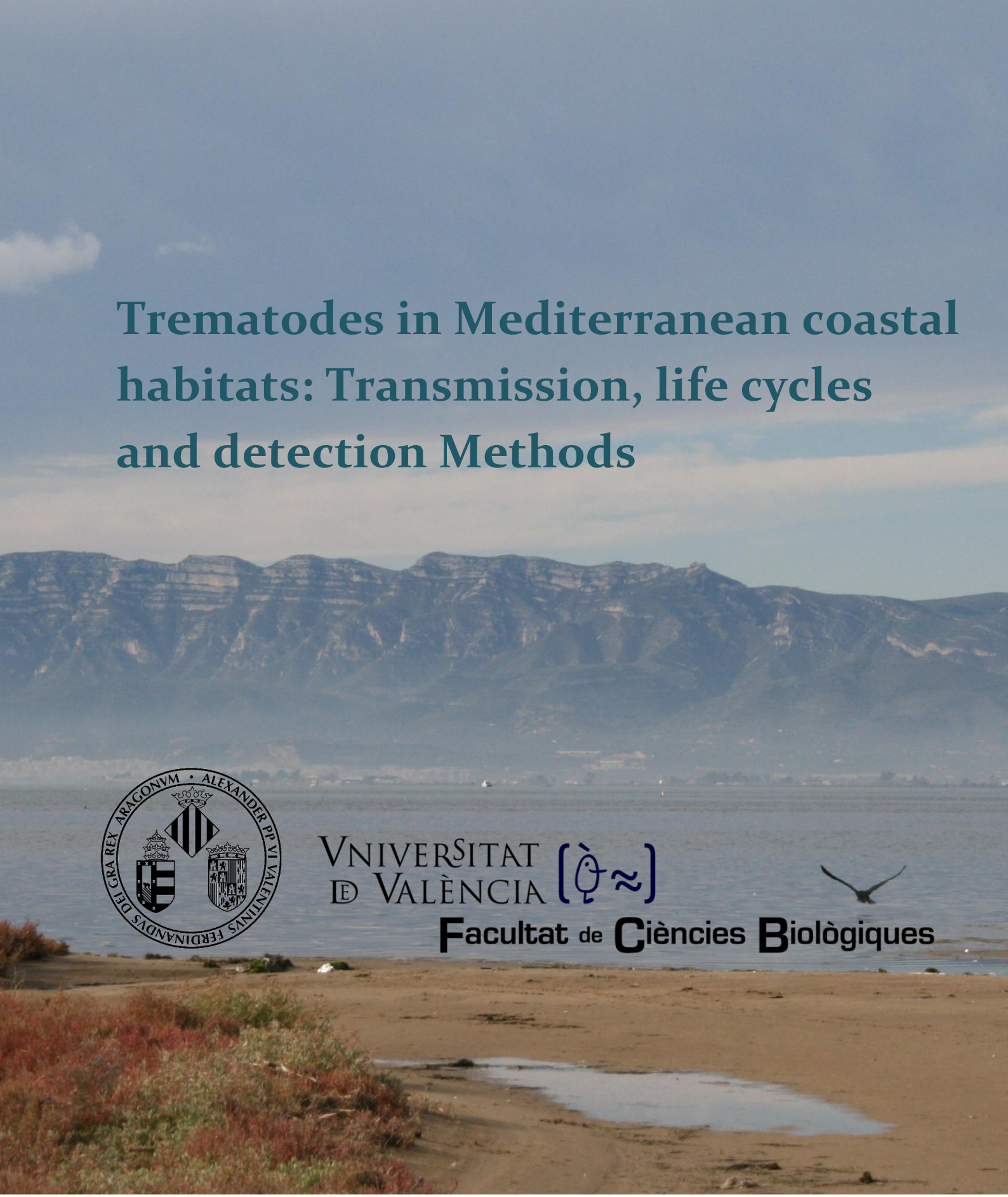
Trematodes in Mediterranean coastal habitats: Transmission, life cycles and detection Methods



Tesis Doctoral por: Ana Isabel Born Torrijos

Directores: Astrid Sibylle Holzer y Juan Antonio Raga Esteve

Valencia, junio 2015

PROGRAMA DE DOCTORADO EN BIODIVERSIDAD 3001

VNIVERSITAT DE VALÈNCIA

Facultat de Ciències Biològiques

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D.ª Astrid Sibylle Holzer, Jefa del Laboratorio de Protistología de Peces del Instituto de Parasitología del Centro de Biología de la Academia de Ciencias de la República Checa, y

D. Juan Antonio Raga Esteve, Catedrático de Zoología de la Facultad de Ciencias Biológicas de la Universitat de València.

CERTIFICAN que **D.ª Ana Isabel Born Torrijos** ha realizado bajo nuestra dirección, y con el mayor aprovechamiento, el trabajo de investigación recogido en esta memoria, y que lleva por título "Trematodes in Mediterranean coastal habitats: Transmission, life cycles and detection Methods", para optar al grado de Doctora en Ciencias Biológicas.

Y para que así conste, en cumplimiento de la legislación vigente, expedimos el presente certificado en Valencia a 18 de Junio de 2015.

Firmado: Astrid Sibylle Holzer Firmado: Juan Antonio Raga Esteve

A mi familia

(y pensar que con unas hormigas empezó todo…)

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Summary

Among metazoan parasites, trematodes (Platyhelminthes) are ubiquitous components in natural systems and widespread in the animal kingdom, thus found in a wide range of conditions and habitats. Understanding their important role in natural ecosystems and the total amount of biomass they account for, contributes to our knowledge on parasites from an ecological point of view. Parasites affect ecosystem processes and play crucial roles in the structure and stability of natural communities and food webs. Furthermore, trematodes are the most common parasites in intertidal soft-sediment habitats, being of major importance in lagoons, a habitat which is characterized by extreme fluctuations in abiotic factors. Lagoons and intertidal habitats gather intermediate and final hosts, so that the transmission of trematode life cycle stages may be facilitated. A wide range of factors play a role in the ecological, physiological and biological peculiarities of each host-parasite system, thus being important in the completion of trematode life cycles. Furthermore, although the Mediterranean is a marine biodiversity hot spot, anthropogenic factors such as pollution, habitat loss and degradation, eutrophication, fishing impacts, discards and climate change affect coastal marine ecosystems and all their organisms, including parasites.

This PhD study aims to compile information on various aspects of the life cycles of digenean trematodes, focusing on host-parasite systems common in Mediterranean lagoons, and studying the maturation and emergence of larval stages, their transmission, different methods for the detection of infections, as well as analysing environmental and anthropogenic factors that could enhance the transmission of these parasites.

The study led to the following findings and conclusions:

The snail *Gibbula adansonii* (Trochidae) is highly abundant in the intertidal habitat of “Els Alfacs” lagoon (Ebro Delta, Spain) and shows high infection levels with two cotylocercous cercariae, belonging to the family Opecoelidae. They were identified as *Macvicaria obovata* (Molin, 1859) and *Cainocreadium labracis* (Dujardin, 1845) and were found to occur in *G. adansonii* with 23.1% and 22.04% prevalence, respectively. Identification was based on ITS ribosomal DNA sequences and a comparative sequence analysis using published sequences of opecoelid larval and adult stages. Additionally, detailed morphological descriptions of the cercariae were produced, and chaetotaxy reconstruction was performed on images produced by scanning electron microscopy (SEM). This study, together with the examination of the related literature, helped to morphologically differentiate both cercariae from other known opecoelid species infecting

gastropods along the Mediterranean and North East Atlantic coasts of Europe, allowing for the elucidation of the life cycle of *M. obovata* and for the confirmation of the morphotype of *C. labracis* cercariae. Partial 28S rDNA sequences were provided to aid future studies on systematic relationships within the Opecoelidae.

Due to the shallow and confined nature of lagoons, these habitats provide excellent conditions for the transmission of digenean trematodes to their next intermediate hosts. Nevertheless, environmental conditions can greatly fluctuate in these habitats, so that an experimental study was performed, to assess the influence of changing abiotic factors, which exhibit marked fluctuations in lagoonal environments, on the emergence of cercariae of *M. obovata* and *C. labracis*. Snails emitting cercariae were exposed to varying temperature, salinity, water levels and photoperiods. Both cercariae use a sit-and-wait downstream host-finding strategy, by which they remain attached to the substrate until they infect small benthic fish, in the case of *C. labracis,* and snails in the case of *M. obovata*. The results of the present study indicated contrasting patterns and rates of larval emergence in *C. labracis* and *M. obovata*, likely an adaptation to ensure spatial and temporal co-occurrence with their next intermediate hosts. Increasing temperature, low water level and darkness increase the emergence of *M. obovata,* facilitating the encounter with the nocturnal mollusc *C. neritea*, probably when tidal pools are formed and cercariae concentrate in a small water volume. On the contrary, daylight hours, a 12-h light: 12-h dark photocycle and increased salinity trigger the emergence of *C. labracis*, probably to coincide with small gobiids present in the lagoons during the early summer. Both, exogenous control of parasite transmission by these abiotic factors as well as endogenous control associated with the different transmission pathways of both opecoelid cercariae was proven.

