a) and accurately described in Guilan (ASHRAFI et al., 2006a). The existence of these intermediate forms has been molecularly verified to be not only common but even the rule in these overlap areas (MAS-COMA et al., 2009a) and poses a question mark on whether metric characteristics are suitable as a tool for the differential diagnosis of fascioliasis caused by either species. So far, there are only few studies that have quantitatively analysed the influence that the definitive host species has on adult and egg morphology of fasciolids (BORAY, 1969; AKAHANE et al., 1970, 1974; SRIMUZIPO et al., 2000; VALERO et al., 2001a, 2009a), having mainly focused on *F. hepatica* from areas where only this species is present. Thus, appropriate studies on *F. gigantica* are needed. Moreover, no comparative study has been carried out until now with adults from different host species originating from geographical areas where both species co-exist, whenever possible in human endemic areas. The present study in Guilan province addresses all these aspects for the first time, with the aim to obtain a model which might be subsequently applied to other zonal overlap endemic areas of fascioliasis in Iran or other countries and thereby assess up to which level overlap areas may be similar or different. This knowledge is crucial when control measures in the different overlap areas have to be applied, either being the same or made to measure for each overlap area. In modern morphometrics, the estimate of size is contained in a single variable reflecting variation in many directions, as many as there are landmarks under study, and shape is defined as their relative positions after correcting for size, position and orientation. With these informative data, and the corresponding software freely available to conduct complex analyses, significant biological and epidemiological features can be quantified more accurately (DUJARDIN, 2008). Through this methodology, the analysis of the morphometric characterisation of populations of *F. hepatica*-like and *F. gigantica*-like specimens from Guilan province, taking into account standardized measurements, parasite growth, definitive host species influence and its correlation with the fascioliasis transmission patterns, has been carried out.

4.1.7.2. Geographical distribution of fasciolids

In Guilan province, hitherto studies on the presence of *F. hepatica* and *F. gigantica* have mainly been undertaken in cattle, because this livestock species is by far the most slaugthered in the area of Rasht and Bandar Anzali cities. Sheep breeding is not usually found in lowland areas. Until now, no comparative study has been carried out with fasciolid materials from lowland and highland areas where both species co-exist. Moreover, studies on this question are also needed in human endemic localities, although the analysis of the presence of *F. hepatica* and/or *F. gigantica* in the animal host species can only be an approximation, given the possibility of livestock mobility along time. The results of the present study demonstrate that, in Guilan, fascioliasis follows a zonal overlap transmission pattern, with *F. hepatica*-like transmission taking place mainly in the highlands and *F. gigantica*-like transmission mainly in the lowlands. The co-existence of the two fasciolids in livestock in Guilan suggests a complicated scenario of possible ways of circulation of the causal agents, e.g. through altitudinal livestock transhumance or animal transportation in both past and recent times (MAS-COMA et al., 2009a). Transhumance was practised in many parts of Guilan in the past and nowadays it still occurs on a yearly basis in areas such as those of Talesh and Masal in the west, Rodsar in the south-east and many others where people live near mountains. In these areas, it is traditional to move livestock to highlands for the summer months and back to the lowlands for overwintering. This, animals which harbour *F. gigantica* in the highlands may have been infected most probably during their stay in the lowlands in the cold season. Similarly, livestock presenting *F. hepatica* in the lowlands may have been infected most probably during their stay in the highlands in the hot season. This is, however, not the case in the flatland areas located very far away from the mountains, such as in the districts surrounding the cities of Bandar Anzali and Rasht where livestock transhumancy is not practised. Regarding human infection, the similar infectivity of fasciolid isolates from the different animal reservoir species (VALERO & MAS-COMA, 2000; VALERO et al., 2001a) but mainly the presence and characteristics of their vectors, should be considered. The two fluke species, the smaller *Fasciola hepatica* and the larger *F. gigantica*, are transmitted by respective specific freshwater lymnaeid vector species (BARGUES & MAS-COMA, 2005). *Fasciola*

hepatica is transmitted mainly by species of the *Galba/Fossaria* group which are small lymnaeids preferring mild-cold temperatures, surface small water bodies and highlands (BARGUES et al., 2007, 2011a), whereas *F. gigantica* is transmitted by lymnaeids of the *Radix* group which are usually larger snails preferring warmer temperatures, deeper and larger water bodies and lowlands (BARGUES et al., 2001; MAS-COMA et al., 2009a). The different geographical distributions of these snail vectors explain the distributional differences at continental level, *F. hepatica* presenting a worldwide distribution and *F. gigantica* only covering many regions of Africa and Asia (MAS-COMA et al., 2009a). Lymnaeid vectors also define the geographical distributions of the fasciolids both inside countries and at local level (BARGUES et al., 2011b, c, 2012; ARTIGAS et al., 2011). Therefore, the fauna and ecological characteristics of the lymnaeid vectors present are crucial when defining the different transmission patterns and epidemiological situations of this disease (MAS-COMA et al., 2009a). In several of the regions of Africa and Asia, the distributions of *F. hepatica* and *F. gigantica* overlap due to the coexistence of lymnaeid vector species of the *Galba/Fossaria* and *Radix* groups in the same endemic area. Diagnosis in these areas is troublesome, due to the existence of intermediate forms between the phenotypes of the "pure"species (PERIAGO et al., 2006) in the morphology of the adult stage and in the morphometry of the adult stage as well as eggs, in both animals and humans (VALERO et al., 2005, 2009a; MAS-COMA et al., 2009a, 2014a). These intermediate forms, usually identified as *Fasciola* sp., are the consequence of hybridisation processes between adult specimens of *F. hepatica* and *F. gigantica* in the biliary canals and gallbladder of their hosts (MAS-COMA et al., 2009a) and have already been described in several countries such as Egypt (PERIAGO et al., 2008) in Africa and Iran (ASHRAFI et al., 2006a) in Asia. Our results suggest the presence of intermediate form of fasciolids in Guilan. Interestingly, of the 69 livers analysed, 22 were co-infected with both fasciolids, which means that there are possibilities for hybridization. Hybridization phenomena are known in Trematodes. Within *Schistosoma*, experimental studies have demonstrated that there are no reproductive isolating barriers between *Schistosoma* species belonging to the same group. These hybrids share morphological and biological features with their parental species, and some hybrid generations may even have many reproductive advantages over their parental

Schistosoma species, which in turn has epidemiological consequences as well (PAGES & THERON, 1990; WEBSTER & SOUTHGATE, 2003; FAN & LIN, 2005). Thus, in *Fasciola hepatica* it*Schistosoma*, hybridization has been shown to exhibit several enhanced phenotypic characteristics such as faster maturation time, higher infectivity, higher fecundity, increased pathology and the ability to infect both intermediate snail hosts of the parental species, thereby widening their intermediate host spectrum (WRIGHT & ROSS, 1980; WEBSTER et al., 2013). Therefore, the presence of different types of morphs in Guilan suggests the likelihood of some degree of hybridization between *F. hepatica* and *F. gigantica*. Future experimental studies will elucidate if these intermediate specimens have a better fitness, i.e. infecting both species of the *Galba/Fossaria* group and species of the *Radix* group in order to characterise their different biological aspects of transmission and epidemiology as to establish appropriate fascioliasis control measures. Two different overlap situations were distinguished by MAS-COMA et al. (2009a) in areas where both *Fasciola* species coexist:

Local overlap: in places where climatic characteristics throughout the year enable the coexistence of Galba and *Radix* species in the same locality, nearby water bodies and even sometimes the same water body, thus allowing transmission and consequently infection of livestock and humans living sedentarily in the locality by both *F. hepatica* and *F. gigantica*; The low flatlands of the Nile Delta region in Egypt is a typical example.

- Zonal overlap: in areas with different altitudes so that highlands offer the necessary cold-mild weather conditions for *Galba/Fossaria* and *F. hepatica*, and lowlands offer the warm-hot climate necessary for *Radix* species and *F. gigantica*, definitive hosts becoming co-infected by both fasciolids when moving from lowlands to neighbouring highlands and vice-versa (animals because of interzonal transhumance, transportation and trade; humans when moving around for different reasons).

Among the many different species of Lymnaeidae reported from Iran, the species *Galba truncatula*, Lymnaea *(Stagnicola palustris)*, and (*L.Radix gedrosiana*) were noted to be present in the human endemic lowlands around the cities of Bandar-Anzali and Rasht in Guilan province (MANSOORIAN, 2000; MOGHADDAM et

al., 2004b). In this Guilan lowland area, the two fasciolid species were thought to be involved, because of the presence of the lymnaeid vector species *Galba truncatula*, main vector of *F. hepatica*, and *Lymnaea gedrosiana* (recently molecularly proved to be a synonym of *Radix auricularia* - see ASHRAFI et al. (2014), vector of *F. gigantica*. This situation was assumed because of the northern latitude, temperatures entering the survival range of these two vectors, and the mildering influence of the neighbouring Caspian Sea, whose water surface is at -29 m altitude. Such a transmission pattern and epidemiological situation were initially thought to be similar to those known in the Nile Delta region of Egypt, although studies soon demonstrated that the Guilan and Nile Delta endemic areas do in fact pronouncedly differ from the disease point of view (see MAS-COMA et al., 2009a, p. 92, Fig. 2.3) despite sharing the same vector species and similar lowland endemiotopes. However, another recent study on the lymnaeids present in the Bandar-Anzali and Rasht endemic areas contributed unexpected results related to fascioliasis transmission in the Guilan area. It has been demonstrated that specimens previously ascribed to *Galba truncatula* in that lowland endemic area indeed belong to another species without transmission capacity, *Lymnaea schirazensis*, a lymnaeid of the *Galba/Fossaria* group morphologically similar and hitherto confused with *Galba truncatula* (BARGUES et al., 2011a). This indicates that *F. hepatica* may not be transmitted in that area. Such a situation suggests that only *F. gigantica* may be transmitted in the aforementioned endemic lowlands, similarly as suggested by other previous studies on natural infections of lymnaeid snails and cattle by fasciolids throughout the year (ASHRAFI et al., 2004, 2014a). Thus, it was definitively demonstrated that the Guilan endemic area is in fact a zonal overlap area and not a local overlap area as previously believed, explaining the differences found when compared to the Nile Delta local overlap area (MAS-COMA et al., 2009a). This new discovery was, however, adding confusion to the understanding of fascioliasis in the Guilan lowland area, as indeed *F. gigantica* has never been reported to cause such large epidemics in humans (emerging human fascioliasis situations in southeastern Asia initially noted to be due to *F. gigantica* are indeed caused by *Fasciola* hybrids - see LE et al. (2008). In Guilan, *G. truncatula* has been found transmitting *F. hepatica* in the Talesh mountains (ASHRAFI et al., 2007), whereas *R. auricularia* (= *L. gedrosiana*) appears well distributed in the lowlands around the cities of Bandar Anzali and Rasht (ASHRAFI & MAS-COMA, 2014).

These data correlate with results of previous studies suggesting *F. gigantica* to be the most prevalent species (91.1 %) in these human endemic lowlands around the two aforementioned cities (ASHRAFI et al., 2004). At the highest altitudes (1,000-2,000 m), *F. hepatica*-like specimens dominate infecting sheep and goats, whereas *F. gigantica*-like specimens are only sporadically found in cattle, which suggest their presence in bovines most probably as a consequence of animal movements. However, a sporadic presence of isolated populations of *R. auricularia* (= *L. gedrosiana*) may not a priori be ruled out and is perhaps likely to enable a short transmission season of *F. gigantica* at such altitudes in Guilan during the summer months. Indeed, *R. auricularia* has been noted to be present and transmit *F. gigantica* in altitudes of 1,332 m in the northwestern Azerbaijan province of Iran, where average temperature ranges from 9.4°C to 11.6°C but reaches up to 22.6°C in summer (IMANI-BARAN et al., 2011, 2012, 2013). At the lowest altitudes (from -27 m to 0 m), *F. gigantica*-like specimens dominate infecting cattle and buffaloes, whereas *F. hepatica*-like specimens appear only sporadically in cattle, buffaloes and goats. Again, movements of these livestock species may underlie these findings, although isolated transmission of *F. hepatica* may occur thanks to the presence of scattered populations of *L. (S.) palustris* in the lowland coastal strip (MANSOORIAN, 2000) *L. (S.) palustris* is a species which has been observed to be able to transmit *F. hepatica* under given circumstances (mainly if infected during the first few days of the snail's life) and in the absence of the main vector G. truncatula in different latitudes in other countries (KENDALL, 1970; MCREATH et al., 1982; DREYFUSS et al., 1994; NOVOBILSKY et al., 2013). However, the absolute absence of G. truncatula populations in this lowland coastal strip is still pending confirmation, as not all populations might have been confused with L. schirazensis (BARGUES et al., 2011a). In fact, the mild temperatures during the cooler months in this lowland coastal strip (ASHRAFI & MAS-COMA, 2014) do not a priori exclude the survival capacity of G. truncatula nor a transmission of *F. hepatica* in isolated foci (see temperature thresholds in AFSHAN et al. (2014). At the interface between the highest and lowest altitudes, there appears a strip of land between 1 m and 999 m (lowland areas above sea level at 1-99 m and hilly areas at 100-999 m) in which both *Fasciola* forms are present: *F. hepatica*-like specimens in cattle and sheep at 1-999 m and in goats at 100-999 m, and *F. gigantica*-like specimens in cattle at 1-999 m, in buffaloes only at 1-99 m and in

sheep at 100-999 m. These results indicate that the transition between highlands and lowlands is gradual, most probably related to a distributional overlap of the lymnaeid vector species transmitting the two fasciolids. The two cities and their surrounding areas where most of the patients were detected during the two large human fascioliasis outbreaks in the past are located in the Guilan lowlands, the coastal city of Bandar Anzali between - 28 m and - 25 m and the large city of Rasht extending from -9 m on the seaside to +9 m on the inland side.