Trematode communities often consist of different species exploiting the same host population, so that two or more trematodes can co-occur in the same individual host. Multiple infections usually occur in very low prevalences suggesting that within-host competition may be regulating co-infections. The commonly used diagnostic method for the detection of larval trematode infections in snail intermediate hosts is based on cercarial shedding; however, this method has been often criticized as inaccurate, e.g. in comparison with the prevalence obtained by snail dissection. In order to achieve a precise estimation of the prevalence of intramolluscan stages, a species-specific duplex PCR was designed for the detection of single and double infections in two host-parasite systems in

the intertidal habitat: i) the two co-occuring opecoelid trematode species in the Ebro Delta lagoon (Spain), for which within-host interactions have never been studied, and ii) two co-occurring trematode species (*Maritrema novaezealandensis* and *Philophthalmus* sp) in the snail *Zeacumantus subcarinatus* (Batillariidae) in intertidal habitats in Otago (New Zealand), with interspecific competition known to occur. The molecular assays were shown to detect between 0.6% and 54.2% more single as well as between 2.4% and 9.5% more double infections, thus showing a higher accuracy than methods based on cercarial shedding. The duplex PCR assay furthermore provides the advantage to be able to differentiate between the morphologically similar opecoelid species co-infecting a single snail host, as well as detecting and identifying immature infections. According to the expected frequency of multiple infections and the comparison with observed prevalences, the higher accuracy of the duplex PCR suggested that double infections were more frequent than expected. However, interspecific competition in double infections may eliminate early infections by the first established species, a scenario likely occurring in *Z. subcarinatus*. Thus, the frequency of double infections detected by the more sensitive duplex PCR may overestimate that of naturally occurring mature infections resulting from the release of cercariae. Hence these results have to be interpreted with caution and are best considered together with emission rates. A more realistic assessment of the frequency of larval trematodes infecting snails helps us to understand the community structure and potential interspecific interaction within the host. The species-specific ITS-based PCR assay can be extrapolated to other systems and should be considered as an important additional tool for determining prevalences of larval stages with high accuracy.

Cercarial development and transmission is affected by many factors, with the host's distribution being of particular importance. Cercarial release from the first intermediate host has to be temporally and spatially adapted to maximise the encounter with the downstream hosts, especially in sessile cercariae such as those of *C. labracis* and *M. obovata*. The development of intramolluscan larval stages and parasite maturation inside sporocysts of these two species was monitored during the spawning-related upshore residency of the snail *G. adansonii*, i.e. from March to May, by quantifying different maturation stages inside the sporocysts. Furthermore, the effect of sporocyst size and snail size on the number of developmental stages was estimated. Both sporocyst and snail size act as proxies of the reproductive capacity of *M. obovata* but not that of *C. labracis*. Surprisingly, we did not observe variation in the number of germinal balls or cercarial embryos in either parasite species, during these months, however, a higher number of

mature stages and the highest maturity index (average maturity of each sporocyst) were found in April. Thus, the production of mature cercariae of both species is continuous from March to May, when the snail *G. adansonii* inhabits the upshore habitat and it coincides with the spawning period of the host and the presence of small benthic fish and the snail *C. neritea* in the habitat Spawning-related upward migration is common among trochid snails, as well as the manipulation of host behaviour by trematodes, with parasitized individuals changing their geographic distribution to enhance transmission probabilities of the trematodes to the next host. Thus, a link between larval maturation and snail upshore residency during migration can be hypothesised, since the synchronisation of larval and host maturation in time and space guarantees the completion of the life cycle of *C. labracis* and *M. obovata*. Thus, this snail-parasite system opens a door to further studies on parasite-induced behaviour changes as a true adaptation for transmission.

Trophically-transmitted parasites play an important role in host community structure, exerting strong effect on food web structure when infecting a wide range of host species. However, information on host specificity is often scarce or unavailable. The aim of this study was to extend the knowledge of the host-spectrum and host-specificity of a common parasite in the Mediterranean, the heteroxenous digenean *Cardiocephaloides longicollis*, considering all host levels, in order to characterise its life cycle ecology. For this, 3351 molluscs (24 species), 2108 fish (25 species), 154 birds (17 species) and 158 faecal samples from birds were screened for *C. longicollis*. New host records at the first intermediate host level (1 taxon), but especially the second intermediate host level (12 taxa, including two new families) were detected, and, most importantly, considerably higher infection levels than previously reported were detected in first intermediate and definitive hosts. The infection level in fish was highest in autumn and winter, possibly related to a parasite-induced mortality in spring. Demersal and benthic fish seem to be targeted more frequently by cercariae and hence their parasite loads were higher than that of pelagic fish. A cumulative effect of metacercariae over time, i.e. with increasing host size, was observed, in the fish brain. At the same time, a decline of parasite aggregation in the largest fish size classes was detected, thus indicating parasite-induced mortality by which the most infected fish are eliminated from the population. The accumulation of metacercariae of *C. longicollis* may increase the manipulation of the host behaviour, causing fish to swim in the upper part of the water column, making them more susceptible to predation by the definitive host and thus enhancing their transmission. During this

study, high infection rates were found in hosts from areas with high fishing activity, suggesting an enhanced transmission of *C. longicollis*. Scavengers such as seagulls are attracted by bycatch, i.e. discarded fish, which, in the Mediterranean, is composed of fish sizes and species found to have the highest infection rates. These fish inhabit demersal and benthic habitats and would under natural conditions be less accessible to the birds. Moreover, metacercariae of *C. longicollis* were found in the gilthead sea bream *Sparus aurata* from aquaculture installations with 54% prevalence, and it is possible that aquaculture activities impact further on *C. longicollis* transmission. As a trophically-transmitted generalist species with a complex life cycle that affects a high number of host species, *C. longicollis* likely has a strong effect on the energy flow through the food web, even under natural conditions. This feature makes *C. longicollis* an ideal candidate to compare future web structures between natural areas and those impacted by fisheries.

Resumen

0.1 Introducción general

Los metazoos trematodos (Platyhelminthes) son componentes ubicuos de los ecosistemas naturales entando temporal o permanentemente presentes en más de la mitad de las especies animales (Bush *y col.* 2001, Prietrock & Marcogliese 2003). Los parásitos trematodos pueden encontrarse en un amplio rango de condiciones y hábitats, teniendo un gran impacto en los procesos ecológicos: mediante la regulación de las poblaciones de hospedadores (Hudson *y col.* 2006) y afectando a la estructura de las comunidades y redes tróficas (ej. Mouritsen & Poulin 2002, Thompson *y col.* 2005, Lafferty *y col.* 2006, 2008). Son sobre todo, los parásitos que son transmitidos a través de la cadena trófica los que afectan a un gran número de especies de hospedadores, condicionan la estabilidad de las redes tróficas, sus interacciones, longitud y flujo de energía (Thompson *y col.* 2005, Lafferty *y col.* 2008).

.En el Mediterráneo Occidental existe una gran riqueza de especies, encontrándose la mayor diversidad de peces e invertebrados en aguas de la plataforma continental. Las masas de agua de la plataforma continental constituyen el 20% del Mar Mediterráneo, por lo que tienen un papel importante en los procesos ecológicos (Bartoli & Gibson 2007, Coll *y col.* 2008). Los platelmintos constituyen el 6,7% del total de la diversidad de especies del Mediterráneo, siendo sin embargo poco conocidos, con menos del 50% descritas (Coll *y col.* 2008). Bartoli & Gibson (2007) aumentaron en gran medida nuestro conocimiento sobre platelmintos trematodos lagunares, al recopilar información sobre 72 especies nominales de lagunas del Mediterráneo Occidental, de las cuales aportaron el ciclo vital completo de 56.

Los trematodos son los parásitos más comunes en hábitats intermareales de sedimento blando (Sousa 1991, Mouritsen & Poulin 2002, Poulin & Mouritsen 2006), siendo además de gran importancia en lagunas (Bartoli & Boudouresque 2007). El hábitat intermareal se caracteriza por fluctuaciones extremas de los factores abióticos (Palacín *y col.* 1991, Koutsoubas *y col.* 2000), con pequeños cambios en el nivel del agua que, por la evaporación, pueden exponer a los moluscos a altas temperaturas y salinidades elevadas (6°C a 30°C y de 21‰ a 38‰, respectivamente, Solé *y col.* 2009). Los moluscos, que constituyen del 15 al 25% de la macrofauna bentónica (Coll *y col.* 2008), son hospedadores de una gran variedad de parásitos, normalmente estados larvales de trematodos digeneos (Lauckner 1980). Las lagunas suelen ser utilizadas como zonas de alimentación y cría de aves y peces, por lo que la transmisión de los estados vitales de los trematodos entre los

diferentes hospedadores podría verse facilitada gracias a su proximidad (Bartoli & Prévot 1986, Bouchereau & Guelorget 1998¸ Koutsoubas *y col.* 2000, Soppelsa *y col.* 2007).

Durante el siglo pasado, las actividades pesqueras han evolucionado desde la producción a pequeña escala a la explotación industrializada y extensiva. Esta evolución de las pesquerías también ha sido evidente en el Mediterráneo. Por otra parte se sabe que tanto las costas marinas como la biodiversidad del Mediterráneo se ven amenazadas por el impacto antropogénico, como el cambio climático o la sobreexplotación (IPCC 2001, Coll *y col.* 2008). Debido a la mortalidad de organismos intermareales a causa de cambios extremos de temperatura (Harley *y col.* 2006) y su efecto sobre la abundancia y distribución de estos organismos (Harley *y col.* 2006, Helmuth *y col.* 2006), estos hábitats han sido propuestos como sistemas tempranos de alerta para la detección del cambio climático. Además, la alta sensibilidad de los parásitos a los cambios de temperatura y la contaminación también podrían ayudar a monitorizar el impacto ecológico del cambio climático (Marcogliese 2001, Poulin & Mouritsen 2006). Además, los descartes de las pesquerías facilitan el acceso de las aves marinas a grandes cantidades de comida, particularmente peces demersales que de manera habitual serían inaccesibles (Tasker *y col.* 2000, Oro & Ruiz 1997). Por lo tanto, aunque se conoce el impacto de estas prácticas sobre las poblaciones de hospedadores (ej. en láridos, Oro 1996, Oro *y col.* 1995, 1996), los efectos sobre la transmisión de los parásitos no se ha estudiado en profundidad.