4.1.7.3. Phenotypic variation under host species influence, with emphasis on *F. gigantica*

Another aim of the present study was to quantitatively characterise the influence of the host species on the morphometric traits of *F. hepatica* adults (from cattle, buffaloes, sheep and goats) and *F. gigantica* (from cattle, buffaloes and sheep) in natural liver fluke populations from Guilan, taking into account standardised measurements and allometric growth (VALERO et al., 2012c). It should be considered that there is no apparent relationship between the shape of fasciolid adults with regard to altitudinal difference or geographical origin and that allometry-free shape appears as a more stable trait than size in fasciolid species (VALERO et al., 2012c). This is the first study on the decisive influence exercised by the host species on metric traits of *F. gigantica* adults. Morphological variation has been quantified by geometrical morphometrics (ROHLF & MARCUS, 1993). The animal host species can strongly influence the phenotype of the adult stage as well as the egg, mainly due to the different size of the liver duct microhabitat (VALERO et al., 2001a,b, 2002, 2009a). The ability of organisms to produce different phenotypes under different environmental conditions (phenotypic plasticity) has been an object of evolutionary and ecological studies since the Neo-Darwinian synthesis (PIGLIUCCI, 2005). Yet, another direction of future research certainly lies at the interface between genetics and environments, in the epigenetic machinery that somehow translates genetic effects and environmental influences (host species, geographical location) into coherent fasciolid phenotypes. Studies on isoenzymes, protein sequences and mitochondrial DNA sequences suggest that fasciolids are able to develop a capacity for definitive host species selection (MILLER et al., 1993; PANACCIO & TRUDGETT, 1999; SPITHILL et al., 1999). In India, some degree

of genetic variation among *F. gigantica* isolates derived from cattle, buffaloes and goats has been found by RAPD analysis (GUNASEKAR et al., 2008). Differences in the ionic composition between liver flukes from cattle and sheep detected (CASEBY et al., 1995) may reflect physiological adaptations by the fluke to different environments rather than genetically determined traits (PANACCIO & TRUDGETT, 1999). Proteomic analysis of embryonic *F. hepatica* by characterisation of a developmentally regulated heat shock protein shows plasticity in expression of major proteins, particularly a prominently expressed 65kDa protein cluster between natural populations of embryonating *F. hepatica* eggs, suggesting that liver fluke embryogenesis is a plastic process (MOXON et al., 2010). Consequently, research must be carried out to define phenotypic measurements complementing the molecular tools that would enable the analysis of parasite plasticity. Surprisingly, however, only very few studies have quantitatively assessed the influence that the definitive host species exercises on adult and egg morphology of these two fasciolids (BORAY, 1969; AKAHANE et al., 1970, 1974), having mainly focused on *F. hepatica*. Thus, conclusive data have shown the presence of certain morphological traits that characterise *F. hepatica* adult size in different host species: a) cattle, sheep, pigs and donkeys in the northern Bolivian Altiplano (VALERO et al., 2001a,b); b) cattle, sheep and wild boars in Galicia, Spain (MEZO et al., 2013). Likewise, fasciolid eggs shed by humans show morphological traits different from eggs shed by animals (VALERO et al., 2009a), and *F. hepatica* egg size correlates with naturally infected host species such as the house mouse *M. musculus*, the black rat *R. rattus* and cattle on the island of Corsica (VALERO et al., 2002). Nevertheless, little is known about *F. gigantica*. Based on the marked variation in adult dimensions of a mixed *F. gigantica* strain from cattle in Thailand, the comparative morphometry of adults and eggs, adult surface topography under scanning electron microscopy (SEM), and testicular metaphase karyotypes of the provisionally classified races, i.e. body length <25 mm (designated small race), 25-35 mm (designated medium-sized race), >35 mm (designated big race) were carried out to search for differences among the three tentative races. The results showed that the morphological races of *F. gigantica* from Thailand are not the consequence of a possible species complex but the result of plasticity, i.e. similar genotypes give birth to several phenotypes (SRIMUZIPO et al., 2000).

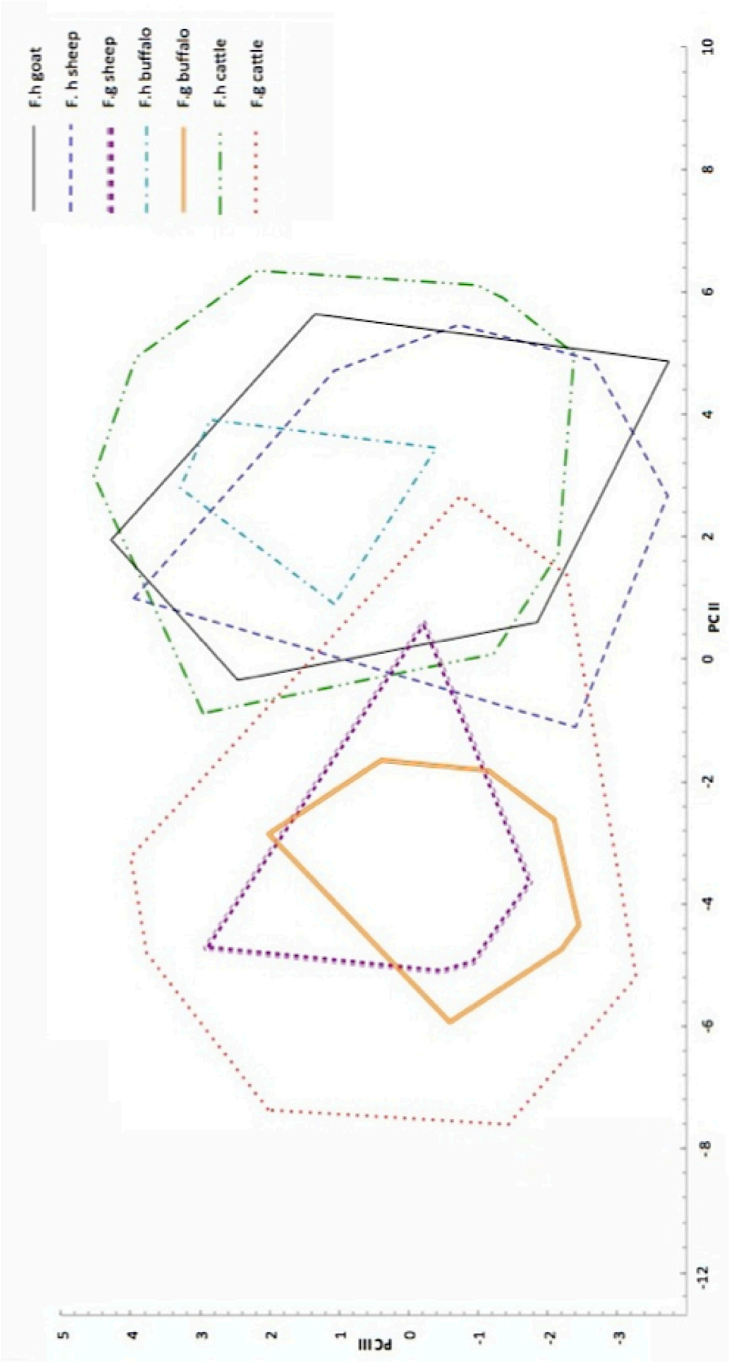

FIGURA 4.5: Host influence on fasciolid shape: Factor map showing principal component analysis of adult fasciolids from natural infections in livestock in Guilan province, Iran. F.h. = *Fasciola hepatica*-like specimens from cattle, buffaloes, sheep and goats; F.g. = *Fasciola* gigantica-like specimens from cattle, buffaloes and sheep. Samples are projected on to the second (PCII) and third (PCIII) principal components. Each group is represented by its perimeter.

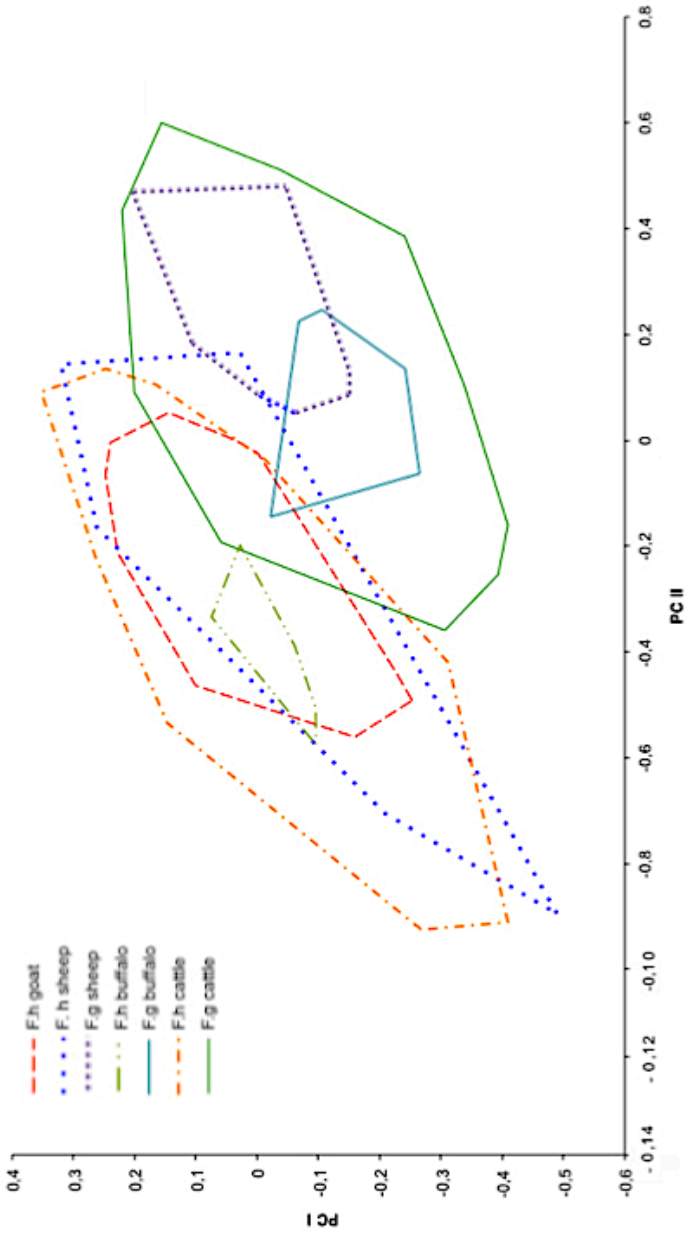

FIGURA 4.6: Host influence on fasciolid shape: Factor map showing principal component analysis of adult fasciolids from natural infections in livestock in Guilan province, Iran. F.h. = *Fasciola hepatica*-like specimens from cattle, buffaloes, sheep and goats; F.g. = *Fasciola* gigantica-like specimens from cattle, buffaloes and sheep. Samples are projected on to the second (PCII) and third (PCIII) principal components. Each group is represented by its perimeter.

Capítulo 5

Fenotipaje de fasciólidos adultos en el jabalí (*Sus scrofa)*

Phenotyping of adult fasciolids in Wild boar

5.1. Phenotyping of adult fasciolids in Wild boar

In Galicia (NW, Spain), the wild boar is the main wild ungulate in terms of abundance and distribution. Its population has continuously increased over the past decades (SCHMALENBERGER et al., 2004) and this population growth has been accompanied by a reduction of habitats, so that the wild boar populations encroach more and more frequently onto agricultural lands. The increase of the interface area between livestock and the wild boars frequently involves the sharing of pastures and water sources, so that the circulation of common pathogens is propitiated. It must be highlighted that in Galicia, the prevalence of fasciolosis in livestock is very high and that some cases of natural infections by *F. hepatica* have been observed in wild boars hunted close to cattle farms (MEZO et al., 2008). However, the role of this wild ungulate in the maintenance and transmission of fasciolosis has not been studied until now.

5.1.1. Materials and Methods

5.1.1.1. Material

The liver-fluke material analyzed in the present chapter was obtained from a study carried out by Dr. M. MEZO from Centro de Investigaciones Agrarias de Mabegondo, Instituto Galego da Calidade Alimentaria-Xunta de Galicia (Spain) in livers from 358 hunted wild boars in the time period between 2007-2010 in Galicia (Spain). The Material used is detailed in Section 2.1.2 in the Material & Methods. The study area is described in 2.1.1.1 Section.

5.1.1.2. Morphometrics

All measurements of adult worms and eggs were made according to a previously described standardised methodology (VALERO et al., 2005, 2009a; PERIAGO et

al., 2006, 2008). After egg collection from the uteri of flukes, standardised measurements were taken using a microscope and images captured by a digital camera (Nikon Coolpix), which were then analysed by image analysis software (Image-Pro plus version 5.0 for Windows, Media Cybernetics, Silver Spring, USA) (see 2.2.2.8 Section). For adult fasciolids, the standardised measurements described in 2.2.2.8 Section were taken (Fig. 2.7). Furthermore, the uterus area was calculated using the methodology previously described by PANOVA (2002) and VALERO et al. (2011). Since the morphometric maximum values are characteristic for each population, they are considered the comparative base of this study.

5.1.1.3. Data analysis

Morphological variation is quantified by geometrical morphometrics (ROHLF & MARCUS, 1993). The principal component analysis is used to summarize most of the variations in a multivariate dataset in few dimensions (DUJARDIN & LE PONT, 2004) and is described in 2.3 Section. The following non-redundant measurements (one measurement is not included in another) used for fasciolid adults were BL, BW, BP, OS max, OS min, VS max, VS min, A-VS, VS-Vit, Vit-P, PhL, PhW and TL, where at least one dimension was measured among the most important morphological characters. The remaining variables were all significantly correlated with the first principal component (PC1), contributing 72 % to overall variations. PC1 could therefore be accepted as a general indicator of size (BOOKSTEIN, 1989), so that the resulting factor maps (Fig. 5.1) clearly illustrate global size differences in the populations analysed.

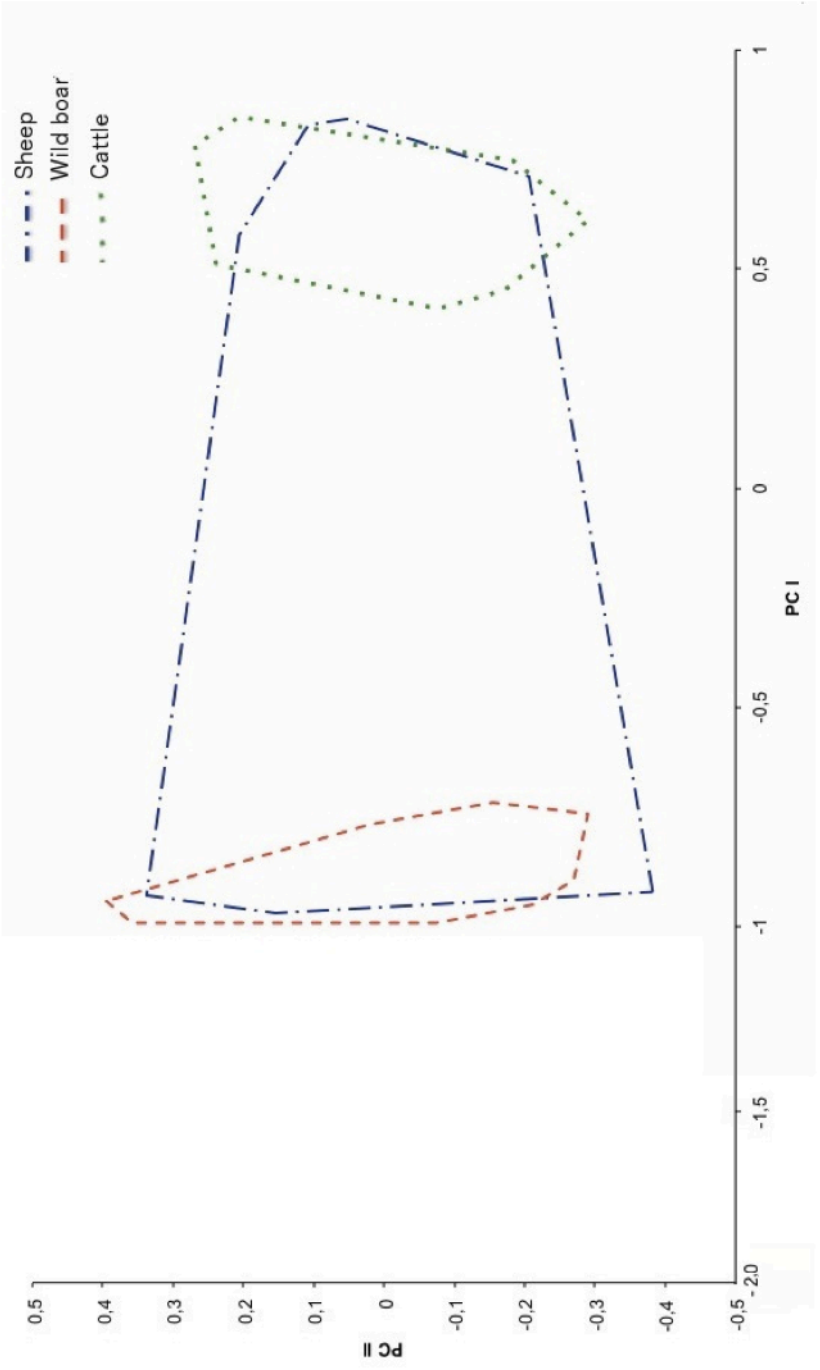

FIGURA 5.1: Principal component analysis of adult *F. hepatica* from natural infections from Galicia in wild boar (dotted line) compared with *F. hepatica* in sheep (dashed line) and *F. hepatica* in cattle (uninterrupted line); samples are projected on to the first (PCI, 91.0 %) and second (PCII, 3.5 %) principal components. Each group is represented by its perimeter.

5.1.2. Results

Comparative morphometric data of *F. hepatica* from cattle, sheep and wild boar are shown in (Table 5.1 and Table 5.2). The development and gravidity of liver flukes from wild boars were similar to those of liver flukes from sheep and cattle (Fig. 5.2). Within-comparison of the values of the *F. hepatica* populations shows a general overlap between them regardless of the different definitive host species of origin. size and shape of liver-fluke bodies were studied by multivariate analyses. A scatter plot of the first two principal components (PC) is shown in (Fig. 5.1).

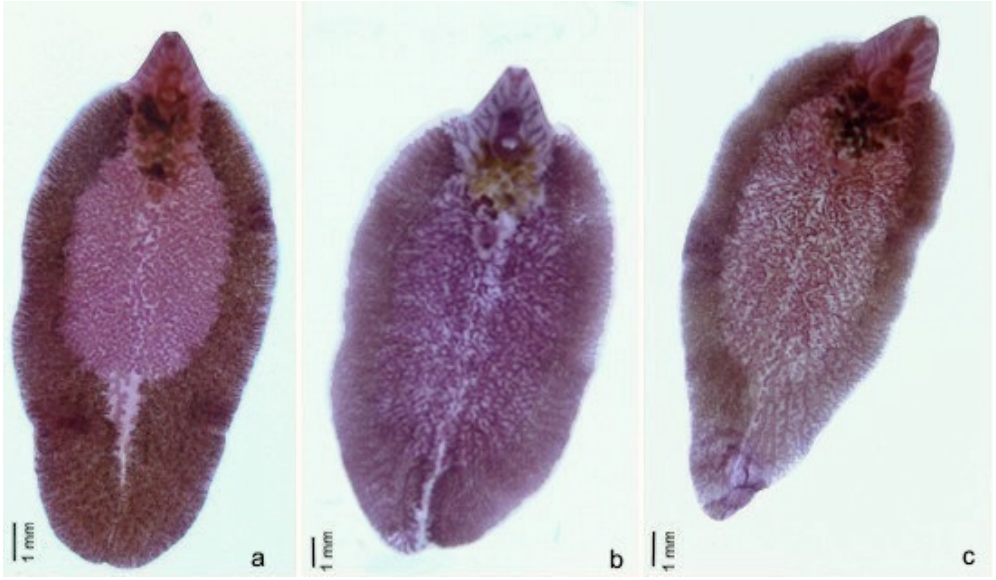

FIGURA 5.2: Gravid *Fasciola hepatica* adults from Galicia (NW, Spain) in: (a) sheep; (b) cattle; (c) wild boar.

The remanning variables were all significantly correlated with the first principal component (PC1), contributing 91 %and with the second principal component (PC2), contributing 3.5 % to overall variations (BOOKSTEIN, 1989), i.e., the resulting factor maps (Fig. 5.1) clearly illustrate global size differences in the populations analyzed. The results show that the bodies of *F. hepatica* populations from sheep are of smaller size than those from cattle. The liver fluke population from wild boars presents an intermediate size between sheep and cattle populations (Fig. 5.1). Our results indicate that liver fluke bodies from wild boars present their own morphometric pattern in size and shape.

Adult measurements (mm)	Sheep n= 88	Board n= 35	Cattle n= 133
Body area, **BA**	38,3-149,80 93,56± 22,81	34,06-138,83 81,30± 26,40	62,00-190,31 117,00±26,60
Body length, **BL**	10,25 - 21,06 16,25 ± 2,13	11,93-25,29 17,01 ± 3,47	12,36-21,94 16,25 ± 2,05
Body width, **BW**	4,96 -11,10 8,15 ±1,31	3,98-9,09 7,03 ± 1,19	6,87-13,05 9,71 ± 1,25
BW at ovary level, **BWOv**	1,91-8,47 6,20±1,05	4,32-7,89 5,81±0,90	5,75-13,95 7,73 ± 1,17
Body perimeter, **BP**	25,76-52,84 40,54± 5,11	28,90-55,25 40,04±7,25	32,35-59,04 44,60 ± 5,40
Body roundness, **BR**	1,17-1,75 1,42± 0,11	1,20-2,15 1,62± 0,23	1,21-1,90 1,42 ±0,14
Cone length, **CL**	1,19-2,39 1,80±0,27	0,93-2,87 1,97±0,52	1,02-2,35 1,74±0,25
Cone width, **CW**	1,97-3,36 2,66±0,31	1,76-3,86 2,70±0,62	1,88-3,96 3,17 ±0,32
BWOv/CW ratio	0,89-3,81 2,34±0,40	1,30-3,50 2,23 ±0,59	1,64-4,35 2,48±0,46
Oral sucker area, **OSA**	0,35-1,22 0,75±0,22	O,21-0,54 0,37±0,08	0,23-1,30 0,41 ±0,12
Maximum diameter of the oral sucker, **OSmax**	0,66 -1,87 1,02 ± 0,19	0,58-1,27 0,88 ± 0,14	0,66-1,09 0,81 ± 0,06
Minimum diameter of the oral sucker, **OSmin**	0,48 -1,31 0,87 ± 0,16	0,31-1,10 0,65 ± 0,20	0,38-0,80 0,56 ± 0,08
Ventral sucker area, **VSA**	0,23-1,08 0,48±0,20	0,67-1,13 0,88 ±0,12	0,35-2,25 0,98± 0,28
Maximum diameter of the ventral sucker, **VSmax**	0,58 -1,32 0,83 ± 0,16	0,58-1,27 0,97 ± 0,16	0,93-1,34 1,15 ± 0,07

TABLA 5.1: Comparative morphometrics with mean values ± SD (range in size) of 82 adult liver fluke uteri in sheep, 17 in wild boars and 124 in cattle from Galicia; n. number of adult liver flukes measured; lineal biometric characters in mm. areas in mm^2.

Adult measurements (mm)	Sheep n= 88	Board n= 35	Cattle n= 133
Minimum diameter of the ventral sucker, **VSmin**	0,45 -1,1 0,71 ± 0,15	0,48-1,13 0,86 ± 0,20	0,82-1,24 1,03 ± 0,07
OSA/VSA ratio	0,36-3,13 1,85 ± 0,76	0,25-2,97 1,15± 0,77	0,20-1,09 0,47 ± 0,19
Distance between the anterior end of the body and the ventral sucker, **A-VS**	0,74-2,05 1,44±0,23	1,20-2,95 2,04± 0,41	1,22-3,62 1,88± 0,29
Distance between the oral sucker and the ventral sucker, **OS-VS**	0,15-0,55 0,39±0,08	0,16-2,62 0,76± 0,73	0,69-1,63 1,28 ± 0,20
Distance between the ventral sucker and the union of the vitelline glands, **VS-Vit**	8,40-14,70 11,74±1,48	7,01-17,58 10,63±2,61	7,60-15,48 10,64 ± 1,61
Distance between the union of the vitelline glands and the posterior end of the body, **Vit-P**	2,81-7,59 4,64±1,02	2,10-5,97 3,93±1,08	1,72-7,31 4,04 ± 0,90
Distance between the ventral sucker and the posterior end of the body, **VS-P**	12,33-20,22 14,14±2,63	10,59-23,11 13,93±3,37	10,62-20,07 14,14± 2,14
BL/VS-P ratio	0,67-1,96 1,15 ±0,25	0,68-1,94 1,08±0,22	0,73-1,69 1,14 ±0,17
Pharynx area, **PhA**	0,12-0,34 0,23± 0,04	0,15-0,34 0,30± 0,07	0,13-0,33 0,21± 0,04
Pharynx length, **PhL**	0,49 -0,92 0,72 ± 0,07	0,43-0,97 0,75± 0,11	0,56-0,89 0,71 ± 0,06
Pharynx width, **PhW**	0,29 -0,52 0,41 ± 0,04	0,22-0,51 0,40 ± 0,07	0,29-0,57 0,39 ± 0,05
Testicular length, **TL**	3,64 -10,43 7,01 ± 1,02	4,15 - 13,17 7,75 ± 1,71	4,56 - 10,99 7,15 ± 0,92
Testicular width, **TW**	2,64-6,79 4,76± 0,90	2,06-6,43 4,32± 0,99	3,86-8,04 6,31± 1,12
Testicular area, **TA**	10,25-48,76 27,08± 7,46	10,76-47,69 24,22± 9,95	16,93-65,92 36,81± 9,16
Testicular perimeter, **TP**	13,15-45,03 22,00± 5,51	14,27-34,45 22,04 ±5,74	18,73-37,39 26,83 ±3,50

TABLA 5.1: Comparative morphometrics with mean values ± SD (range in size) of 82 adult liver fluke uteri in sheep, 17 in wild boars and 124 in cattle from Galicia; n. number of adult liver flukes measured; lineal biometric characters in mm. areas in mm^2.

Liver fluke uteri of adults were studied by multivariate analyses. A scatter plot of the first two principal components (PC) is shown in (Fig. 5.3). The remaining variables were all significantly correlated with the first principal component (PC1) contributing 94 % and the second principal component (PC2) contributing 4 % to overall variations. PC1 could therefore be accepted as a general indicator of uteri size. The results show that the uteri of F. hepatica populations from sheep, cattle and wild boars showed small size differences between the three host species analyzed.

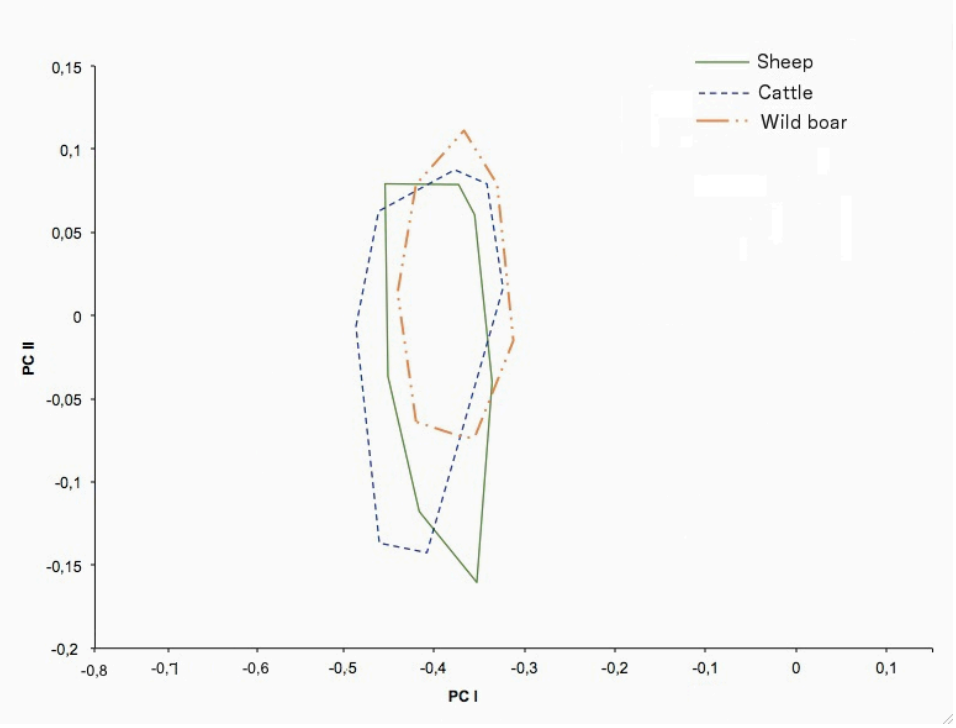

FIGURA 5.3: Principal component analysis of uterus *Fasciola hepatica* from natural infections from Galicia in wild boar (dotted line) compared with *F. hepatica* in sheep (dashed line) and *F. hepatica* in cattle (uninterrupted line); samples are projected on to the first (PCI, 94.0 %) and second (PCII, 4 %) principal components. Each group is represented by its perimeter.

Adult measurements (mm)	Sheep n=82	Wild boar n=17	Cattle n=124
Uterus length, UL	3.12±0.58 1.72-4.41	3.12±0.70 1.71-4.12	3.05±0.41 1.98-4.20
Uterus width, UW	2.12±0.34 1.38-3.05	2.19±0.72 1.10-3.44	2.53±0.41 1.58-3.59
Uterus perimeter, UP	14.30± 2.26 8.53-19.51	14.30±3.13 92-18.25	14.14±2.76 9.44-21.35
Uterus area, UA	3.69±1.16 1.01-6.90	4.10±2.05 1.02-8.03	4.71±1.27 1.49-7.74

TABLA 5.2: Comparative morphometrics with mean values ± SD (range in size) of 82 adult liver fluke uteri in sheep, 17 in wild boars and 124 in cattle from Galicia; n. number of adult liver flukes measured; lineal biometric characters in mm. areas in mm^2.