La subclase Digenea (Trematoda) comprende alrededor de 18.000 especies nominales (Cribb *y col.* 2001, Bartoli & Gibson 2007), lo que representa el mayor número de endoparásitos presentes en todas las clases de vertebrados (Bush *y col* 2001). La mayoría de los trematodos digeneos tienen un ciclo vital complejo que involucra tres hospedadores y diferentes estados de desarrollo (Galaktionov & Dobrovolskij 2003).Los hospedadores definitivos son por lo general vertebrados (aves o peces). Los huevos maduros se liberan al agua, normalmente a través de las heces, emergiendo una larva ciliada, el miracidio, que infecta al primer hospedador intermediario, por lo general un gasterópodo. Dentro del gasterópodo, los trematodos se reproducen de manera asexual en un esporocisto o redia, dando lugar a un gran número de cercarias que son liberadas al medio una vez maduras. Estos estados de vida libre no se alimentan, y parasitan al segundo hospedador intermediario, generalmente otro molusco o un pez, en un corto período de tiempo (menos de 24 horas). Después de la penetración en el segundo hospedador intermediario, la cercaria se enquista y se transforma en un metacercaria. De manera general, el

hospedador definitivo adquiere las metacercarias infectivas a través de la cadena trófica. Cada etapa del ciclo vital del parásito muestra una estrategia de transmisión diferente y específica para completar con éxito el ciclo vital (Combes *y col.* 2002). Las etapas de vida libre de trematodos, es decir, los huevos, miracidios y cercarias, dependen de sus propios recursos energéticos y están directamente expuestos a los cambios ambientales, lo que condiciona la interacción entre los parásitos y sus potenciales hospedadores. Por lo tanto, los factores ambientales abióticos y bióticos influyen en el éxito de transmisión de los estados larvales de vida libre, en especial de las cercarias durante la búsqueda del siguiente hospedador, alterando su supervivencia o infectividad (Fingerut *y col.* 2003, Pietrock & Marcogliese 2003, Thieltges *y col.* 2008). Cabe destacar que los hábitats lagunares están caracterizados por grandes cambios ambientales y una alta heterogeneidad interna. La comprensión de los efectos de estos factores ambientales sobre la emergencia de cercarias es importante para estimar el impacto de las condiciones cambiantes en la transmisión y el ciclo vital de un parásito.

Las instalaciones de acuicultura y los descartes provocan que diferentes grupos de hospedadores (peces, Bozzano & Sarda 2002; aves, Oro & Ruiz 1997 y gasterópodos, Morton & Yuen 2000) se concentren en la misma zona, por lo que la transmisión del parásito se ve facilitada. La atracción hacia instalaciones de acuicultura de potenciales hospedadores definitivos, como aves piscívoras (Witt *y col.* 1981, Arcos *y col.* 2001, Christel *y col.* 2012), puede, por lo tanto, intensificar la transmisión de parásitos. El impacto ambiental de las actividades pesqueras e instalaciones de acuicultura hacen que sea primordial estudiar posibles ciclos vitales que podrían verse favorecidos bajo estas condiciones.

La coexistencia de dos organismos en un sistema parásito-hospedador se caracteriza por la obtención de beneficios por parte del parásito (ej. energía o espacio) a expensas del hospedador, por lo que un parásito, por definición, afectará a su hospedador de diferentes maneras (Lauckner 1980, 1983, Price 1980). Por lo general, el tamaño del hospedador es un factor importante para los trematodos, existiendo un compromiso entre el número de cercarias que emergen de un gasterópodo y su tamaño, proveyendo más espacio y nutrientes aquellos hospedadores más grandes (Loker 1983, McCarthy *y col.* 2002, Poulin & Morand 2004). Asimismo, se ha observado en varias especies la existencia de un efecto acumulativo de parásitos relacionado con el tamaño del hospedador (ej. Thomas *y col.* 1995, Karvonen *y col.* 2003, Osset *y col.* 2005). La mortalidad inducida por la acumulación de parásitos, por la cual los individuos más infectados son eliminados de la

población (Rousset *y col.* 1996, Poulin 2001), se ha descrito tanto en peces como en invertebrados.

Dada la corta vida de las cercarias, la sincronización de la emergencia tiene que estar optimizada para facilitar la transmisión al siguiente hospedador intermediario (Combes *y col.* 1994). La distribución de los hospedadores es igualmente importante, existiendo incluso parásitos que manipulan el comportamiento del hospedador en el que se encuentran para aumentar las probabilidades de alcanzar al siguiente hospedador (Poulin 2007). En cualquier sistema, la combinación de varios factores influye en la emergencia del parásito, así como a su transmisión y ciclo vital, teniendo que tener en cuenta la interdependencia de los factores a la hora de estudiar un sistema.

La combinación de diferentes técnicas, como el empleo de herramientas moleculares, el desarrollo de experimentos y la implementación de técnicas nuevas para la detección de infecciones, es necesaria para obtener una visión holística del sistema parásito-hospedador estudiado.

0.2 Justificación y objetivos

La presente tesis tiene cuatro propósitos generales: (i) describir un sistema parásito-hospedador común en lagunas del Mediterráneo que se ajusta a las condiciones en el hábitat intertidal, (ii) aumentar el conocimiento sobre los factores que juegan un papel importante en la emergencia, transmisión y desarrollo de las diferentes etapas larvales, (iii) proporcionar nuevas evidencias sobre la mayor precisión de los métodos moleculares para detectar infecciones en gasterópodos, teniendo también en cuenta sus desventajas, y (iv) ampliar nuestro conocimiento sobre el rango de hospedadores y la transmisión de las fases del ciclo vital de *Cardiocephaloides longicollis*, un parásito digeneo que está altamente presente en el Mediterráneo y que está integrado en sus redes tróficas.

Los objetivos específicos a desarrollar son los siguientes:

I.- Identificación morfológica y molecular de dos morfotipos diferentes de cercarias emergidas del caracol *Gibbula adansonii*, común en el hábitat intermareal de la laguna del Delta del Ebro, determinando las tasas de infección, así como su relación filogenética con especies de trematodos cercanas.

II.- Evaluación experimental del efecto de los cambios en la temperatura, la salinidad, el fotoperiodo y el nivel de agua sobre la emergencia de larvas de *Cainocreadium labracis* y *Macvicaria obovata,* desde su primer hospedador intermediario *G. adansonii*, mediante técnicas *in vitro*, en el contexto de sus estrategias de encuentro del siguiente hospedador intermediario.

III.- Comparación de métodos tradicionales de detección de infecciones en el primer hospedador intermediario, por emergencia y herramientas moleculares, con una PCR dúplex específicamente diseñada para la detección de estados larvales en gasterópodos, tanto en infecciones simples como dobles. Se utilizarán dos sistemas de parásito-hospedador presentes en el hábitat intermareal: i) dos especies de trematodo, *C. labracis* y *M. obovata,* que co-habitan en gasterópodo *G. adansonii*, desarrollándose en esporocistos y cuya interacción interespecífica no se ha estudiado, y ii) dos especies de trematodo, *Maritrema novaezealandensis* y *Philophthalmus* sp. , que co-habitan en el gasterópodo *Zeacumantus subcarinatus*, desarrollándose en esporocistos y redias, respectivamente, del cual se conoce su competencia interespecífica.

IV.- Diferenciación morfológica y cuantificación de los estados larvales de desarrollo en esporocistos, y subsecuente descripción de los patrones de maduración en esporocistos de *C. labracis* y *M. obovata* infectando a *G. adansonii* durante los meses de primavera, cuando este gasterópodo se encuentra en el hábitat intermareal de la laguna del Delta del Ebro. Se determinará si existe un vínculo entre la maduración de las larvas y la migración del gasterópodo.

V.- Detección a gran escala de potenciales hospedadores (moluscos, peces y aves) para documentar el espectro de hospedadores y la carga parásita de *C. longicollis* en el Mediterráneo, determinando también el microhábitat marino al que se dirigen las cercarias, comparando los niveles de infección en peces de diferentes hábitats.

VI.- Cuantificación de las metacercarias de *C. longicollis* en los encéfalos de espáridos para analizar el efecto del tamaño de los peces en su acumulación a lo largo del tiempo, y explorar el papel de los factores antropogénicos como la pesca y la acuicultura, que puedan intensificar la transmisión de metacercarias al hospedador final.

0.3. Materiales y métodos generales

En este resumen los materiales y métodos se describen por separado para cada una de las secciones correspondientes. A continuación se resumen los estudios realizados, estructurados por capítulos.

0.4. Identificación molecular y morfológica de opecoélidos larvales (Digenea: Opecoelidae) parasitando caracoles prosobranquios en una laguna del Mediterráneo Occidental

0.4. 1. Introducción

Las hábitats costeros de las lagunas mediterráneas son áreas donde se lleva a cabo la reproducción y crecimiento de muchas especies de peces (Franco *y col.* 2006, 2011, Ribeiro *y col.* 2006, Verdiell-Cubedo *y col.* 2007). Además, los parásitos trematodos representan una parte importante de las comunidades marinas de los hábitats intermareales, ya que la naturaleza confinada de las lagunas permite que existan las condiciones propicias para la transmisión de los parásitos con ciclos vitales complejos, es decir, con dos o más hospedadores intermediarios. A pesar de que Bartoli y Gibson (2007) dilucidaron, en su revisión bibliográfica sobre digeneos del Mediterráneo Occidental, el ciclo vital de un total de 56 especies de trematodos, entre las referencias empleadas no existen muchos datos sobre los rasgos morfológicos que podrían ayudar a la identificación de las larvas en futuros estudios. Opecoelidae es una familia de parásitos cosmopolita, con al menos 25 especies parasitando peces marinos en el Mediterráneo Occidental (Bartoli *y col.* 2005). Sin embargo, sólo se conoce el ciclo vital completo de cuatro de ellas, probablemente por la falta de empleo de técnicas moleculares. Entre los ciclos conocidos se encuentra el de la especie *Cainocreadium labracis* (Dujardin, 1845) (Maillard 1971, 1974, 1976).

En un estudio sobre los digeneos en moluscos de la laguna de "Els Alfacs" en el Delta del Ebro (España), se encontraron infecciones en dos moluscos: *Gibbula adansonii* (Payraudeau, 1826) (Prosobranchia, Trochidae), que contenía esporocistos con dos morfotipos de cercarias cotilocercas, y *Cyclope neritea* (L., 1758) (Prosobranchia, Nassariidae), que albergaba metacercarias. Durante el presente trabajo presentamos evidencias moleculares y morfológicas para la identificación de los estados larvales de dos especies de opecoélidos encontrados en dos moluscos.