5.1.3. Discussion

The natural infection by *F. hepatica* has been previously reported in different wild animal species (MENARD et al., 2000, 2001; VALERO et al., 2002; SHIMALOV & SHIMALOV, 2003; VENGUST et al., 2003; SOARES et al., 2007; CAPUCCHIO et al., 2009; ISSIA et al., 2009). In this sense, although some sporadic cases of parasitation have also been described in the wild boar (BARUTZKI et al., 1990; SHIMALOV & SHIMALOV, 2000; THOMPSON et al., 2009), the importance of this hos tspecies in the transmission of fasciolosis has hardly been studied until now. Herein, the prevalence and intensity of this hepatic parasitosis in wild boars in Spain is analyzed. The amount and viability of eggs excreted in feces, as well as the morphometric characteristics of the adult flukes parasitizing the liver are also given. Several authors reported that another Swidae species, the pig, has a considerable natural resistance to fasciolosis (ASHIZAWA et al., 1966; OSHIMA et al., 1971; SHIMIZU et al., 1994). In the most recent European study, *F. hepatica* was found in only 79 out of 3021 feral pigs in Sicily (Italy) (CAPUCCHIO et al., 2009). Nevertheless, the study of MEZO et al. (2013),the prevalence of infection in the total wild boar population (11.2 %) was only slightly lower than that found in adult cattle (16 %) in the same region (MEZO et al., 2008), this difference being

even lower when only wild boars older than 30 months were considered (13.6 %). It must be taken into account that this study was carried out in an area with endemic fasciolosis where the density of grazing cattle is high. Moreover, in recent years, the expanding wild boar population frequently encroaches into livestock pastures where there is a high risk of infection with *F. hepatica* metacercariae. Data relative to the intensity of the infection in wild boars are very scarce. THOMPSON et al. (2009) reported infections with 15–25 adult flukes in wild boars slaughtered in western Scotland. Also, CAPUCCHIO et al. (2009) detected some massive infections of up to 50 flukes in feral pigs in the aforementioned study, although most parasitized livers (89 %) harbored parasite burdens ranging between 1–10 flukes, similar to those found in MEZO et al. (2013) study (mean = 2.3 flukes; max = 14). These parasite burdens are significantly lower than those observed in cattle (up to 244 flukes) in our region, which could be due, according to NANSEN & ANDERSEN (1974), to a low susceptibility to fasciolosis of swine. Nevertheless, other causes such as variations in feeding habits might also be implicated. In relation to the contribution of wild boars to the transmission of fasciolosis, MEZO et al. (2013) data show that they are very likely to contribute to the environmental contamination with parasite eggs. In fact, by analyzing a single sample, MEZO et al. (2013) detected *F. hepatica* eggs in the feces of 40 % of para-sitized wild boars, with a mean of 6.1 epg and individual values of up to 49 epg. The non-detection of eggs in wild boars infected with liver flukes located in the bile ducts suggests that in some cases egg shedding does not take place or only in a very reduced manner, making infection undetectable by microscopic techniques. In this sense, once the worms have matured, diagnosis is sometimes difficult because, although commonly employed, microscopic techniques for quantitative diagnosis of *Fasciola* eggs are very specific but they are rather insensitive. In addition, in some cases diagnosis is also difficult during the biliary stage, due to the intermittent excretion of parasite eggs. Fecal egg counts are known to follow inter- and intraindividual variations in fasciolosis (VALERO et al., 2009b, 2011). It must be kept in mind that the fasciolid egg shedding pattern is not linear but fluctuates between maximum and minimum values (VALERO et al., 2009b). One possible explanation for the negative results obtained in the coprological analysis carried out by MEZO et al. (2013) is that samples were from wild boars with chronic infections, in which egg excretion is probably more erratic. However, in the same study, among the

parasitized wild boars in which the MM3-COPRO ELISA test could be applied (n = 27), most of them were demonstrated to excrete *Fasciola* coproantigens, in spite of the fact that the parasite eggs were not detected in their feces. This suggests that wild boars shed a much reduced, undetectable number of eggs with the technique used, even despite presenting alive and metabolically active flukes in their biliary canals. In cows with *F. hepatica* burdens similar to those found in wild boars (1–10 flukes), we observed that both the percentage of animals shedding parasite eggs (47.7 %) and the epg counts (mean = 6.1 ± 4.4) were similar (MEZO et al., 2004). These results agree with those obtained by MAS-COMA et al. (1997) who, in an interesting study carried out in the Bolivian Altiplano, concluded that the *F. hepatica* egg output capacity of pigs does not greatly differ from that of sheep and cattle in the same area. In addition, MEZO et al. (2013) founded considerable amounts of viable parasite eggs within the gallbladders of all parasitized livers, so that the wild boar can be considered an additional source for environmental contamination by *F. hepatica* eggs in this endemic area. According to DUJARDIN et al. (2009), quantitative morphological variation informs about both genetic variation and external influences. In the case of endoparasites, it is necessary to distinguish between macrohabitat (external environment according to geography) and microhabitat (parasitized organ inside the host) (VALERO et al., 2012c). Given that the entire material in this study comes from one geographical area, external environment according to geography is not a variable to be considered, and only the study of the host species effect on the size and shape of the parasite populations was analyzed. The results of this study show that *F. hepatica* in Galicia has a normal development in wild boars, presenting its own characteristics in shape and size in comparison with other host species. Thus, results obtained in the principal component analysis show that liver flukes from wild boars present both an intermediate size and shape when compared to parasites from sheep and cattle. The maximum size reached by *F. hepatica* in wild boars is smaller than that in cattle but bigger than that in sheep. Additionally, uterus size has been demonstrated to be proportional to both body size (VALERO et al., 2001b) and number of eggs shed per gram of feces (VALERO et al., 2012c). VALERO et al. (2001b) compared uterine development in Bolivian liver flukes from cattle, sheep and pig, showing that uterine allometry of the *F. hepatica* adult with respect to BA follows a pattern, which is independent of the

host species. It is of great interest that it follows the same pattern in Bolivian pigs as it does in hosts considered normal, such as sheep or cattle, because, as above-mentioned, in other geographical areas the pig shows substantial natural resistance to infection by *Fasciola* species (ASHIZAWA et al., 1966; OSHIMA et al., 1971; SHIMIZU et al., 1994), or is even considered to be non-viable as a host for *F. hepatica* (POLYAKOVA-KRUSTEVA & GORCHILOVA, 1972). It can be concluded that in Galicia flukes from the wild boar present a minimum uterus size similar to that in flukes from sheep and cattle. Furthermore, VALERO & MAS-COMA (2000) found that there were no differences in the infectivity and viability of the metacercariae among sheep, cattle and pig isolates from the Bolivian Altiplano and deduced that the pig has a potentially high transmission capacity in the geographical area in question. Given that the quantification of *F. hepatica* eggs in feces can lead to inaccuracies. VALERO et al. (2012c) proposed *F. hepatica* uterus size as a quantitative biomarker of average epg shed, which is consistent with similar findings in other helminths. In this sense, a positive correlation between worm size and egg production has been described in nematodes of animals and humans (DEZFULI et al., 2002; IRVINEET al., 2001; RICHARDS & LEWIS, 2001; UGLAND et al., 2004; WALKER et al., 2009).

Capítulo 6

Conclusiones

Conclusions

The present research aims at the phenotypic characterization of fasciolid adults from endemic areas with different epidemiological characteristics and heterogeneous transmission, i.e. areas where both fasciolid species co-exist and areas where only *F. hepatica* is present. The conclusions obtained from our work are numerous and varied according to their different contents.

To facilitate their presentation, they are listed according to two groups depending on the transmission model:

- With regard to the results obtained in the study on areas where both species of fasciolids co-exist: This is the first time that intermediate forms, as well as the *F. hepatica*-like and *F. gigantica*-like adult worms from bovines are described in Pakistan and Bangladesh.

  The detection of *F. hepatica*-like forms in these geographical areas is crucial, given the well-known capacity of *F. hepatica* to infect humans which is markedly higher than that in *F. gigantica*.

  In relation to the liver-fluke material from Pakistan, the intermediate forms, as well as the *F. hepatica*-like and *F. gigantica*-like adult worms, found in buffaloes from the Central Punjab area, may be explained by a zonal overlap transmission pattern. In fact, nomads move their animals from the highlands to the lowlands in the winter season looking for grazing lands. Similarly, animals are moved from the lowlands to the cooler highlands when warmer conditions return. Further work on this very complex orographic and transhumance scenario is required to understand the epidemiology of the disease in each locality, to assess how transmission of fascioliasis occurs throughout the Central Punjab area and thus establish appropriate control measures for each endemic subzone in the future.

  In relation to the liver-fluke material from Bangladesh, the intermediate forms, as well as the *F. hepatica*-like and *F. gigantica*-like adult worms, found in zebu from the entire country, may be explained by the traditional trade links between these countries.

In relation to the liver-fluke material from Bangladesh, present widespread fascioliasis with high pathogenicity in ruminants is a good indicator for the disease likely to be diagnosed in humans if properly investigated. The higher proportion of *F. gigantica*-like forms in that country may suggest more severe pathogenic characteristics related to the larger size of *F. gigantica* if it infects humans.

In relation to the liver-fluke material from Iran, the presence of different types of morphs in Guilan suggests the likelihood of some degree of hybridization between *F. hepatica* and *F. gigantica*.

Furthermore, the present study in Guilan province addresses to obtain a transmission model which might be subsequently applied to other zonal overlap endemic areas of fascioliasis in Iran or other countries and thereby assess up to which level overlap areas may be similar or different. This knowledge is crucial when control measures in the different overlap areas have to be applied, either being the same or made to measure for each overlap area. The results of the present study demonstrate that, in Guilan, fascioliasis also follows a zonal overlap transmission pattern, with *F. hepatica*-like transmission taking place mainly in the highlands and *F. gigantica*-like transmission mainly in the lowlands. The co-existence of the two fasciolids in livestock in Guilan suggests a complicated scenario of possible ways of circulation of the causal agents, e.g. through altitudinal livestock transhumance which does not take place in Guilan nowadays, but might have been practised in the past, or animal transportation in recent times. In Guilan, *G. truncatula* has been found to transmit *F. hepatica* in the Talesh mountains, whereas *R. auricularia* (= *L. gedrosiana*) appears well distributed in the lowlands around the cities of Bandar Anzali and Rasht. These data correlate with results of previous studies suggesting *F. gigantica* to be the most prevalent species in these human endemic lowlands around the two aforementioned cities. At the highest altitudes (1,000-2,000 m), *F. hepatica*-like specimens dominate infecting sheep and goats, whereas *F. gigantica*-like specimens are only sporadically found in cattle, which suggest their presence in bovines most probably as a consequence of animal movements. However, a sporadic presence of isolated populations of *R. auricularia* (*L. gedrosiana*) may not a

priori be ruled out and is perhaps likely to enable a short transmission season of *F. gigantica* at such altitudes in Guilan during the summer months. At the lowest altitudes (from -27 m to 0 m), *F. gigantica*-like specimens dominate infecting cattle and buffaloes, whereas *F. hepatica*-like specimens appear only sporadically in cattle, buffaloes and goats. At the interface between the highest and lowest altitudes, there appears a strip of land between 1 m and 999 m (lowland areas above sea level at 1-99 m and hilly areas at 100-999 m) in which both Fasciola forms are present: *F. hepatica*-like specimens in cattle and sheep at 1-999 m and in goats at 100-999 m, and *F. gigantica*-like specimens in cattle at 1-999 m, in buffaloes only at 1-99 m and in sheep at 100-999 m. These results indicate that the transition between highlands and lowlands is gradual, most probably related to a distributional overlap of the lymnaeid vector species transmitting the two fasciolids.

In relation to the liver-fluke material from Iran, the present study has quantified for the first time the different developmental courses of *F. hepatica* in four different host species (cattle, buffaloes, sheep and goats) and those of *F. gigantica* in three different host species (cattle, buffaloes and sheep) using geometrical morphometric techniques. The analysis of the influence of the host species on the size and shape of *F. hepatica*-like and *F. gigantica*-like specimens shows that the definitive host species has a decisive influence on the size and shape of the liver-flukes studied. Nevertheless, this host influence does not follow the same pattern in the phenotype of each fasciolid species.

- With regard to the results obtained in the study on areas where only *F. hepatica* is present:

  In relation to the liver-fluke material from Galicia, the present study has quantified for the first time the different developmental courses of *F. hepatica* in three different host species (cattle, buffaloes, and wild boar) from the same geographical area, using geometrical morphometric techniques. The analysis of the influence of the host species on the size and shape of *F. hepatica* specimens shows that the definitive host species has a decisive influence on the size and shape of the *F. hepatica* adults studied.

  PC analysis enabled the characterization of *F. hepatica* specimens found in the wild boar and their comparison with *F. hepatica* specimens originating

from host species considered main reservoir hosts of the disease in the area, with particular emphasis on the uterus development. Our study shows for the first time that in Galicia the *F. hepatica* uterus from the wild boar presents an intermediate size between that found in primary reservoir hosts such as cattle and sheep, i.e., the individual potential egg output capacity of the wild boar does not greatly differ from that detected in Galician livestock. The normal development of the flukes in the liver of the wild boar suggests a possible role of this species as a secondary reservoir in this Spanish region.

The global results obtained do not only underline the extraordinary plasticity and adaptability of these trematode species to different mammalian hosts, but also highlight the utility of the phenotypic characterization of the fasciolid adults in epidemiological studies in endemic areas. Future experimental studies will elucidate if intermediate specimens have a better fitness, i.e. infecting both species of the *Galba/Fossaria* group and species of the *Radix* group in order to characterise their different biological aspects of transmission and epidemiology as to establish appropriate fascioliasis control measures

Capítulo 7

Bibliografía

Bibliography

A

AFSHAN (K.), VALERO (M.A.), QAYYUM (M.), PEIXOTO (R.V.), MAGRANER (A.) & MAS-COMA (S.), 2013.- Phenotypes of intermediate forms of *Fasciola hepatica* and *F. gigantica* in buffaloes from Central Punjab. *Journal of Helminthology*, 88: 417-426.

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.

AKAHANE (H.), HARADA (Y.) & OSHIMA (T.), 1970.- Patterns of the variation of the common liver fluke *(Fasciola)* sp. in Japan. III. Comparative studies on the external form, size of egg and number of eggs in uterus of the fluke in cattle, goat and rabbit. *Japanese Journal of Parasitology*, 6: 619-627.

AKAHANE (H.), HARADA (Y.), OSHIMA (T.), TAKAYAMA (A.) & ASHIZAWA (H.), 1974.- Patterns of the variation of the common liver-fluke (*Fasciola* sp.) in Japan. IV. Comparative studies on the external form, size of egg and number of eggs in the uterus of fluke in pig and horse. *Japanese Journal of Parasitology*, 23: 207-212.

ALVI (A.S.) & SHARIF (M.), 1995.- Arid zone agriculture and research in Pakistan, *AZRC Research Plan for 1995/1996*.

AMOR (N.), HALAJIAN (A.), FARJALLAH (S.), MERELLA (P.), SAID (K.) & BEN SLIMANE (B.), 2011.- Molecular characterization of *Fasciola* spp. from the endemic area of northern Iran based on nuclear ribosomal DNA sequences. *Experimental Parasitology*, 128: 196-204.

ANONYMOUS, 1988.- Parasitic diseases: hepatic distomiasis caused by *Fasciola hepatica*. *The Weekly Epidemiological Record*, 63: 109-111.

ANONYMOUS, 2001.- The proven solution for image analysis. 8/1-8/16 pp. Image-Pro® Plus, Version 4.5 for WindowsTM, Start-Up Guide. Media Cybernetics, Inc., Silver spring, MD.

ANTOLOVA (D.), REITEROVA (K.) & DUBINSKY (P.), 2006.- The role of wild boars *(Sus scrofa)* in circulation of trichinellosis, toxocarosis and ascariosis in the Slovak Republic. *Helminthologia*, 43: 92-97.

ARTIGAS (P.), BARGUES (M.D.), MERA Y SIERRA (R.), 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: 245-257.

ASHRAFI (K.), MASSOUD (J.), HOLAKOUEI (K.), MAHMOODI (M.), JOAFSHANI (M.A.), VALERO (M.A.), FUENTES (M.V.), KHOUBBANE (M.), ARTIGAS (P.), BARGUES (M.D.) & MAS-COMA (S.), 2004.- Evidence suggesting that *Fasciola gigantica* might be the most prevalent causal agent of fascioliasis in northern Iran. *Iranian Journal of Public Health*, 33: 31-37.

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

ASHRAFI (K.), VALERO (M.A.), PANOVA (M.), PERIAGO (M.V), MASSOUD (J.) & MAS-COMA (S.), 2006b.- Phenotypic analysis of adults of *Fasciola hepatica*, *Fasciola gigantica* and intermediate forms from the endemic region of Gilan. *Iranian Journal of Parasitology*, 55: 249-260.

ASHRAFI (K.), MASSOUD, (J.), HOLAKOUEI NAINEI, (K.), JO-AFSHANI, (M.A.), MAHMOODI, (M.), EBEDATI, (N.), REZVANI, (S.M.), ARTIGAS, (P.), BARGUES, (M.D.) & MAS-COMA (S.), 2007.- Nuclear ribosomal DNA ITS-2 sequence characterization of *Fasciola hepatica* and *Galba truncatula* Iranian. *Journal of Public Health*, 36: 42-49.

ASHRAFI (K.), VALERO (M.A.), QAYYUM (M.), PEIXOTO (R.V.), MAGRANER (A.) & MAS-COMA (S.), 2013.- Phenotypes of intermediate forms of *Fasciola hepatica* and *F. gigantica* in buffaloes from Central Punjab. *Journal of Helminthology*, 88: 417-426.

ASHRAFI (K.) & MAS-COMA (S.), 2014.- *Fasciola gigantica* transmission in the zoonotic fascioliasis endemic lowlands of Guilan, Iran: Experimental assessment. *Veterinary Parasitology*, 205: 96-106.

ASHRAFI (K.), BARGUES (M.D.), O'NEILL (S.) & MAS-COMA (S.), 2014a.- Fascioliasis: A worldwide parasitic disease of importance in travel medicine. *Travel Medicine and Infectious Disease*, 12: 636-649.

ASHRAFI (K.), FORTES-LIMA (C.A.), ARTIGAS (P.), VALERO (M.A.), QAYYUM (M.) & MAS-COMA (S.), 2014b.- 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.

ASHRAFI (K.), VALERO (M.A.), PEIXOTO (R.V.), ARTIGAS (P.), PANOVA (M.) & MAS-COMA (S.), 2015.- Distribution of *Fasciola hepatica* and *F. gigantica* in the endemic area of Guilan, Iran: Relationships between zonal overlap and phenotypic traits. *Infection, Genetics and Evolution*, 31: 95-109.

ASHIZAWA (A.H.), NOSAKA (D.) & OSATO (K.), 1966.- Pathological studies on fascioliasis. VI. Lesions in pig livers infected with *Fasciola* in Japanese. *Faculty of Agriculture University of Miyazaki*, 13: 1-63.

ASSMAR (M.), MILANINIA (A.), AMIR-KHANI (A.), YADEGARI (D.), FORGHANPARAST (K.), NAHRAVANIAN (H.), PIAZAK (N.), ESMAEILI (A.), HOVANESIAN, (A.) & VALADKHANI (Z.), 1991.- Seroepidemiological investigation of fascioliasis in northern Iran. *Medical Journal of The Islamic Republic of Iran*, 5: 23-27.

B

BANGLADESH ACADEMY OF SCIENCE (BAS)-USDA, 2012.- Annual Project Report of LS 07/2011 project, Mondal MMH. Bangladesh Agricultural University, Mymensingh.

BARGUES (M.D.), 1986.- Contribución al conocimiento de la evolución de los Digénidos de la familia Brachylaimoidea Joyeux et Foley, 1930 (Trematoda: *Brachylaimoidea*) a nivel de Molusco Gasterópodo primer hospedador intermediario. Tesis Doctoral, Facultad de Farmacia, Universidad de Valencia, 968 pp.

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

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: 621-641.

BARGUES (M.D.), ARTIGAS (P.), KHOUBBANE (M.), FLORES (R.), GLÖER (P.), ROJAS-GARCÍA (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 (33 pp. + 3 Suppl. Tables + 5 Suppl. Figures).

BARGUES (M.D.), GONZALEZ (C.), 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. *Parasites & Vectors*, 4: 200-218.

BARGUES (M.D.), ARTIGAS (P.), KHOUBBANE (M.) & MAS-COMA (S.), 2011c.- 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*, 4: 132-154.

BARGUES (M.D.), ARTIGAS (P.), KHOUBBANE (M.), ORTIZ P., NAQUIRA (C.) & MAS-COMA (S.), 2012.- Molecular characterisation of *Galba truncatula*, *Lymnaea neotropica* and *L. schirazensis* from Cajamarca, Peru and their potential role in transmission of human and animal fascioliasis. *Parasites & Vectors*, 5: 174-190.

BARUTZKI (D.), SCHOIERER (R.) & GOTHE (R.), 1990.- Helminth infections in wild boars in enclosures in southern Germany: species spectrum and infection frequency. *Tierarzliche-Praxis*, 18: 529-534.

BAYU (B.), ASNAKE (S.), WORETAW (A.), ALI (J.), GEDEFAW (M.), FENTE (T.), GETACHEW (A.), TSEGAYE (S.), DAGNE (T.) & YITAYEW (G.), 2005.- Cases of Human fascioliasis in north-west Ethiopia. *The Ethiopian Journal of Health Development*, 19: 237-240.

BERGEON (P.) & LAURENT (M.), 1970.- Différences entre la morphologie testiculaire de *Fasciola hepatica* et *Fasciola gigantica*. *Revue d'Elevage et de Medecine Veterinaire des Pays Tropicaux*, 223-227.

BHUIYAN (M.A.), 1970.- A survey of helminth parasitizing the liver of domesticated ruminants in East Pakistan. M. Sc. Thesis, Department of Parasitology, Faculty of Veterinary Science, Bangladesh Agricultural University (Former EPAU, Mymensingh.

BJORLAND (J.), BRYAN (R.T.) & STRAUSS (W.), 1995.- An outbreak of acute fascioliasis among Aymara Indians in the Bolivian Altiplano. *Clinical Infectious Diseases*, 21: 1228-1233.

BOOKSTEIN (F.L.), 1989.- Size and shape: a comment on semantics. *Systematic Zoology*, 38: 173-180.

BORAY (J.C.), 1969.- Experimental fascioliasis in Australia. *Advances in Parasitology*, 7: 95-210.

BORAY (J.), 1982.- Fascioliasis. En: *C.R.C. Handbook series in zoonoses*. C.R.C. Press. (Hiller & Hopla eds.). Florida: 77-88 pp.

BOCH (J.) & SUPPERER (R.), 1982.- Parasitología en Medicina Veterinaria. Buenos Aires: Hemisferio Sur, S.A. 627 pp.

BORCHET (A.), 1981.- Parasitología Veterinaria. Zaragoza: Acribia. 745 pp.

C

CAPUCCHIO (M.T.), DEBORAH (C.), VINCENZO (D.M.), MIRIAM (R.), VINCENZO (A.), AMEDEO (T.), ALESSANDRO (L.), STEFANO (A.), ELEONORA (S.F.), BRUNO (D.) & FRANCO (G.), 2009.- Natural trematode infestation in feral Nebrodi Black pigs: pathological investigations. *Veterinary Parasitology*, 159: 37-42.

CASEBY (R.H.), HARRIOT (M.) & FAIRWEATHER (I.), 1995.- Ionic composition of the liver fluke *Fasciola hepatica* from different mammalian hosts and comparison with host bile. *Parasitology Research*, 81: 394-397.

CHAUDHRY (A.H.) & NIAZ (M.), 1984.- Liver fluke – a constant threat to livestock development. *Pakistan Veterinary Journal*, 4: 42-43.

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: 1-38.

CHOWDHURY (S.A), SHILL (B.K.) & HOSSAIN (S.M.J.), 2003.- Chagol palon mannul. En: *Bangladesh Livestock Research Institute*, Savar, Dhaka-1341, January.

CHU (J.K.) & KIM (Y.K.), 1967.- Taxonomical study on the Fasciolidae in Korea. *Korean Journal of Parasitology*, 5: 139-146.

CIA WORLD FACTBOOK, 2014.- Available from: https://www.cia.gov/library /publications/the-world-factbook/geos/ir.html.

CONSELLERÍA DO MEDIO RURAL E DO MAR, MAR.- Available from: https://www.medioruralemar.xunta.es.

COBBOLD (T.S.), 1855.- Description of a new trematode worm (*Fasciola gigantica*). Edinbourg N. Phil. J. N. S., 2: 262-266.

CORDERO DEL CAMPILLO (M.), CASTAÑÓN ORDÓÑEZ (L.) & REGUERA FEO (A.), 1994.- Índice-catálogo de zooparásitos ibéricos. Segunda edición. Secretariado de publicaciones, Universidad de León.

CRUZ (R.V.), HARASAWA (H.), LAL (M.), WU (S.), ANOKHIN (Y.), PUNSALMAA (B.), HONDA (Y.), JAFARI (M.), LI (C.) & HUU NINH (N.), 2007.- Asia. pp. 469-506 in Parry, M.L., Canziani, O.F., Palutikof, J.P., van der Linden, P.J. & Hanson, C.E. (Eds) Climate Change 2007: Impacts, Adaptation and Vulnerability.

CWIKLINSKI (K.), DALTON (J. P.) , DUFRESNE (P. J.), COURSE (J.), WILLIAMS (D. JL.), HODGKINSON (J.), & PATERSON (S.), 2015.- The *Fasciola hepatica* genome: gene duplication and polymorphism reveals adaptation to the host environment and the capacity for rapid evolution. *Genome Biology*, 16: 71.

D

DE SOUSA, (B.C.), DE CARVALHO (M.), FAZENDEIRO (I.), CASTRO REGO (F.) & AFONSO-ROQUE (M.M.), 2004.- Contribution for the knowledge of Wild Boar *(Sus scrofa L.)* helmintic fauna in Tapada Nacional de Mafra, an enclosured hunting area. *Revista Ibérica de Parasitología*, 64: 3-7.

DEZFULI (B.S.), VOLPONI (S.), BELTRAMI (I.) & POULIN (R.), 2002.- Intra- and interspecific density-dependent effects on growth in helminth parasites of the cormorant. *Phalacrocorax. Parasitology*, 124: 537-544.

DREYFUSS (G.), MOUKRIM (A.), RONDELAUD (D.) & VAREILLE-MOREL (C.), 1994.- Field observations concerning infection of *Lymnaea palustris* by *Fasciola hepatica. Journal of Helminthology*, 68: 115-118.

DUJARDIN (J.P.), 2002.- BAC software. Institute de Reserches pour le Développement (IRD, France) Available from: http://www.fsf.org/copyleft/gpl.html.

DUJARDIN (J.P.), SCHOFIELD (C.J.) & PANZERA (F.), 2002.- Les vecteurs de la maladie de Chagas. En: *Reserches taxonomiques, biologiques et génetiques.* Academie Royale des Sciencies d'Outre-Mer. Bruxelles (Belgique): 189 pp.

DUJARDIN (J.P.) & LE PONT (F.), 2004.- Geographical variation of metric properties within the neotropical sandflies. *Infection, Genetics and Evolution*, 4: 353-359.

DUJARDIN (J.P.), 2008.- Morphometrics applied to medical entomology. *Infection, Genetics and Evolution*, 8: 875-890.

DUJARDIN (J.P.), COSTA (J.), BUSTAMANTE (D.), JARAMILLO (N.) & CATALA (S.), 2009.- Deciphering morphology in Triatominae: the evolutionary signals. *Acta Tropica*, 110: 101-111.

E

ENIGK (K.) & HILDEBRANDT (J.), 1964.- Zur Lebensdauer der Metacercarien von *Fasciola hepatica* im Heu. *Tierärztliche Rundschau*, 19: 592-599.

ESTEBAN (J. G.), BARQUES (M. D.) & MAS-COMA (S.), 1998.- Geographical distribution, diagnosis and treatment of Human fascioliasis; a review. *Research and Reviews in Parasitology*, 58: 13-48.

ESTEBAN (J.G.), FLORES (A.) & ANGLES (R.), 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: 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 & International Health*, 7: 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: 429-437.

EUZEBY (J.), 1984.- Les parasites humains d origine animal. Caracteres epidemiologiques. Flammarion. *Medicine-Science*, 324.

F

FAIRWEATHER (I.), THREADGOLD (L.T.) & HANNA (R.E.B.), 1999.- Development of *Fasciola hepatica* in the mammalian host. En: *Fasciolosis*. CAB International, 47-111 pp.

FAN (P.C.) & LIN (L.H.), 2005.- Hybridization of *Schistosoma mansoni* and *Schistosoma japonicum* in mice. *Southeast Asian Journal of Tropical Medicine and Public Health*, 36: 89-96.

FAROOQ (A.B.) & KHAN (A.H.), 2004.- Climate change perspective in Pakistan. *Proceedings of the Capacity Building APN Workshop on Global Change Research*, Islamabad, 39:46.

FENTIE (T.), ERQOU (S.), GEDEFAW (M.) & DESTA (A.), 2013. Epidemiology of Human fascioliasis and intestinal parasitosis among schoolchildren in Lake Tana Basin, northwest Ethiopia. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 107: 480-486.