0.4. 2. Materiales y métodos

Se recogieron un total de 514 *G. adansonii* y 129 *C. neritea* en la laguna de "Els Alfacs" (Delta del Ebro, España) desde marzo a julio de los años 2010 y 2011. Después de recoger manualmente los caracoles del sedimento fangoso y sobre las hojas de *Zostera noltii* Hornemann, 1832, se transportaron al laboratorio en agua marina aireada.

Para la extracción de ADN se utilizaron de dos a cuatro cercarias de cada morfotipo y dos metacercarias, fijadas en etanol 100% y disueltas en 300µl de tampón de extracción TNES-urea. Estas muestras se disolvieron durante la noche a 55ºC en 100µg ml^{-1} de proteinasa K, siguiendo después un protocolo fenol-cloroformo para extracción de ADN (Holzer *y col.* 2004). El ADN se resuspendió y disolvió en 40µl de agua nanopura durante la noche. Las amplificación del ADN por reacción en cadena de la polimerasa (PCR) se llevó a cabo con un termociclador en un volumen final de 30µl, conteniendo ≈0.5 unidades de polimerasa ThermoPrime Plus DNA y el correspondiente tampón (10x), que contiene 1.5mM $MgCl_2$ (ABgene), 0.2 mM de cada dNTP, 0.5µM de cada primer, y aproximadamente 100ng de la muestra de ADN. Se amplificaron y purificaron dos fragmentos de ADN ribosomal: la secuencia completa de la región ITS (ITS1+5.8S+ITS2) y la secuencia parcial de la región 28S. Después de ensamblar las secuencias con Bioedit v7.0.5. (©1997–2005; Hall 1999), las secuencias resultantes de ITS se alinearon con secuencias de otros opecoélidos obtenidas en GenBank (Mafft v5.531 (Katoh *y col.* 2002), Gblocks v 2.0 (Castresana 2000)). Para estimar la posición de las secuencias generadas en relación a otros miembros de la familia Opecoelidae se llevaron a cabo análisis de Neighbour-joining (NJ) con MEGA5 (Tamura *y col.* 2011) e inferencia filogénica con máxima verosimilitud (ML) con RAxML (Stamatakis 2006). Se usó el programa FigTree v1.3.1 (Rambaut 2009) para la visualización de los árboles generados.

Después de la emisión espontánea de cercarias de los caracoles *G. adansonii*, se estudió inicialmente la morfología de las cercarias con la tinción *in vivo* rojo neutro. Después, las muestras se fijaron en etanol al 70% y se tiñeron con carmín alumínico o acetocarmín férrico, se deshidrataron en series de etanol y se clarearon en dimetilftalato, para ser montados en una preparación con bálsamo de Canadá. Las metacercarias encontradas en *C. nerita* se sacaron del quiste y se estudiaron bajo el microscopio. Se tomaron fotografías con una cámara Leica DC300 acoplada a un microscopio DMR. Para el estudio de cercarias en el microscopio electrónico de barrido (SEM), se fijaron muestras en glutaraldehido al 2.5% o en etanol al 70%. Después de lavar los especímenes con tampón

fosfatos 0.1M (pH 7.4), se deshidrataron en series de acetona, se secaron hasta punto crítico con CO_2 y se revistieron por bombardeo iónico con oro. Las muestras se estudiaron con un voltaje de aceleración de 4kV (JEOL JSM 7401-F).

0.4. 3. Resultados

Se obtuvieron secuencias completas de ITS ADNr y parciales de 28S ADNr de los dos morfotipos de cercarias emergidas de *G. adansonii* y de las metacercarias encontradas en *C. neritea*. Después del alineamiento de las 20 secuencias disponibles de ITS de otros opecoélidos, se seleccionaron las regiones no ambiguas para llevar a cabo el análisis filogenético (con NJ y ML). Los dos análisis revelaron la misma posición relativa de las secuencias nuevas respecto a las más cercanas filogenéticamente: las secuencias del morfotipo pequeño y de la metacercaria encontrada en *C. neritea* formaron un clado diferenciado, junto a muestras de *Macvicaria obovata*. El morfotipo grande también formó un clado monofilético separado, junto a secuencias de *Cainocreadium labracis*, siendo la divergencia en ambos casos inferior a la divergencia interespecífica de los géneros *Macvicaria* spp. (2,8–11,9%) y *Cainocreadium* spp. (5,3-9,4%).

Se tomaron medidas para la descripción morfológica de los dos morfotipos de cercarias, así como las de los esporocistos que las contenían, y de la metacercaria encontrada en *C. neritea*. Además, se describió el comportamiento de las cercarias y su patrón de receptores sensoriales, utilizando fotografías con microscopía electrónica de barrido (SEM) para destacar algunos rasgos morfológicos de las dos cercarias, como por ejemplo las papilas que forman parte del sistema sensorial. Dependiendo del mes de muestreo, la prevalencia de *C. labracis* fue de 17,6-30,8%, mientras que la de *M. obovata* fue de 0,9-23,1%. Tanto los esporocistos como las cercarias de *M. obovata* resultaron tener un tamaño menor que los de C. *labracis* (*M. obovata*, longitud esporocitos (µm) 600-1650, longitud cercarias (µm) 196-296 (media 248); *C. labracis*, longitud esporocitos (µm) 900-2377, longitud cercarias (µm) 208-402 (media 294). Ambas cercarias, al tener una cola de tipo cotilocerco, se sujetaron al sustrato después de su emergencia desde *G. adansonii*, mostrando *C. labracis* movimientos laterales pendulares, más vigorosos en presencia de luz. *Macviacria obovata,* con movimientos más lentos, contraía y expandía el cuerpo, balanceándose en presencia de luz.

0.4. 4. Discusión

La identificación de digeneos se suele basar en la morfología de los adultos, siendo la dilucidación de sus ciclos vitales una tarea difícil. Por lo tanto, el uso de herramientas moleculares es de gran ayuda para la identificación de estados larvales. Los estudios de Jousson y otros autores (Jousson y col. 1999, 2000, Jousson & Bartoli 2000, 2001, Bartoli & Jousson 2003) aportaron 17 secuencias de opecoélidos adultos encontrados en peces del Mediterráneo con los que comparar e identificar estados larvales de esta familia. La detallada descripción morfológica junto con los análisis moleculares que se han llevado a cabo, han permitido la identificación de dos opecoélidos que infectan al caracol *G. adansonii* como primer hospedador intermediario como *Macvicaria obovata* (Molin, 1859) (morfotipo pequeño) y *Cainocreadium labracis* (Dujardin, 1845) (morfotipo grande). Además, la metacercaria de *C. neritea* se identificó como *M. obovata*, por lo que también sabemos que este parásito especie utiliza a este gasterópodo como segundo hospedador intermediario.

Se recopilaron datos de otras cercarias cotilocercas descritas en el Mediterráneo y las costas del Atlántico nororiental, para comparar las principales características. Las cercarias de *C. labracis* diferían del resto al presentar glándulas de penetración mucho mayores, coincidiendo además con la descripción de Maillard (1971) y Dufour & Maillard (1974). Aunque el número de glándulas de penetración de *M. obovata* coincide con las de *Cercaria linearis* Lespès, 1857, exhibe una variación mayor en el límite superior de algunas medidas morfométricas, por lo que harían falta análisis moleculares para confirmar la coespecificidad.

Este trabajo ha permitido dilucidar el ciclo vital de *M. obovata*, ya que su segundo hospedador intermediario era hasta ahora desconocido. Hasta el momento, sólo se conocía el gasterópodo *Calliostoma striatum* como primer hospedador intermediario de *C. labracis,* por lo que se han aportado evidencias moleculares para una segunda especie de molusco que actúa como primer hospedador intermediario. Asimismo, se han proporcionado descripciones morfológicas detalladas de los dos estados larvales, siendo además la primera descripción de la cercaria de *M. obovata*.

0.5. Mismo hospedador, misma laguna, diferentes vías de transmisión: efectos de los factores exógenos en la emergencia de las larvas de dos parásitos digeneos marinos

0.5.1. Introducción

Las lagunas representan sistemas típicos de hábitats costeros, con pequeños cambios en el nivel de agua. Dada su naturaleza confinada y de sus aguas someras, son hábitats con condiciones propicias para la transmisión de parásitos trematodos. Sin embargo, las lagunas sufren cambios ambientales que afectan sobre todo a los estados de vida libre de los trematodos digeneos. Durante la búsqueda del segundo hospedador intermediario, las cercarias están a merced de los cambios de los factores abióticos. Las estrategias de transmisión adoptadas por las cercarias deben asegurar la sincronización temporal y espacial de los hospedadores intermediarios para aumentar la probabilidad de éxito del ciclo vital (Combes *y col.* 1994, 2002, Pietrock & Marcogliese 2003).

A pesar de la importancia de las cercarias en los ecosistemas marinos, se han llevado a cabo relativamente pocos estudios sobre el efecto de los factores abióticos en la emergencia de éstas desde el primer hospedador intermediario. Por eso, en este estudio se han realizado experimentos para saber el efecto que tienen los cambios de factores abióticos como la temperatura, la salinidad, el fotoperiodo y el nivel del agua. Para eso se han usado las dos cercarias que emergen desde el molusco *G.adansonii* en una laguna del Mediterráneo Occidental: *Cainocreadium labracis* y *Macvicaria obovata*. Ambas cercarias tienen una cola cotilocerca en forma de copa que les permite adherirse a una superficie y esperar a infectar al siguiente hospedador de manera pasiva; peces bentónicos (*Gobius niger* L.; ver Maillard 1971) en el caso de *C. labracis* y caracoles (*Cyclope neritea* (L.); ver Born-Torrijos *y col.* 2012) en el caso de *M. obovata*. Dado que comparten el primer hospedador intermediario y la misma estrategia de búsqueda del siguiente hospedador, ambas cercarias deberían exhibir respuestas similares en cuanto al efecto de los cambios de factores abióticos sobre su emergencia desde *G. adansonii*. De manera contraria, respuestas diferentes indicarían la influencia de factores endógenos (por ejemplo, el agotamiento de sus limitadas reservas energéticas) asociados a las vías de transmisión de los parásitos.

0.5.2. Materiales y métodos

Se recogieron un total de 514 *G. adansonii* en la primavera del año 2010 en las aguas someras de la laguna "Els Alfacs" (Delta del Ebro). Esta laguna semicerrada está conectada con el Mar Mediterráneo, y tiene una profundidad máxima de 4 metros y una masa de agua de aproximadamente 200 millones de litros cúbicos. Además, está caracterizada por una gran heterogeneidad de los factores ambientales: la superficie de la laguna y las áreas someras sufren cambios diarios de temperatura y salinidad, causados por los cambios en el nivel del agua, la evaporación, las lluvias o las descargas de agua dulce desde los canales de drenaje de los arrozales.

Después de mantener los caracoles durante 24h en acuarios en la Unidad de Zoología se comenzó el experimento. Para identificar a los caracoles infectados, se les mantuvo durante 24h a 25ºC y un fotoperiodo de 12h luz: 12h oscuridad. Después de la emergencia de cercarias desde los *G. adansonii* infectados, se llevó a cabo la identificación de las dos especies de cercarias (Born-Torrijos *y col.* 2012). Los caracoles infectados se repartieron en placas individuales para el recuento individualizado de las cercarias emergidas de cada caracol cada 24h. Las condiciones control fueron 20ºC, nivel de agua alto, 14h luz:10h oscuridad, y se siguieron los siguientes patrones para las condiciones experimentales: descenso y aumento de temperatura, entre 25ºC y 15ºC (cambios de 5ºC diarios); descenso de salinidad desde 45psu a 35psu y después 25psu; nivel de agua de 3ml (sin cubrir al caracol completamente) y 6ml (cubriendo el caracol completamente); por último, mantenimiento de los caracoles con un fotoperiodo de 12h luz: 12h oscuridad y posteriormente de 15h luz: 9h oscuridad. Estas condiciones se eligieron en base a los cambios naturales que se pueden dar en el hábitat intermareal de una laguna. Las diferencias en el número de cercarias emitidas durante 24h en cada condición experimental se evaluaron con modelos lineales generalizados (GLM) de medidas repetidas ANOVA (RM-ANOVA) (STATISTICA 7.0 (StatSoft, Inc. 1984–2004)).

0.5.3. Resultados

Las dos especies, *C. labracis* y *M. obovata,* alcanzaron un máximo de 318 y 1145 cercarias por caracol y día a 20ºC, respectivamente, aunque, de manera general, las dos especies emitieron mayor número de cercarias en temperaturas altas. El patrón de respuesta de ambas cercarias fue diferente dependiendo de la dirección del gradiente de temperatura (aumento o descenso). Mientras que ambas especies mostraron una emergencia

disminuida con temperatura descendiente, cuando ésta aumentó, *C. labracis* no experimentó cambios significativos, mientras que *M. obovata* mostró un aumento brusco de cercarias emitidas en torno a los 15ºC. La emergencia diaria alcanzó un máximo de 506 cercarias de *C. labracis* por caracol y día en la salinidad más alta (45 psu), y 660 cercarias de *M. obovata* en la salinidad más baja (25 psu). La emergencia de cercarias de *C. labracis* descendió notablemente al bajar la salinidad, mientras que no se observaron diferencias en *M. obovata*. Aunque la emergencia de *C. labracis* fue mayor en el nivel de agua alto (756 cercarias por caracol y día), esta diferencia no resultó significativa respecto al número emitido durante el nivel de agua bajo. Por el contrario, *M. obovata* emergió significativamente en mayor número durante el nivel de agua bajo (832 cercarias por caracol y día). La respuesta de las dos especies a los cambios de fotoperiodo fue diferente*:* *C. labracis* emergió en mayor número durante el fotoperiodo de 12h luz: 12h oscuridad, sobre todo durante el día, mientras que *M. obovata* no mostró diferencias significativas entre los dos fotoperiodos, aunque su emergencia fue mayor durante los períodos de oscuridad.

0.5.4. Discusión

Hasta donde conocemos, este es el primer estudio en documentar experimentalmente los efectos de las variaciones de los factores ambientales característicos de las lagunas sobre la emergencia de cercarias, y el primero en examinar especies de digeneos con ciclos vitales complejos, pero con limitada capacidad de dispersión. Las dos especies comparten ciertas similitudes, como el primer hospedador intermediario, el hábitat lagunar, la proximidad filogenética y su estrategia de búsqueda del siguiente hospedador intermediario mediante la fijación al sustrato. Sin embargo, y en contra de nuestras expectativas iniciales, los resultados muestran que las cercarias exhiben respuestas diferentes a la variación de los factores ambientales.

Las temperaturas altas suelen afectar al desarrollo de estados embrionarios y por lo tanto a la emergencia y transmisión de parásitos (Galaktionov & Dobrovolskij 2003, Poulin 2006). El descenso de las temperaturas podría acarrear efectos contrarios, sin embargo no ha sido demostrado experimentalmente. En general, la emergencia de cercarias en respuesta a cambios rápidos de temperatura parece estar bajo control exógeno en *M. obovata*, de manera similar a otras especies (Mouritsen & Jensen 1997, Mouritsen 2002, Thieltges & Rick 2006, Koprivnikar & Poulin 2009, Studer *y col.* 2010, Prinz *y col.* 2011). La falta de respuesta de *C. labracis* al aumento de la temperatura podría deberse al

agotamiento de las cercarias maduras listas para emerger. Estudios previos han demostrado la tolerancia de cercarias marinas a un rango amplio de salinidades dependiendo de la especie (Stunkard & Shaw 1931, Prokofiev 1999, Mouritsen 2002). *Cainocreadium labracis* emergió en menor número con salinidades bajas, mostrando así un control exógeno y su adaptación a niveles altos de salinidad, mientras que *M. obovata*, al no mostrar cambios significativos, parece mejor adaptada a la salinidad variable de la laguna, en especial a las salinidades bajas alrededor de zonas de afluencia de agua dulce.

Dada su estrategia de las cercarias de esperar fijadas al sustrato hasta el contacto con su siguiente hospedador intermediario, la dispersión de ambas especies no depende de los cambios en el nivel del agua. De esta manera, *C. labracis* no mostró cambios significativos con los cambios en el nivel del agua, probablemente debido a que su siguiente hospedador intermediario son peces bentónicos que permanecen durante todo el año en la laguna, como los góbidos. En el caso de *M. obovata*, el siguiente hospedador intermediario es el caracol *C. neritea*, que permanece al bajar el nivel del agua, por lo que la concentración de cercarias en un volumen de agua pequeño favorece el encuentro con estos caracoles. Ambas cercarias mostraron ritmos circadianos de emergencia con control endógeno, pero tasas de emergencia diferentes durante los períodos de luz y oscuridad. La mayor emergencia de *C. labracis* durante el día está relacionado con la mayor actividad de los peces, mientras que *M. obovata* emerge más durante la noche, cuando los caracoles *C. neritea* son más activos.

Por lo tanto, este estudio ha demostrado patrones contrarios en la emergencia de las dos cercarias dependiendo de los cambios de factores exógenos simulando las condiciones ambientales de las lagunas. Las cercarias de *M. obovata* exhibieron un aumento de las tasas de emergencia con temperatura elevada, disminución de la salinidad y nivel de agua bajo, mientras que la emergencia de las larvas de *C. labracis* no se vio afectada por la variación de la temperatura ni el nivel de agua, y mostró una disminución de su emergencia a niveles bajos de salinidad. Estos resultados muestran la complejidad de los patrones bajo control exógeno que influyen tanto en la transmisión del parásito como en su abundancia. Asimismo, los diferentes ritmos de emergencia de cercarias indican un control endógeno asociado a las diferentes vías de transmisión de estos dos opecoélidos.

0.6. Estimación de la prevalencia de trematodos en caracoles hospedadores usando una PCR dúplex de un solo paso: ¿cuánto subestima la emergencia de cercarias las tasas de infección?

0.6.1. Introducción

Las comunidades de trematodos normalmente abarcan diferentes especies que explotan una misma población de caracoles, con dos o más especies coexistiendo en el mismo hospedador individual (Sousa 1993, Kuris & Lafferty 1994). La prevalencia de infecciones múltiples es normalmente baja, sugiriendo que pueden darse mecanismos de competencia interespecífica que limiten la coexistencia de las especies en un mismo hospedador. Otros mecanismos, como la supresión de los mecanismos de resistencia del caracol (Sousa 1992) o su fisiología pueden impedir o predisponer a algunos moluscos a ser infectados (Ewers 1960). Por lo tanto, cualquier inferencia sobre las interacciones interespecíficas o la estructura de las comunidades de trematodos en caracoles hospedadores depende de métodos precisos para detectar infecciones en caracoles.

Uno de los métodos más habituales para la detección de infecciones larvales en el primer hospedador intermediario es la emergencia de cercarias. Los caracoles se colocan en pocillos individuales y se incuban con una iluminación y temperatura constantes; después de la emergencia de cercarias, éstas se identifican y se calcula su prevalencia. Para aumentar la precisión, algunos autores además diseccionan los caracoles (Gambino 1959, Studer & Poulin 2012b). Sin embargo, este método clásico ha sido calificado como impreciso por varios autores (DeCoursey & Vernberg 1974, Curtis & Hubbard 1990, Cucher *y col.* 2006, Martínez-Ibeas *y col.* 2011, Studer & Poulin 2012b), ya que cuando se han comparado prevalencias obtenidas por emergencia de cercarias y por disección de los caracoles, las primeras han resultado estar subestimadas (Miller & Northup 1926, Cucher *y col.* 2006, Martínez-Ibeas *y col.* 2011, Lambert *y col.* 2012), incluyendo las infecciones múltiples e infecciones inmaduras (Stunkard & Hinchliffe 1952, Vernberg *y col*. 1969, Curtis & Hubbard 1990).