FLUX (J.E.C.) & ANGERMANN (R.), 1990.- The hares and Jackrabbits. In: J.A. Chapman and J.E.C. Flux (eds) Rabits, Hares and Pikas: status survey and conservation action plan: chapter 4: 61-94. *International Union for Conservation of Nature and Natural Resources*, Gland, Switzerland.

FORGHANPARAST (K.) & ASHRAFI (K.), 2001.- Comparision of the formalin-ether and Kato Katz in the parasitological diagnosis of Human fascioliasis. *Journal of Guilan University of Medical Sciences*, 9: 1-6.

FORGHANPARAST (K.), YADEGARI (D.) & ASSMAR (M.), 1993.- Clinical epidemiology of Human fascioliasis in Guilan. *Journal of Guilan University of Medical Sciences*, 2: 4-11.

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.

G

GIBBS (E.P.J.), 1997.- The public health risks associated with wild and feral swine. *Revue Scientifique et Technique* (*International Office of Epizootics*), 16: 594-598.

GIRONES (N.), VALERO (M.A.), GARCIA-BODELON (M.A.), CHICO-CALERO (M.I.), PUNZON (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: 1504-1512.

GORTÁZAR (C.), VICENTE (J.) & GAVIER-WIDEN (D.), 2003.- Pathology of bovine tuberculosis in the European wild boar. *Veterinary Record*, 152: 779-780.

GUÍA PAÍS PAKISTÁN, 2006.- Elaborada por la Oficina Económica y Comercial de España en ISLAMABAD. Available from: http://www.comercio.es/tmpDocsCanalPais/A3BD46F1CDE827EA23405579DBB0E0ED.pdf

GUNASEKAR (K.R.), TEWARI (A.K.), SREEKUMAR (C.), GUPTA (S.C.) & RAO (J.R.), 2008.- Elucidation of genetic variability among different isolates of *Fasciola gigantica* (giant liver fluke) using random-amplified polymorphic DNA polymerase chain reaction. *Parasitology Research*, 103: 1075-1081.

H

HASEEB (A.N.), EL SHAZLY (A.M.), ARAFA (M.A.) & MORSY (A.T.), 2002.- A review on fascioliasis in Egypt. *Journal of the Egyptian Society of Parasitology*, 32: 317-354.

HOPE CAWDERY (M.J.), GETTINBY (G.) & GRAINGER (J.N.R.), 1978.- Mathematical models for predicting the prevalence of liver-fluke disease and its control from biological and metereological data. In: Weather and Parasitic Animal Disease. *World Metereological Organisation Technical*, 159: 21-38.

HOSSAIN (M.M.), PAUL (S.), RAHMAN (M.M.), HOSSAIN (F.M.A.), HOSSAIN (M.T.) & ISLAM (M.R.), 2011.- Prevalence and economic significance of caprine fascioliasis at Sylhet district of Bangladesh. *Pakistan Veterinary Journal*, 3: 113-116.

HUDSON (P.J.), RZZOLI (A.), GRENFELL (B.T.) & HEESTERBEEK (H.), DOBSON, 2002.- AP. The Ecology of Wildlife Diseases. Oxford, Great Britain: Oxford University Press.

I

ICHIKAWA (M.), BAWN (S.), MAWD (N.N.), HTUN (L.L.), THEIN (M.), GYI (A.), SUNN (K.), KATAKURA (K.) & ITAGAKI (T.), 2011.- Characterization of *Fasciola* sp. in Myanmar on the basis of spermatogenesis status and nuclear and mitochondrial DNA markers. *Parasitology International*, 60: 474-479.

IMANI-BARAN (A.), YAKHCHALI (M.), MALEKZADEH VIAYEH (R.) & FARHANGPAJUH (F.), 2011.- Prevalence of cercariae infection in *Lymnaea auricularia* (Linnaeus, 1758) in NorthWest of Iran. *Veterinary Research*, 2: 121-127.

IMANI-BARAN (A.), YAKHCHALI (M.) & MALEKZADEH VIAYEH (R.), 2012.- Molecular analysis of 28S rDNA for detecting the infection with *Fasciola gigantica* (Cobbold, 1855) in *Radix auricularia* (Linnaeus, 1785) snails from northwestern Iran. *Veterinary Parasitology*, 189: 374-377.

IMANI-BARAN (A.), YAKHCHALI (M.), MALEKZADEH VIAYEH (R.) & FARAHNAK (A.), 2013.- Seasonal and geographic distribution of cercarial infection in *Lymnaea gedrosiana* (Pulmunata: *Lymnaeidae*. In North West Iran. *Iranian Journal of Parasitology*, 8: 423-429.

IPCC. 2007. Intergovernmental Panel on Climate Change. Climate Change: Synthesis Report; Summary for Policymakers. Working Group contributions to the Fourth Assessment Report, IPCC Plenary XXVII.

IRVINE (R.J.), STIEN (A.), DALLAS (J.F.), HALVORSEN (O.), LANGVATN (R.) & ALBON (S.D.), 2001.- Contrasting regulation of fecundity in two abomasal nematodes of Svalbard reindeer (Rangifer tarandus platyrhynchus). *Parasitology*, 122: 673-681.

ISSIA (L.), PIETROKOVSKY (S.), SOUSA-FIGUEIREDO (J.), STOTHARD (J.R.) & WISNIVESKY- COLLI (C.), 2009.- *Fasciola hepatica* infections in livestock flock, guanacos and coypus in two wildlife reserves in Argentina. *Veterinary Parasitology*, 165: 341-344.

ITAGAKI (T.) & TSUTSUMI (K.I.), 1998.- Triploid form of Fasciola in Japan: genetic relationships between *Fasciola hepatica* and *Fasciola gigantica* determined by ITS-2 sequence of nuclear rDNA. *International Journal for Parasitology*, 28: 777-81.

ITAGAKI (T.), SAKAGUCHI (K.), TERASAKI (K.), SASAKI (O.), YOSHIHARA (S.) & VAN DUNG (T.), 2009.- Occurrence of spermic diploid and aspermic triploid forms of *Fasciola* in Vietnam and their molecular characterization based on nuclear and mitochondrial DNA. *Parasitology International*, 58: 81-85.

K

KAMARUDDIN (M.), 2003.- Goat farming as a means of poverty alleviation. In: proceedings of BSVER symposium. Goats farming in Bangladesh; Problems and prospects. BAU. *Bangladesh Society for Veterinary Education & Research*, 25: 26-34.

KENDALL (S.B.), 1954.- Fascioliasis in Pakistan. *Annals of Tropical Medicine and Parasitology*, 48: 307-313.

KENDALL (S.B.) & PARFITT (J.W.), 1959.- Studies on the susceptibility of some species of Lymnaea to infection with *Fasciola gigantica* and *F. hepatica*. *Annals of Tropical Medicine and Parasitology*, 53: 220-227.

KENDALL (S.B.), 1965.- Relationships between the species of *Fasciola* and the molluscan hosts. *Advances in Parasitology*, 3: 59-98.

KENDALL (S.B.), 1970.- Relationships between the species of *Fasciola* and their molluscan hosts. *Advances in Parasitology*, 9: 251-258.

KHAN (M.K.), SAJID (M.S.), KHAN (M.N.), IQBAL (Z.) & IQBAL (M.U.), 2009.- Bovine fasciolosis: prevalence, effects of treatment on productivity and cost benefit analysis in five districts of Punjab, Pakistan. *Research in Veterinary Science*, 87: 70-75.

KHAN (M.K.), SAJID (M.S.), KHAN (M.N.), IQBAL (Z.) & HUSSAIN (A.), 2010.- Gastrointestinal helminthiasis: prevalence and associated determinants in domestic ruminants of district Toba Tek Singh, Punjab, Pakistan. *Parasitology Research*, 107: 787-794.

KIMURA (S.), SHIMIZU (A.) & KAWANO (J.), 1984.- Morphological observation on liver fluke detected from naturally infected carabaos in the Philippines. *The Science Reports of the Faculty of Agriculture*, 16: 353-357.

KLEIMAN (F.), GONZÁLEZ (N.), RUBEL (D.) & WISNIVEKSY (C.), 2004.- *Fasciola hepatica*(Linnaeus, 1758) (Trematoda, Digenea) in the hare *Lepus europaeus*, Pallas 1778 *(Lagomorpha, Leporidae)* from the Andean Patagonian Region, Chubut Province, Argentina. *Parasitología latinoamericana*, 59: 68-71.

KLINGENBERG (C.P.), 1996.- Multivariate allometry in Marcus, L.F., Corti, M., Loy, A., Naylor, G.J.P. & Slice, D. (Eds) Advances in Morphometrics. En: *Proceedings of the 1993 NATO-ASI on Morphometrics*, NATO ASI, Ser. A, Life Sciences. New York, Plenum Publishers. 23-49 pp.

KUKIELKA (E.), BARASONA (J.A.), COWIE (C.E.), DREWE (J.A.), GORTAZAR (C.), COTARELO (I.) & VICENTE (J.), 2013.- spatial and temporal interactions between livestock and wildlife in South Central Spain assessed by camera traps. *Preventive Veterinary Medicine*, 112: 213-221.

L

LA MONCLOA, GOVIERNO DE ESPAÑA, 2014.- Available from: http://www.lamoncloa.gob.es/Paginas/i

LIGDA (D.J.), 1998.- Water Buffalo Facts (on-line). Available from: http://animals .nationalgeographic.com/animals/mammals/water-buffalo.html.

LUCCINI (V.), MEIJAARD (E.), DIONG (C.H.), GROVES (C.P.) & RANDI (E.), 2005.-New phylogenetic perspectives among species of South-East Asian wild pigs*(Sus)* sp. based on mtDNA sequences and morphometric data. *Journal of Zoology*, 266: 25-35.

M

MAQBOOL (A.), ARSHAD (M.J.), MAHMOOD (F.) & HUSSAIN (A.), 1994.- Epidemiology and chemotherapy of fascioliasis in buffaloes. *Assiut Veterinary Medical Journal*, 30: 115-123.

MAQBOOL (A.), HAYAT (C.S.) & AKHTAR (T.), 2002.- Hashmi Epidemiology of fasciolosis in buffaloes under different managemental conditions. *Veterinarski arhiv*, 72: 221–228.

MARCILLA (A.), BARGUES (M.D.) & MAS-COMA (S.), 2002.- A PCR- RFLP assay for the distinction between *Fasciola hepatica* and *Fasciola gigantica. Molecular and Cellular Probes*, 16: 327-33.

MARCOS (M.D.), 1993.- Ontogenia del adulto de *Fasciola hepatica*(Linnaeus, 1758) (Trematoda: *Fasciolidae*) en infestaciones experimentales y naturales de macromamíferos y micromamíferos continentales e insulares. Tesis Doctoral, Facultad de Ciencias Biológicas, Universidad de Valencia, 475 pp.

MANSOORIAN (A.), 2000.- Some freshwater snails from Northern Iran.*Iranian Journal of Public Health*, 29: 77–82.

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.), ANGLES (R.) & ESTEBAN (J.G.), 1999.- The Northern Bolivian Altiplano: a region highly endemic for Human fascioliasis. *Tropical Medicine & International Health*, 4: 454-467.

MAS-COMA (S.), BARGUES (M.D.), MARTY (A.M.) & NEAFIE (R.C.), 2000.- Hepatic trematodiases. En: textitPathology of Infectious diseases, Vol. 1 Helminthiases (Meyers (W.M.), Neafie (R.C.), Marty (A.M.) & Wear (D.J.) edit., Armed Forces Institute of Pathology and American Registry of Pathology, Washington D.C.: 69-92.

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: 115-27.

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. En: *Taxonomy, Ecology and Evolution of Metazoan Parasites* (Combes (C.) & Jourdane (J.) edit., Presses Universitaires de Perpignan, Perpignan, 2: 81-123.

MAS-COMA (S.) COTRUVO (J.A.), DUFOUR (A.), REES (G.), BARTRAM (J.), CARR (R.), CLIVER (D.O.), CRAUN (G.F.) & FAYER (R.), 2004.-. VPJ (eds) *World Health Organization* (WHO), waterborne zoonoses: identification, causes and control. En: *Human fascioliasis*, IWA, London.

MAS-COMA (S.), BARGUES (M.D.) & VALERO (M.A.), 2005.- Fascioliasis and other plant-borne trematode zoonosis. International. *Journal of Parasitology*, 35: 1255-1278.

MAS-COMA (S.), VALERO (M.A.) & BARGUES (M.D.), 2008.- Effects of climate change on animal and zoonotic helminthiases. *Revue scientifique et technique cientifique*, 27: 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: 264-280.

MAS-COMA (S.), AGRAMUNT (V.H.) & VALERO (M.A.) 2014a. Neurological and ocular fascioliasis in humans. *Advances in Parasitology*, 84: 27-149.

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: 1918-1946.

MAS-COMA (S.), VALERO (M.A.) & BARGUES (M.D.), 2014c.- Fascioliasis. En: Digenetic Trematodes, Advances in Experimental Medicine and Biology. Toledo (R.) & Fried (B.) eds., 77-114 pp.

MASSOUD (J.), 1990.- Fascioliasis outbreak of man and drug test (triclabendazol) in Caspian littoral, northern part of Iran, 1989. *Bulletin of France Society for Parasitology*, 8: 438.

MASUD (F.S.) & MAJID (A.),1984.- Incidence of fascioliasis in buffaloes and cattle of Multan division. *Pakistan Veterinary Journal*, 4: 33-34.

MCREATH (A.M.), READER (T.A.J.) & SOUTHGATE (V.R.), 1982.- The development of the host-response in juvenile Lymnaea palustris to invasion by *Fasciola hepatica*. Z. *Parasitenkde*, 67: 175-184.

MEEUSEN (E.) & PIEDRAFITA (D.), 2003.- Exploiting natural immunity to helmith parasites for the development of veterinary vaccines. *International Journal for Parasitology*, 33: 1285-1290.

MÉNARD (A.), L'HOSTIS (M.), LERAY (G.), MARCHANDEAU (S.), PASCAL (M.), ROUDOT (N.), MICHEL (V.) & CHAUVIN (A.), 2000.- Inventory of wild rodents and lago- morphs as natural hosts of *Fasciola hepatica* on a farm located in a humid area in Loire Atlantique (France). *Parasite*, 7: 77-82.

MÉNARD (A.), AGOULON (A.), L'HOSTIS (M.L.), RONDELAUD (D.), COLLARD (S.) & CHAUVIN (A.), 2001.- Myocastor coypus as a reservoir host of *Fasciola hepatica* in France. *Veterinary Record*, 32: 499-508.

MEEUSEN (E.) & PIEDRAFITA (D.), 2003.- Exploiting natural immunity to helminth parasites for the development of veterinary vaccines. *International Journal for Parasitology*, 33: 1285-1290.

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 & Vector*, 4: 104.

MEZO (M.), GONZÁLEZ-WARLETA (M.), CASTRO-HERMIDA (J.A.) & UBEIRA (F.M.), 2008.- Evaluation of the flukicide treatment policy for dairy cattle in Galicia (NW Spain). *Veterinary Parasitology*, 157: 235-243.