Para cuantificar la subestimación de la prevalencia de trematodos, en este estudio se han utilizado dos sistemas de parásito-hospedador con infecciones dobles, comparando, por primera vez, la prevalencia determinada por emergencia de cercarias con la aquella obtenida por métodos moleculares. Se desarrolló un método de PCR dúplex capaz de diferenciar entre infecciones simples (una de las especies) y dobles (dos especies en la

misma muestra). Los dos sistemas utilizados son: (i) Muestras del Ebro (España), con las especies *Cainocreadium labracis* y *Macvicaria obovata* (Opecoelidae), que se desarrollan en esporocistos en la glándula digestiva y gónadas del caracol *Gibbula adansonii* (Trochidae). La prevalencia de *C. labracis* en el Delta del Ebro varía entre 17,6 y 30,8%, mientras que la de *M. obovata* varía de 0,9% a 23,1% (Born-Torrijos *y col.* 2012). (ii) Muestras de Otago (Nueva Zelanda), con las especies *Maritrema novaezealandensis* (Microphallidae) y *Philophthalmus* sp. (Philophthalmidae), que infectan al caracol *Zeacumantus subcarinatus* (Batillariidae). La prevalencia de *M. novaezealandensis,* que desarrolla esporocistos en las gónadas, varía entre 5 y 80% (Studer & Poulin 2012b, Martorelli *y col.* 2004, Keeney *y col.* 2008), mientras que *Philophthalmus* sp., que desarrolla redias en la glándula digestiva y gónadas, varía de menos de 5% hasta casi 30% de prevalencia (Studer & Poulin 2012b). La prevalencia de infecciones dobles de normalmente inferior a 2% (Studer & Poulin 2012b).

0.6.2. Materiales y métodos

Se recolectaron *G. adansonii* en la laguna de "Els Alfacs" (Delta del Ebro, España) y *Z. subcarinatus* en las bahías de "Lower Portobello" y "Oyster" (Otago, Nueva Zelanda). Se incubaron los caracoles con las condiciones propicias para la emergencia de cercarias y se identificaron para calcular su prevalencia, obtenida por "el método clásico". Estos mismos caracoles se diseccionaron y se fijaron la glándula digestiva y las gónadas en etanol al 100% para el segundo método de detección, el método "PCR dúplex".

Para el diseño de primers específicos para cada especie se utilizaron secuencias de las regiones de ITS. En el caso de *C. labracis* [Genbank JQ694148] y *M. obovata* [GenBank JQ694145] se utilizaron las publicadas en Born-Torrijos *y col.* (2012), mientras que para las dos especies de cercarias de Otago se produjeron secuencias nuevas. Después de la extracción de ADN mediante un protocolo con Chelex, se llevaron a cabo reacciones en cadena de la polimerasa (PCR) con los primers 3S (Morgan & Blair 2008) e ITS2.2 (Cribb *y col.* 1998). Las muestras de ADN se secuenciaron y se alinearon con Bioedit v7.0.5 (©1997–2005) (Hall 1999), y más tarde se les asignó un número de acceso de GenBank [*M. novaezealandensis* KJ540203 y *Philophthalmus* sp. KJ540204]. Se diseñaron primers específicos para las cuatro especies de cercaria, comparando sus secuencias con secuencias de otros taxones cercanos, para así identificar regiones variables que permitan la identificación específica y a la vez se diferencien de otras especies cercanas. Con el programa Primer3 (Rozen & Skaletsky 2000) se eligieron las características para los primers, tanto los iniciadores (“forward”) como los inversos (“reverse”). Los primers se

diseñaron para generar secuencias de diferentes longitudes para cada especie. Por lo tanto, después de hacer la PCR dúplex con los 4 primers correspondientes a las muestras del Ebro o Otago (2 primers "forward" más 2 "reverse"), las bandas permitieron diferenciar las dos especies visualizando el gel de agarosa hecho, diferenciando las infecciones simples (solamente muestran la banda correspondiente a la infección con una especie) de las dobles (se observan dos bandas de dos tamaños diferentes, correspondientes a las dos especies).

Los resultados de las prevalencias obtenidas mediante el método clásico y el método de la PCR dúplex se transformaron en tablas bidimensionales de contingencia (2 x 2) para evaluar estadísticamente las diferencias con un test McNemar Chi-cuadrado para proporciones emparejadas ($\chi 2$, P-valor <0,05) (R, paquete stats, versión 2.15.0) (Agresti 1990). Además, se calculó el Coeficiente kappa de Cohen ("Cohen's kappa statistic") (K, P-valor <0,05) para evaluar el grado de concordancia entre ambas técnicas (R, paquete FMSB, versióno.4.1) (Nakazawa 2013).

0.6.3. Resultados

La tendencia generalizada en los dos sistemas de parásito-hospedador fue una prevalencia mayor detectada con el método de la PCR dúplex en comparación con el método clásico. Dependiendo del año de muestreo (2011 frente a 2013), se aumentó entre 17,9% y 60,1% la detección del número de caracoles infectados de las muestras del Ebro usando el método molecular. En las muestras de Otago, la diferencia entre prevalencias basadas en el método clásico y PCR dúplex no era tan pronunciada, aunque sí importante, con el 9,9% y el 20,6% más de las infecciones detectadas por PCR, dependiendo del sitio de muestreo (bahías de "Lower Portobello" y "Oyster").

Las estimaciones de la prevalencia de las infecciones fueron significativamente mayores con el método de PCR dúplex en la mayoría de las muestras (7 de 12), siendo el aumento de la detección entre 0,6% y 54,2% para infecciones simples y 2,4% a 9,5% para infecciones dobles. Los valores de Kappa indicaron de manera general una concordancia de razonable a sustancial entre el método clásico y el molecular (22% a 78% de concordancia). Es importante destacar que estos valores fueron más altos para las muestras de Otago en comparación con las del Ebro (más concordancia), y que entre las infecciones dobles había menos concordancia entre métodos en comparación con las infecciones simples.

0.6.4. Discusión

Hasta donde llega nuestro conocimiento, éste es el primer estudio en el que han sido diseñados primers específicos para usar en una PCR dúplex para la evaluación precisa de infecciones simples y dobles con una muestra ciega de tejido de caracol. El porcentaje de muestras de PCR positivos no detectados previamente por emisión de cercarias es alto, especialmente para las infecciones dobles. Las diferencias entre prevalencias obtenidas por el método clásico y de PCR dúplex, estadísticamente comparadas, demuestran una mayor precisión de la detección molecular sobre el método clásico. El método clásico puede pasar por alto infecciones inmaduras, subestimando así la prevalencia verdadera. La diferencia entre los métodos de detección fue mayor en las muestras del Ebro, mostrando también una gran variabilidad entre años (17,9% en 2011 frente al 60,1% en 2013), que podría deberse a infecciones inmaduras de *C. labracis* durante el 2013, no detectadas por el método clásico. La diferencia porcentual entre los dos métodos en las muestras de Otago (9,9% en bahía "Lower Portobello" y 20,6% en bahía "Oyster") destaca las diferentes prevalencias de infección entre los dos sitios de muestreo, como ya se había sugerido con anterioridad (Martorelli *y col.* 2008).

Un hallazgo notable es la mayor detección de infecciones dobles con el método de PCR, probablemente debido a la detección fallida de infecciones inmaduras con el método clásico, ya que las cercarias no emergen del caracol y sólo se contabilizan con el método molecular. Además, la PCR dúplex evita errores de identificación en el caso de especies morfológicamente similares que infectan al mismo hospedador, como es el caso de las dos cercarias de las muestras del Ebro. Por el contrario, la concordancia entre los dos métodos en las muestras de Otago es mayor, lo que confirma que se trata de especies fáciles de diferenciar. Sin embargo, esta concordancia no es perfecta, por lo que se demuestra que incluso en el caso de especies morfológicamente diferentes, el método de la PCR dúplex muestra una mayor detección. No obstante, la interpretación de la mayor sensibilidad en el caso infecciones dobles debe hacerse con cautela, debido a posibles infecciones que se pierden después de un breve período de coexistencia en el caracol, por ejemplo a raíz de competencia interespecífica.

Este estudio basado en una PCR dúplex para la detección de regiones ITS específicas, con el objetivo de calcular prevalencias 'verdaderas', puede ser extrapolado a otros sistemas, incluso aquellos que incluyen más de dos especies de parásitos. Este método podría ser considerado por lo tanto como una herramienta adicional para

determinar con gran exactitud la prevalencia de estados larvarios. Además, los resultados presentados nos permiten una mayor comprensión de la estructura de la comunidad de larvas de trematodos en caracoles, motivando así la revisión de anteriores conclusiones basadas en estudios de emisión de cercarias, en relación con la competencia interespecífica en infecciones mixtas, parámetros poblacionales de parásitos (prevalencia e intensidad en caracoles infectados) o fluctuaciones estacionales de larvas en caracoles.

0.7. Patrones de maduración de trematodos en un caracol hospedador migratorio: ¿Qué sucede durante su permanencia en el hábitat intermareal en una laguna mediterránea?

0.7.1. Introducción

Los gasterópodos son un componente importante de las lagunas europeas, donde actúan como primer hospedador intermediario de muchas especies de trematodos (Torchin *y col.* 2002, Bartoli & Boudouresque 2007, Bartoli & Gibson 2007). Durante el ciclo complejo de los trematodos digeneos, el primer hospedador intermediario, normalmente un gasterópodo, alberga esporocistos en cuyo interior, mediante reproducción asexual, se desarrolla un gran número de cercarias. Éstas, una vez maduras, emergen del gasterópodo para infectar, en un plazo corto de tiempo (de horas a días), al segundo hospedador intermediario, normalmente otro molusco o un pez, donde formarán una metacercaria antes de ser consumidos por el hospedador definitivo.

Las vías de transmisión y los factores que afectan a la emergencia y dispersión de las cercarias se han estudiado en profundidad, al igual que la heterogeneidad espacial y estacional de las cercarias (Lang & Dennis 1976, Hughes & Answer 1982, Curtis 1987, Bao-Zhen *y col.* 1997, McCurdy *y col.* 2000, Miura & Chiba 2007, Thieltges 2007, Born-Torrijos *y col.* 2014a; Pietrock & Marcogliese 2003 y Thieltges *y col.* 2008 para revisiones). Sin embargo, aunque sí que existen descripciones detalladas de las generaciones partenogenéticas dentro de esporocistos (ej. Touassem & Théron 1986, Dobrovolskij *y col.* 2000), la información sobre la variación de las proporciones de estados de desarrollo en el tiempo es escasa (Korniychuk 2008, Fermer *y col*. 2010, Prinz *y col.* 2010), a pesar de su importancia en el contexto de la transmisión de los parásitos.

Cada asociación parásito-hospedador se ve afectada por las peculiaridades ecológicas y fisiológicas de cada especie (Gorbushin & Levakin 1999). El parásito puede

ocupar o incluso destruir el tejido reproductivo del hospedador, castrándolo, por lo que la energía destinada a reproducción podría desviarse hacia el crecimiento somático del caracol (Baudoin 1975, Poulin 2007) o en beneficio propio del parásito, afectando así su rendimiento reproductivo o desarrollo (Cort *y col.* 1954, Baudoin 1975, Gérard *y col.* 1993, Poulin 2007).

Para entender los patrones y ciclos de maduración de las larvas en un hospedador intermediario en relación a su transmisión a los siguientes hospedadores, se analizaron los estados larvales y patrones de maduración de *Macvicaria obovata* y *Cainocreadium labracis* (Opecoelidae) en el gasterópodo *Gibbula adansonii* (Trochidae) durante la primavera, período en el que éste se encuentra en el hábitat intermareal de la laguna "Els Alfacs" en el Delta del Ebro. Ambas cercarias poseen una cola cotilocerca con la que se fijan al sustrato para esperar a que el segundo hospedador intermediario se acerque o deprede sobre ellas para infectarlo (estrategia "sit and wait", ver Born-Torrijos *y col.* 2014 a). Estos segundos hospedadores intermediarios serían pequeños peces bentónicos en el caso de *C. labracis* y gasterópodos en el caso de *M. obovata*. Dada la baja movilidad de las cercarias, el primer y segundo hospedador intermediario deben estar sincronizados espacial y temporalmente, siendo a veces esencial el desplazamiento del caracol para optimizar el éxito de la transmisión del parásito. Las migraciones relacionadas con el desove son comunes en los gasterópodos de la familia Trochidae (Williams 1965, Underwood 1973, Kendall y col. 1987), al igual que la manipulación del caracol por parte de trematodos para modificar su microhábitat, aumentando así sus probabilidades de transmisión (Curtis 1987, 1993, Lowenberger & Rau 1994, McCarthy *y col.* 2000, McCurdy *y col.* 2000, Miura & Chiba 2007, Averbuj & Cremonte 2010); esto sugiere una posible relación entre la maduración de las larvas y la residencia del caracol en el hábitat costero durante su migración. Asimismo, se estudió la relación entre el tamaño del hospedador y de los esporocistos y el número total de estados larvales, dado su importante papel en el éxito reproductivo de los trematodos. Los resultados obtenidos se discutirán en el contexto de la coexistencia espacial y temporal del primer y segundo hospedador intermediario para la transmisión exitosa de estas cercarias sésiles.

0.7.2. Material y métodos

Se recolectaron caracoles *G. adansonii* en el hábitat intermareal de la laguna "Els Alfacs" del Delta del Ebro, con muestreos mensuales en los años 2011 y 2013, recogiendo hasta 600

G. adansonii (8,02 ± 1,26 mm (5,73-11,83)) durante marzo, abril y mayo, meses en los que los caracoles abundan. Durante el resto del año sólo se encontraron ocasionalmente caracoles (<3mm de altura) sin emergencia de cercarias, por lo que probablemente pertenecieran a la nueva generación.

Se diseccionaron caracoles escogidos al azar para observar la glándula digestiva y las gónadas bajo la lupa, midiendo antes la altura de la concha (0,1 mm de error). Sólo se incluyeron infecciones simples, confirmadas por el método descrito en Born-Torrijos *y col.* (2014b), y dependiendo de la carga parasitaria, se seleccionaron aleatoriamente de 5 a 25 esporocistos. Bajo el microscopio se contabilizó el número de estados de desarrollo que contenían los esporocistos, siguiendo las categorías propuestas por Prinz *y col.* (2010) y Fermer *y col.* (2010), es decir: (1) bolas germinales, (2) embriones de cercarias en diferentes estados de desarrollo, y (3) cercarias maduras Se midió la longitud de esporocistos de una submuestra, usando imágenes con el programa ImageJ (v 1.44, National Institutes of Health, USA).

La maduración está determinada por el embrión más avanzado (Rakotondravao *y col.* 1992), por lo que para tener una idea intuitiva que resuma la madurez de cada esporocisto, ponderando los diferentes estados de desarrollo, se calculó un índice de maduración (IM = ((No. Cercarias maduras x 3) + (No. Embriones de cercaria x 2) + (No. Bolas germinales x 1)) / (No. total de estados larvales en el esporocisto). Se evaluó el efecto de la infección sobre el tamaño del caracol, comparando caracoles infectados (*C. labracis* y *M. obovata*) y no infectados mediante un modelo lineal. Después, se usaron una serie de modelos (Capítulo 7, Fig. 1, ii-x): modelos lineales para comprobar si existen diferencias interespecíficas en el número total de estados larvales dependiendo del tamaño del caracol o del esporocisto (como indicadores de la capacidad reproductiva de cada especie); modelos lineales separados por especies para ver el efecto del tamaño del esporocisto sobre el IM, y el efecto de los meses sobre el tamaño del caracol o la longitud del esporocisto. Por último se hicieron modelos lineales mixtos para ver el efecto del mes sobre el IM, el número de estados maduros, el número de embriones de cercarias o el número de bolas germinales. La identidad del caracol se usó como factor aleatorio, usando medidas repetidas de caracoles individuales. En los análisis en los que se hicieron comparaciones entre meses, se llevaron a cabo comparaciones múltiples de Tukey (R, paquetes lme4 y multcomp, Hothorn *y col.* 2008, Bates *y col.* 2014).

0.7.3. Resultados

Después de la confirmación molecular del estatus de infección, se incluyeron 36 caracoles infectados con *C. labracis* (407 esporocistos) y 15 con *M. obovata* (202 esporocistos). La mayoría de los caracoles infectados (90,2%, 46/51) contenían los tres estados de desarrollo.

El tamaño medio de caracoles infectados con *M. obovata* era un 14,6% significativamente mayor que aquellos infectados con *C. labracis* (P-valor<0,01), y un 15% mayor que los caracoles sin infección (P-valor=0,49). El tamaño del caracol y la longitud de los esporocistos mostraron una correlación positiva con el número total de estados de *M. obovata*, actuando así como indicadores de su capacidad reproductiva (P-valor=0,01, P-valor=0,03, respectivamente). Esta relación no fue significativa para *C. labracis* (P-valor>0,05). Los modelos lineales no identificaron ninguna variación, en ninguna de las especies, del IM con la longitud de los esporocistos, ni variaciones entre meses en el tamaño del caracol o de los esporocistos (P-valor>0,05). Los modelos lineales mixtos revelaron que el IM de *C. labracis* y *M. obovata* aumentó significativamente en abril (P-valor=0,02 en ambos casos). Sin embargo, sólo el número de estados maduros de *C. labracis* aumentó en abril (P-valor=0,046). Los embriones de cercarias y las bolas germinales no mostraron diferencias significativas entre meses para ninguna de las dos especies. La proporción de la variación explicada por el efecto del caracol en los modelos lineales mixtos fluctuaba entre 13 y 67%.

0.7.4. Discusión

Las condiciones ambientales, al igual que el parasitismo, afectan al crecimiento del caracol, siendo cada sistema de parásito-hospedador diferente (Fernandez & Esch 1991, Gorbushin & Levakin 1999). El mayor tamaño de los caracoles infectados con *M. obovata* en comparación con caracoles no infectados, sugiere que después de la castración del hospedador, la energía antes destinada a su reproducción se destina al crecimiento somático del caracol, como pasa en otras especies (Baudoin 1975, Sousa 1983, Poulin 2007). Por el contrario, los caracoles infectados con *C. labracis* no se diferencian en tamaño de los no infectados, por lo que podrían destinar esa energía a otras funciones, demostrando que el reparto de la energía es diferente en cada especie (Sousa 1983, Fernandez & Esch 1991, Gorbushin & Levakin 1999). El espacio y alimento disponible en el caracol limita la capacidad reproductiva de los esporocistos (Cort *y col*. 1954, Gérard *y col*. 1993, Poulin 2007), por lo que el uso de la energía está también relacionado con el éxito reproductivo

del parásito. El tamaño del esporocisto, y del caracol especialmente, está relacionado con la capacidad reproductiva de *M. obovata*, por lo que esta especie aprovecha los mayores recursos de los caracoles grandes en su propio beneficio. Sin embargo, esta relación no se encuentra en *C. labracis*, cuyos esporocistos y cercarias son mayores que los de *M. obovata* (Born-Torrijos *y col*. 2012), por lo que su desarrollo es más costoso. Al infectar caracoles más pequeños sufre una constricción espacial que limita su capacidad reproductiva (Rakotondravao *y col.* 1992, McCarthy *y col.* 2002).

Se esperaba una relación entre el IM y la longitud de los esporocistos, dado que los esporocistos más grandes y maduros suelen contener cercarias maduras (Byrd 1954, Fermer *y col*. 2010). Sin embargo, esta relación no se detectó, probablemente por un efecto de "vaciado" del esporocisto después de la emergencia de cercarias, así como posiblemente por la simplicidad de la fórmula empleada para calcular el IM. Además, la alta variabilidad de los estados de desarrollo y el IM entre diferentes esporocistos podría provocar que algunos patrones no sean detectados.

El tamaño de los caracoles no varía entre meses, por lo que se trata de individuos adultos que han sido infectados repetidas veces, desarrollando así infecciones maduras. En especies donde no se ha encontrado variación temporal en el tamaño del esporocisto o en el número de estados de desarrollo, se ha sugerido la existencia de un período prepatente de infección corto, lo que explicaría la ausencia de diferencias entre meses en el tamaño de los esporocistos de las especies estudiadas. La mayoría de caracoles (90,2%, 46/51) contenía los tres estados de desarrollo simultáneamente, y ningún esporocisto contenía únicamente bolas germinativas, apoyando la idea de una maduración rápida de las cercarias. El número de estados maduros de *C. labracis* y *M. obovata*, así como su IM, fueron mayores en abril, y no se detectaron diferencias en el número de cercarias inmaduras ni de bolas germinativas. Esto podría indicar la existencia de un equilibrio entre estados larvales maduros y la formación de nuevos embriones, permitiendo así la producción continuada de cercarias maduras durante la residencia de *G. adansonii* en el hábitat intermareal de la laguna en los meses de primavera.