MEZO (M.), GONZALEZ-WARLETA (M.), CARRO (C.) & UBEIRA (F.M.), 2004.- An ultrasensitive capture ELISA for detection of *Fasciola hepatica* coproantigens in sheep and cattle using a new monoclonal antibody (MM3). *Journal of Parasitology*, 90: 845-852.

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.

MILLER (C.M.D.), HOWELL (M.J.) & BORAY (J.C.), 1993.- Host effects of glutathione s-tranferase activity in *Fasciola hepatica*. *Journal of Parasitology*, 23: 1073-1076.

MOGHADDAM (A.S.), MASSOUD (J.), MAHMOODI (M.), MAHVI (A.H.), PERIAGO (M.V.), ARTIGAS (P.), FUENTES (M.V.), BARGUES (M.D.) & MAS-COMA (S.), 2004a.- Human and animal fascioliasis in Mazandaran province, northern Iran. *Parasitology Research*, 94: 61-69.

MOGHADDAM (A.S.), MASSOUD (J.), MAHMOODI (M.), KHOUBBANE (M.), ARTIGAS (P.), PERIAGO (M.V.), FUENTES (M.V.), BARGUES (M.D.) & MAS-COMA (S.), 2004b.- Distributional outline of lymnaeid snails (Gastropoda) in the fascioliasis endemic area of Mazandaran, Iran. *Acta Parasitologica*, 49: 145-152.

MOSHFE (A.A.), BAGHERI (M.) & MOHEBI NOBANDEGHANY (Z.), 2003.- Prevalence of *Fasciola hepatica* in slaughtered livestock in Yasuj's slaughterhouse, 1381–82. Armaghan Danesh. *Journal of Yasuj University of Medical Sciences*, 8: 25-33.

MOXON (J.V.), LACOURSE (E.J.), WRIGHT (H.A.), PERALLY (S.), PRESCOTT (M.C.), GILLARD (J.L.), BARRETT (J.), HAMILTON (J.V.) & BROPHY (P.M.), 2010.- Proteomic analysis of embryonic *Fasciola hepatica*: characterization and antigenic potential of a developmentally regulated heat shock protein. *Veterinary Parasitology*, 169: 62-75.

MOWLAVI, (G.R.), MASSOUD (J.), MOBEDI (I.), SOLAYMANI-MOHAMMADI (S.), GHARAGOZLOU (M.J.) & MAS-COMA (S.), 2006.- Very highly prevalent *Macracanthorhynchus hirudinaceus* infection of wild boar *Sus scrofa* in Khuzestan province, south-western Iran.*Helminthologia*, 43: 86-91.

N

NANSEN (S.) & ANDERSEN (E.), 1974.- Harmer the course of an experimental liver fluke infection in the pig. *Acta Veterinaria Scandinavica*, 5: 138-140.

NOORUDDIN (M.) & ISLAM (K.S.), 1996.- Distribution and body size of *Fasciola gigantica* in livers of Bengal goats in Bangladesh. *Small Ruminant Research*, 19: 189-191.

NOVOBILSKY (A.), KASNY (M.), BERAN (L.), RONDELAUD (D.) & HÖGLUND (J.), 2013.- Lymnaea palustris and Lymnaea fuscus are potential but uncommon intermediate hosts of *Fasciola hepatica* in Sweden. *Parasites & Vectors*, 6: 251-261.

O

ODENING (K.), 1971.- Der grosse Leberegel und seine Verwandten. A. Ziemsen Verlang Wittenberg, Lutherstad.

OLSEN (O.W.), 1977.- Parasitología Animal. 3a ed. Barcelona: Aedos. 719 pp.

OSHIMA (T.), AKAHANE (H.) & SHIMAZU (T.), 1968a.- Patterns of the variation of the common liver fluke (*Fasciola* sp.) in Japan. I. Variations in the sizes and shapes of the worms and eggs. *Japanese Journal of Parasitology*, 17: 97-105.

OSHIMA (K.), ITO (T.) & MIURA (S.), 1971.- Tissue reactions against immature liver-flukes invaded in pigs. *Japanese Journal of Veterinary Science*, 33: 121-126.

OVEREND (D) & BOWEN (F), 1995.- Resistance of *Fasciola hepatica* to Triclabendazole. *Australian Veterinary Journal*, 72: 275-276.

P

PAGES (J.R.) & THERON (A.), 1990.- Analysis and comparison of cercarial emergence rhythms of *Schistosoma haematobium*, *S. intercalatum*, *S. bovis*, and their hybrid progeny. *International Journal for Parasitology*, 20: 193-197.

PANACCIO (M.) & TRUDGETT (A.), 1999.- Molecular Biology. En: *Dalton*, J.P. (Ed.), *Fasciolosis*. CAB International Publishing. Wallingford: 449-464 pp.

PANOVA (M.M.), 2002.- Análisis morfométrico de los estadios de adulto y huevo de especies de Fasciola (Linnaeus, 1758) (Trematoda: Fasciolidae). Tesis Doctoral, Facultad de Farmacia, Universidad de Valencia, 241 pp.

PANTELOURIS (E.), 1965.- The common liver fluke *Fasciola hepatica* L. Pergammon Press, Oxford.

PENG (M.), ICHINOMIYA (M.), OHTORI (M.), ICHIKAWA (M.), SHIBAHARA (T.) & ITAGAKI (T.), 2009.- Molecular characterization of *Fasciola hepatica Fasciola gigantica*, and spermic *Fasciola sp.* in China based on nuclear and mitochondrial DNA. *Parasitology Research*, 105: 809-815.

PERIAGO (M.V.), VALERO (M.A.), PANOVA (M.) & MAS-COMA (S.), 2006.- Phenotipic comparision 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.

PERIAGO (M.V.), VALERO (M.A.), EL-SAYED (M.), ASHRAFI (K.), EL-WAKEEL (A.), MOHAMED (M.Y.), DESQUESNES (M.), CURTALE (F.) & MAS-COMA (S.), 2008.- First phenotypic description of *Fasciola hepatica/ Fasciola gigantica* intermediate forms from the human endemic area of the Nile Delta, Egypt. *Infection, Genetics and Evolution*, 8: 51-58.

PETERS (W.) & PASVOL (G.), 2006.- En: *Atlas of Tropical Medicine and Parasitology.* USA: Mosby. 448 pp.

POPIOLEK (M.), KNECHT (D.), SZCZESNA-STASKIEWICZ (J.) & CZERWINSKA-ROZALOW (A.), 2010.- Helminths of the wild boar *(sus scrofa l.)* in natural and breeding conditions. *Bulletin of the Veterinary Institute in Pulawy*, 54: 161-166.

PIGLIUCCI (M.), 2005.- Evolution of phenotypic plasticity: where are we going now? Trends in *Ecology & Evolution*, 20: 481-486.

POLYAKOVA-KRSTEVA (O.) & GORCHILOVA (L.), 1972.- Morphological study of the liver in experimental fascioliasis in pig (in Russian). *Izv Tsentr Khelmintol Lab Bulg Akad Nauk.*, 15: 181-188.

Q

QUIROZ (H.), 1995.- Impacto económico, epidemiología, control y prevención de *Fasciola hepatica* en la ganadería. En: *XII Congreso Latino Americano de Parasitología.* Santiago De Chile, 20 pp.

QUIROZ (H.), 2000.- Parasitología y enfermedades parasitarias de animals domésticos. México: Uteha, 875 pp.

QURESHI (A.W.), TANVEER (A.), QURESHI (S.W.), MAQBOOL (A.), GILL (T.J.) & ALI (S.A.), 2005.- Epidemiology of human fasciolosis in rural areas of Lahore, Pakistan. Punjab University. *Journal of Zoology*, 20: 159-168.

QURESHI (A.W.), 2008.- Epidemiology, serodiagnosis and therapy of fasciolosis in humans and buffaloes in Punjab, Pakistan. PhD thesis, Department of Zoology, Univerrsity of the Punjab, Quaid-E-Azam, Lahore, 186 pp.

QURESHI (A.W) & TANVEER (A.), 2009.- Seroprevalence of fasciolosis in buffaloes and humans in some areas of Punjab, Pakistan. *Pakistan Journal of Medical Sciences*, 61: 91-96.

R

RICHARDS (D.T.) & LEWIS (J.W.), 2001.- Fecundity and egg output by *Toxocara canis* in the red fox, *Vulpes vulpes*. *Journal of Helminthology*, 75: 157-164.

ROHLF (F.J.) & MARCUS (L.F.), 1993.- A revolution in morphometrics. Trends in *Ecology & Evolution*, 8: 129-132.

ROKNI (M.B.), 2008.- The present status of human helminthic diseases in Iran. *Annals of Tropical Medicine and Parasitology*, 102: 283-95.

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.

S

SAHBA (G.H.), ARFAA (F.), FARAMANDIAN (I.) & JALALI (H.), 1972.- Animal fasciolosis in Khuzestan, Southwestern Iran. *Journal of Parasitology*, 58: 172-176.

SALAHI-MOGHADDAM (A.), HABIBI-NOKHANDAM (M.) & FUENTES (M.V.), 2011.- Low altitude outbreaks of Human fascioliasis related with summer rainfall in Gilan province, Iran. *Geospatial Health*, 6: 133-136.

SALAHI-MOGHADDAM (A.) & ARFAA (F.), 2013.- Epidemiology of Human fascioliasis outbreaks in Iran. *Journal of Archives in Military Medicine*, 1: 6-12.

SCHELL (S.), 1970.- The Trematodes. Dubuque: Brown, Iowa. 355 pp.

SCHMALENBERGER (H.), CARRO MARIÑO (F.) & RODRÍGUEZ LARRINAGA (A.), 2004.- Métodos disuasorios y de minimización de daños de la fauna cinegética en Galicia In: La prevención en los cultivos agrícolas. Jornadas sobre prevención de daños producidos por especies cinegéticas. Consellería do Medio Ambiente, Xunta de Galicia, Spain. 19-43 pp.

SCHWEIZER (G.), BRAUN (U.), DEPLAZES (P.) & TORGERSON (P.R.), 2005.- Estimating the financial losses due to bovine fasciolosis in Switzerland. *Veterinary Record*, 157: 188-193.

SHIMALOV (V.V.) & SHIMALOV (V.T.), 2000.- Findings of *Fasciola hepatica* Linnaeus, 1758 in wild animals in Belorussian Polesie *Parasitology Research*, 86: 527.

SHIMALOV (V.V.) & SHIMALOV (V.T.), 2003.- Helminth fauna of cervids in Belorussian Polesie. *Parasitology Research*, 89: 75-76.

SHIMIZU (A.), FUKUDA (K.), KAWANO (J.) & KIMURA (S.), 1994.- Pathological changes in the liver of a pig experimentally infected with Japanese *Fasciola sp. Science reports of the Hyogo University of Agriculture*, 21: 11-17.

SIDDIQI (M.N.) & SHAH (S.A.U.), 1984.- Natural infection of helminths in liver and respiratory tract of cattle of Peshawar and histology of *Paramohistomum cervi. Pakistan Veterinary Journal*, 4: 100-107.

SOARES (M.P.), DA SILVA (S.S.), NIZOLI (L.Q.), FELIX (S.R.) & SCHILD, (A.L.), 2007.- Chronic fascioliasis in farmed and wild greater rheas *(Rhea Americana). Veterinary Parasitology*, 145: 168-171.

SOLAYMANI-MOHAMMADI (S.), MOBEDI (I.), REZAIAN (M.), MASSOUD (J.), MOHEBALI (M.), HOOSHYAR (H.), ASHRAFI (K.) & ROKNI (M.B.),

2003.- Helminth parasites of the wild boar, *Sus scrofa*, in Luristan province, western Iran and their public health significance. *Journal of Helminthology*, 77: 263-267.

SPITHILL (T.W), SMOOKER (P.M.) & COPEMAN (D.B.), 1999.- *Fasciola gigantica*: epidemiology, control, immunology and molecular biology. En: Dalton JP ed. Fasciolosis. CAB, Wallingford: 465-525 pp.

SRIMUZIPO (P.), KOMALAMISRA (C.), CHOOCHOTE (W.), JITPAKDI (A.), VANICHTHANAKORN (P.), KEHA (P.), RIYONG (D.), SUKONTASAN (K.), KOMALAMISRA (N.), SUKONTASAN (K.) & TIPPAWANGKOSOL (P.), 2000.- Comparative morphometry, morphology of egg and adult surface topography under light and scanning electron microscopies, and metaphase karyotype among three size-races of *Fasciola gigantica* in Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health*, 31: 366-373.

T

TALAIE (H.), EMAMI (H.), YADEGARINIA (D.), NAVA-OCAMPO (A.A.), MASSOUD (J.), AZMOUDEH (M.) & MAS-COMA (S.), 2004.- Randomized trial of a single, double and triple dose of 10 mg/kg of a human formulation of triclabendazole in patients with fascioliasis. *Clinical and Experimental Pharmacology and Physiology*, 31: 777-782.

TAYLOR (E.), 1964.- Fascioliasis and the liver fluke. Agriculture Organization of the United Nations. Rome. 234 pp.

TENDLER (M.) & SIMPSON (A.J.), 2008.- The biotechnology-value chain: development of Sm14 as a schistosomiasis vaccine. *Acta Tropica*, 108: 263-266.

TERASAKI (K.), NODA (Y.), SHIBAHARA (T.) & ITAGAKI (T.), 2000.- Morphological comparisons and hypotheses on the origin of polyploids in parthenogenetic *Fasciola sp. Journal of Parasitology*, 86: 724-729.

THOMAS (A. P.), 1883.- The life history of the liver fluke (*Fasciola hepatica*). Quart. J. Mic. Sci., 23: 99-133.

THOMPSON (H.), IRVINE (R.M.) & PHILBEY (A.W.), 2009- *Fasciola hepatica* infection in wild boar in the UK. *Veterinary Record*, 165: 697-8.

U

UGLAND (K.I.), STRØMNES (E.), BERLAND (B.) & ASPHOLM (P.E.), 2004.- Growth, fecundity and sex ratio of adult whaleworm (Anisakis simplex; Nematoda, Ascaridoidea, Anisakidae) in three whale species from the North-East Atlantic. *Parasitology Research*, 92: 484-489.

URQUHART (G.), ARMOUR (J.), DUNCAN (J.), DUNN (A.) & JENNING (F.), 1987.-. Longaman Scientific la clasificación & Technical, Churchill Livigstone, *Veterinary Parasitology*, NY.

URQUHART (G.) & ARMOUR (J.), 2001.- Parasitología veterinaria. Zaragoza: Acribia. 355 pp.

V

VALERO (M.A.) & MAS-COMA (S.), 1985.- Consideraciones metodológicas sobre la aplicación de técnicas estadísticas en Parasitología. IV Congreso Nacional de Parasitología (Tenerife, España). Resúmenes de las Comunicaciones: 155.

VALERO (M.A.), 1986.- Revisión sistemática de la familia Brachylaimidae Joeux et Foley, 1930 (Trematoda: Digenea: Brachylaimoidea) con aportaciones al conocimiento de la variabilidad intraespecífica de los adultos de sus especies representantes. Tesis Doctoral, Facultad de Farmacia, Universidad de Valencia, 980 pp.

VALERO (M.A.), DE RENZI (M.) & MAS-COMA (S.), 1987.- Propuesta de una metodología para el estudio de las trayectorias ontogénicas de los Digénidos adultos de la familia Brachylaimidae Joeux et Foley, 1930. V Cong. Nac. Parasitol., (Salamanca, España). Resúmenes de las comunicaciones: 21-22.

VALERO (M.A.), MARCOS (M.D.) & MAS-COMA (S.), 1996.- A mathematical model for the ontogeny of *Fasciola hepatica* in the definitive host. *Research and Reviews in Parasitology*, 51: 125-128.

VALERO (M.A.), MARCOS (M.D.), FONS (R.) & MAS-COMA (S.), 1998.- *Fasciola hepatica* development in experimentally infected black rat, *Rattus rattus*. *Parasitology Research*, 84: 188-194.

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 Parasitology*, 47: 17-22.

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.

VALERO (M.A.), PANOVA (M.) & MAS-COMA (S.), 2001b.- Development differences in the uterus of *Fasciola hepatica* between livestock liver fluke populations from Bolivian highland and European lowlands. *Parasitology Research*, 87: 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: 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: 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: 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: 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: 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.- Anaemia in advanced chronic fasciolosis. *Acta Tropica*, 108: 35-43.

VALERO (M.A.), PÉREZ-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: 150-159.

VALERO (M.A.), UBEIRA (F.M.), KHOUBBANE (M.), ARTIGAS (P.), MUIÑO (L.), MEZO (M.), PÉREZ-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: 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: 79-86.

VALERO (M.A.), PERIAGO (M.V.), PÉREZ-CRESPO (I.), RODRÍGUEZ (E.), PERTEGUER (M.J.), GÁRATE (T.), GONZÁLEZ-BARBERA (E.M.) & MAS-COMA (S.), 2012a.- Assessing the validity of an ELISA test for the serological diagnosis of Human fascioliasis in different epidemiological situations. *Tropical Medicine and International Health*, 17: 630-636.

VALERO (M.A.), PERIAGO (M.V.), PÉREZ-CRESPO (I.), ANGLES (R.), VILLEGAS (F.), AGUIRRE (C.), STRAUSS (W.), ESPINOZA (J.R.), HERRERA (P.), TERASHIMA (A.), TAMAYO (H.), ENGELS (D.), GABRIELLI (A.F.) & MAS-COMA (S.), 2012b.- Field evaluation of a coproantigen detection test for fascioliasis diagnosis and surveillance in human hyperendemic areas of Andean countries. *PLoS Neglected Tropical Diseases*, 6: 1812.

VALERO (M.A.), PÉREZ-CRESPO (I.), KHOUBBANE (M.), ARTIGAS (P.), PANOVA (M.), ORTIZ (P.), MAÇO (V.), ESPINOZA (J.R.), & MAS-COMA (S.), 2012c.- *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.

VARMA (A.K.), 1953.- On *Fasciola* n. sp. with some observations on *F. hepatica* and *F. gigantica*. *Journal of Helminthology*, 27: 185-198.

VASQUEZ (P.), 1943.- *Fasciola hepatica*, Reporte del primer caso observado en Cuba, en un niño. *The Arch Medical Practice*, 12:163

VENGUST (G.), KLINKON (M.), BIDOVEC (A.) & VENGUST (A.), 2003.- *Fasciola hepatica*: effects on blood constituents and liver minerals in fallow deer (Dama dama). *Veterinary Parasitology*, 112: 51-61.

W

WALKER (S.M.), MAKUNDI (A.E.), NAMUBA (F.V.), KASSUKU (A.A.), KEY-YU (J.), HOEY (E.M.), PRÖDOHL (P.), STOTHARD (J.R.) & TRUDGETT (A.), 2008.- The distribution of *Fasciola hepatica* and *Fasciola gigantica* within southern Tanzania – constraints associated with the intermediate host. *Parasitology*, 135: 495-503.

WALKER (M.), HAL (L.A.), ANDERSON (R.M.) & BASÁÑEZ (M.G.), 2009.- Density-dependent effects on the weight of female *Ascaris lumbricoides* infections of humans and its impact on patterns of egg production. *Parasites & Vectors*, 2: 11.

WATANABE (S.) & IWATA (S.), 1954.- Fasciola species in Japan. *Journal of Japanese Veterinary Medical Association*, 7: 124-126.

WATANABE (S.), 1962.- Fasciolosis of ruminants in Japan. *Bulletin de l'Office International des Epizooties*, 58: 313-322.

WEBSTER (B.L.) & SOUTHGATE (V.R.), 2003.- Compatibility of *Schistosoma haematobium*, *S. intercalatum* and their hybrids with *Bulinus truncatus* and *B. forskalii*. *Parasitology*, 127: 231-242.

WEBSTER (B.L.), DIAW (O.T.), SEYE (M.M.), WEBSTER (J.P.) & ROLLINSON (D.), 2013.- Introgressive Hybridization of *Schistosoma haematobium* Group species in Senegal: Species Barrier Break Down between ruminant and human schistosomes. *PLoS Neglected Tropical Diseases*, 7: 2110.

WHO, 1995. Control of foodborne trematode infections. WHO Techn. Rep. Ser., *World Health Organization*, Geneva, 849: 1-157.

WILSON (R.A.), 1969.- Fine structure of the tegument of the miracidium of *Fasciola hepatica* L. *Journal of Parasitology*, 55: 124-133.

WILSON (D.E.) & REEDER (D.M.), 1993.- Mammal species of the world. En: *A taxonomic and geographic reference*. Washington: Smithsonian Institution Press.

WIKIPEDIA, 2015.- Available from: www.wikipedia.com.

WORLD HEALTH ORGANIZATION, 2013.- Sustaining the drive to overcome the global impact of neglected Tropical Diseases. *World Health Organization*, WHO Headquarters, Geneva, 138 pp.

WRIGHT (C.A.) & ROSS (G.C.), 1980.- Hybrids between *Schistosoma haematobium* and *S. mattheei* and their identification by isoelectric-focusing of enzymes. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 74: 326-332.

Y

YAMAGUTI (S.), 1958.- The Digenic Trematodes of Vertebrates. Parts 1 and 2. Systema Helminthum. Volume I. *Interscience Publishers*, NY.

YILMA (J.M.) & MALONE (J.B.), 1998.- A geographic information system forecast model for strategic control of fascioliasis in Ethiopia. *Veterinary Parasitology*, 78: 103-127.

ANEXO

ANNEX

This thesis is based on the author's work conducted at the Facultat de Farmacia of the Departamento de Biología Celular y Parasitoloía of the Universitat de València. Parts of it have already been published in articles and proceedings earlier.

Articles

- CUERVO (P.F.), CATALDO (S.D.), FANTOZZI (M.C.), DEIS (E.), ISENRATH (G.D.), VIBERTI (G.), ARTIGAS (P.), PEIXOTO (R.), VALERO (M.A.), SIERRA (R.M.), 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 Parasitology*, 3: 536-43.
- ASHRAFI (K.), VALERO (M.A.), PEIXOTO (R.V.), ARTIGAS (P.), PANOVA (M.), MAS-COMA (S.), 2015.- Distribution of *Fasciola hepatica* and *F. gigantica* in the endemic area of Guilan, Iran: Relationships between zonal overlap and phenotypic traits. *Infection, Genetics and Evolution*, 31: 95-109.
- 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): 274-283.
- AFSHAN (K.), VALERO (M.A.), QAYYUM (M.), PEIXOTO (R.V.), MAGRANER (A.), MAS-COMA (S.), 2013.- Phenotypes of intermediate forms of *Fasciola hepatica* and *F. gigantica* in buffaloes from Central Punjab. *Journal of Helminthology*, 88: 417-426.

FIGURA 7.1: *Fasciola hepatica* adults from France.

FIGURA 7.2: *Fasciola hepatica* adults from Spain.

FIGURA 7.3: *Fasciola gigantica* adults from Burkina Faso.

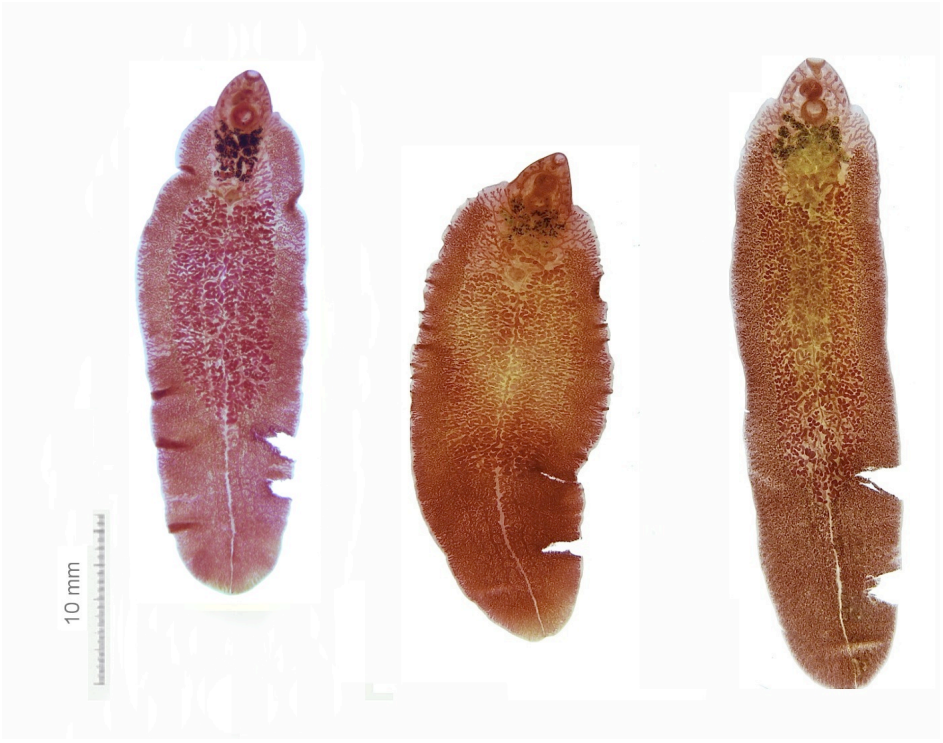

FIGURA 7.4: *Fasciola sp.* adults from Pakistan.

FIGURA 7.5: *Fasciola sp.* adults from Bangladesh.

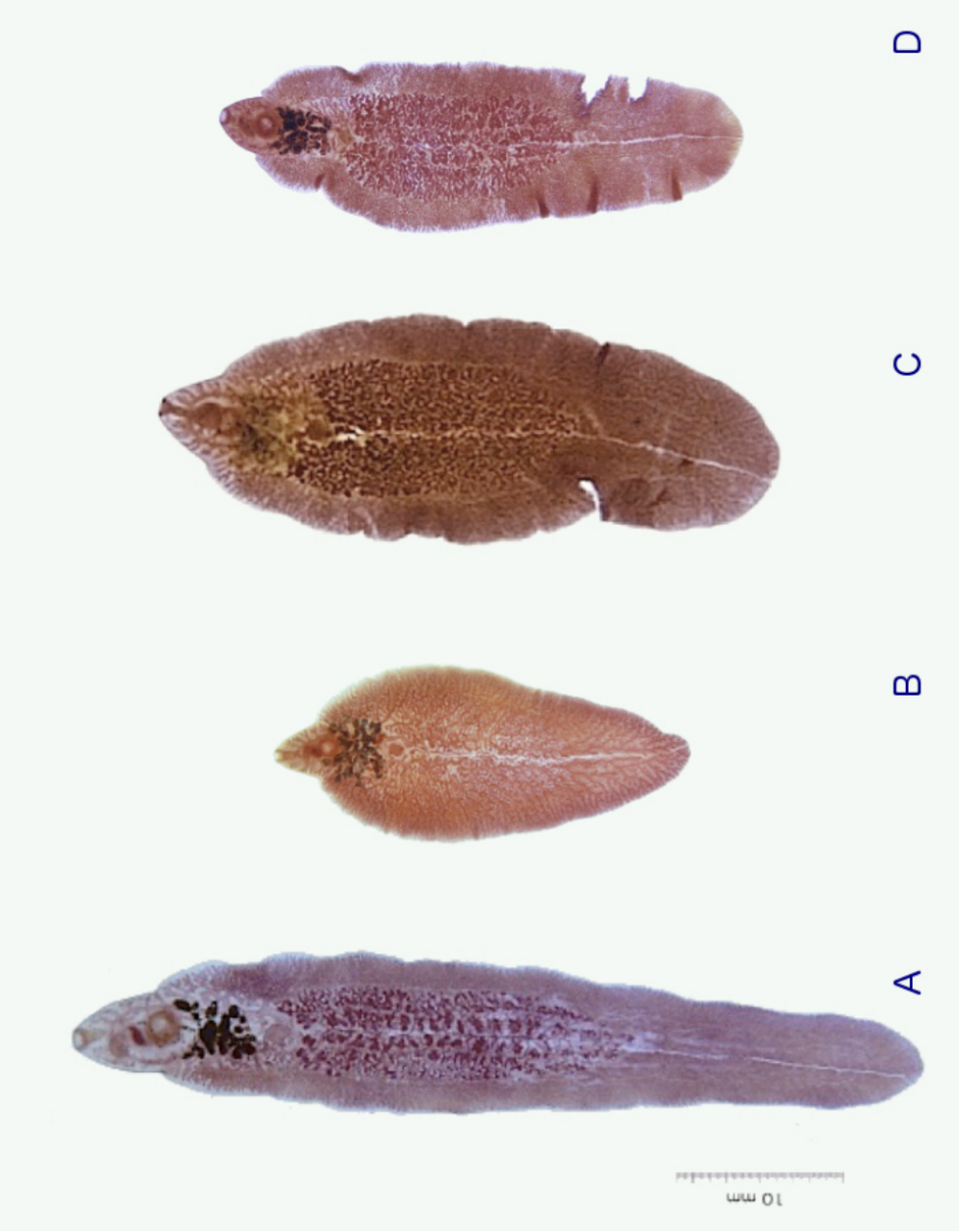

FIGURA 7.6: Fasciola sp. adults from (Iran) A, *Fasciola hepatica* adults from (Corsica, France) B, *Fasciola* sp. adults from (Bangladesh) C and *Fasciola* sp adults from (Pakistan) D.