La eficiencia de este mecanismo debe contemplarse en relación a la estrategia de transmisión de las dos cercarias sésiles, ya que el primer y segundo hospedador intermediario deben coincidir temporal y espacialmente para completar su ciclo vital. Los moluscos intermareales suelen llevar a cabo migraciones con fines reproductores (Fretter & Graham 1962, Williams 1965, Desai 1966, Underwood 1973, Kendall *y col*. 1987). Además,

las aguas someras fuerzan la proximidad de los hospedadores al sedimento, permitiendo que las cercarias les infecten. En este caso, los siguientes hospedadores habitan las aguas someras de lagunas durante los meses de primavera, ya que los peces bénticos pequeños (segundo hospedador intermediario de *C. labracis*) las usan para desovar (Arruda *y col.* 1993, Ribeiro *y col.* 2012, Bouchereau & Guelorget 1998), mientras que el gasterópodo *C. neritea* (segundo hospedador intermediario de *M. obovata*) se encuentra en la parte superior del hábitat intermareal (Koutsoubas *y col.* 2000, Bachelet *y col.* 2004).

Anteriormente, se han observado especímenes infectados con cercarias modificando su distribución vertical para favorecer su transmisión (Curtis 1987, 1990, 1993, Lowenberger & Rau 1994, Miller & Poulin 2001). Los caracoles estudiados conservan su comportamiento migratorio después de su castración o infección con las dos especies, por lo que la búsqueda de un hábitat adecuado para la transmisión de las cercarias podría estar forzada por éstas, siendo un proceso repetitivo y con el objetivo de completar el ciclo vital. Por lo tanto, este trabajo abre las puertas a futuros estudios sobre cambios de comportamiento provocados por parásitos como una adaptación para su transmisión.

0.8. Del bentos a las gaviotas: espectro de hospedadores y estrategias de de transmisión de *Cardiocephaloides longicollis* (Trematoda, Strigeidae) en el Mediterráneo

0.8.1. Introducción

Los parásitos son elementos centrales de las cadenas tróficas acuáticas (Lafferty *y col.* 2006, 2008), ya que juegan un papel importante en la estructura de comunidades del hospedador y afectan a la estructura de la cadena trófica cuando infectan a un alto número de hospedadores (Thompson *y col.* 2005). Sin embargo, la información en este sentido es normalmente escasa (Poulin 2001). En parásitos con vitales complejos, el grado de especialización varía dependiendo del estado del parásito, por lo que se dan estrategias de transmisión específicas para cada especie y para cada estado del trematodo (Sukhdeo & Sukhdeo 2004, Poulin 2007).

El trematodo digeneo *Cardiocephaloides longicollis* (Strigeidae) se encuentra en las costas europeas, y su ciclo vital se completa a través de las cadenas tróficas locales. Los adultos de *C. longicollis* se encuentran en el tracto digestivo del hospedador final (gaviotas), donde se reproducen sexualmente. Los huevos son liberados al agua a través de

las heces, y cuando eclosionan sale un miracidio que infecta al primer hospedador intermediario, los caracoles *Nassarius corniculum* y *N. reticulatus* (Nassariidae). En ellos, se desarrollan esporocistos que contienen cercarias, que emergen del caracol para infectar a los peces. Las cercarias se mantienen en la columna de agua mediante fototactismo positivo para alcanzar a los segundos hospedadores intermediarios e infectarlos (Bartoli & Prévot 1986, Combes *y col.* 1994). Después, las cercarias migran a través de los tejidos del pez y se enquistan en los lóbulos ópticos del cerebro (Prévot & Bartoli 1980), manipulando su comportamiento al influir en su visión y habilidad natatoria (Prévot 1974, Barber & Crompton 1997). Este hecho facilita su transmisión, ya que los peces son más susceptibles a la depredación de las aves marinas (Poulin 2001, Moore 2002, Seppälä *y col.* 2004, Osset *y col.* 2005, Fredensborg & Longoria 2012), incrementando este comportamiento la acumulación de metacercarias en el cerebro (Barber & Crompton 1997, Fredensborg & Longoria 2012). Se han encontrado metacercarias en 7 familias de peces, infectando más frecuentemente a espáridos, blénidos, góbidos y lábridos (Prévot & Bartoli 1980, Bartoli & Prévot 1986, Machkevsky *y col*. 1990, Korniychuk 1997, Zander 2004, Osset *y col.* 2005).Osset *y col.* (2005) sugirió un posible impacto de los descartes en el ciclo vital de C. longicollis, pero no presentaron evidencias. El hecho de que las aves marinas se infecten con *C. longicollis* al consumir peces infectados (Bartoli & Prévot 1986, Fredensborg & Longoria 2012), en zonas con alta actividad pesquera, podría implicar que la transmisión de los parásitos podría verse aumentada si los descartes, sobre los que depredan las aves (Witt *y col.* 1981, Arcos *y col.* 2001, Christel *y col.* 2012) incluyeran a los individuos más parasitados.

A pesar de las citas que existen sobre la aparición y frecuencia de *C. longicollis*, esta información no ha sido resumida con anterioridad, lo que no permite una comprensión holística de las dinámicas de transmisión de este parásito. Por lo tanto, queremos ampliar nuestro conocimiento sobre el ciclo vital de *C. longicollis*, analizando los hospedadores y estrategias de transmisión en todos los niveles, es decir, gasterópodos, peces y aves. Asimismo, se pretende determinar a qué microhábitat se dirigen las cercarias después de emerger del caracol, y determinar el efecto del pez sobre la acumulación de metacercarias, explorando también qué factores antropogénicos podrían aumentar la transmisión de *C. longicollis*.

0.8.2. Material y métodos

Se llevó a cabo un muestreo oportunista de los hospedadores sospechosos de formar parte del ciclo vital de *C. longicollis*. Se recogieron 3351 moluscos (24 especies) en zonas someras del Mediterráneao, se obtuvieron 154 aves (17 especies) de dos centros de recuperación, uno cercano al Delta del Ebro y otro a Valencia; además, se recogieron 158 muestras fecales de la gaviota *Larus michahellis* de la Isla de Benidorm y se muestrearon 2108 peces (25 especies), incluyendo una muestra del teleósteo *Sparus aurata* de una instalación de acuicultura. Todas las muestras se examinaron para encontrar todos los estados de desarrollo de *C. longicollis* (huevos, esporocistos, cercarias, metacercarias y adultos), identificándolos morfológicamente con las descripciones de Prévot & Bartoli (1980) y Dubois (1968). Además, algunos especímenes se analizaron molecularmente con la metodología descrita en Born-Torrijos *y col.* (2012), excepto para la amplificación y secuenciación de la región ITS2 de ADNr, para la que se usaron los primers 3S e ITS2.2 (Morgan & Blair 1995, Cribb *y col.* 1998).

Se calculó la prevalencia de infección, la intensidad media (rango), y la abundancia media (± desviación estándar) (Bush *y col.* 1997). Los datos incluidos de la literatura se obtuvieron después de una búsqueda en Web of Science (ThomsonReuters), completada con una búsqueda bibliográfica manual. Para los análisis estadísticos sólo se incluyeron "valores verdaderos negativos" de prevalencia, es decir, con la prevalencia cero pero existiendo citas previas en las que ese hospedador aparece infectado con *C. longicollis*. Se analizaron los datos mediante modelos generalizados lineales mixtos (GLMM) y modelos lineales mixtos (LMM) si el hábitat del pez (pelágico, demersal o bentónico) o la época de captura tenían un efecto sobre la prevalencia o abundancia de *C. longicollis*. Para acreditar la existencia de patrones congruentes del efecto del hábitat del pez sobre la prevalencia y abundancia de *C. longicollis*, se combinaron los datos de este estudio con los de la literatura, y se analizaron con modelos lineales (LM). La correlación entre la prevalencia e intensidad media se calculó con el coeficiente de correlación de Spearman, incluyendo todas las muestras de peces. Para investigar la relación entre la longitud del pez y la abundancia de *C. longicollis*, se usaron datos de 198 *D. vulgaris* y 196 *D. annularis* estratificados en clases de tamaño, y el efecto del número de metacercarias por pez se analizó con modelos lineales generalizados (GLM binomial negativo). Además, se calculó el grado de agregación con el índice de discrepancia (Poulin 1993) (Quantitative Parasitology, QP web, impulsado por R, versión 1.0.9) (Rozsa *y col.* 2000, Reiczigel *y col.*

2013). Los datos de aves se analizaron para comprobar la relación entre la prevalencia y la abundancia media de *C. longicollis*, incluyendo también datos de otros estudios. El efecto de la estacionalidad sobre el número de huevos de *C. longicollis* en las muestras fecales se analizó con un GLM binomial negativo. Todos los análisis se realizaron con diferentes paquetes de R (R Development Core Team, versión 3.0.1).

0.8.3. Resultados

La prevalencia de *C. longicollis* en gasterópodos nasáridos es relativamente baja, con una prevalencia baja en comparación con otros niveles, añadiéndose sólo una nueva cita a nivel del primer hospedador intermediario (*C. neritea*), indicando así un grado de especificidad alto. A nivel de segundo hospedador intermediario, *C. longicollis* tiene un espectro de hospedadores mucho más amplio: se han añadido 12 nuevas citas de peces pertenecientes a las familias Sparidae, Labriidae, Gobiidae, Pomacentridae y Serranidae, siendo las dos últimas nuevas citas a nivel de familia. Además, se han encontrado metacercarias con una prevalencia de 53,9% en *S. aurata* de instalaciones de acuicultura. Las infecciones encontradas en tres especies de láridos, hospedadores finales, sobrepasan el 50% de prevalencia, siendo hasta 22 veces mayor que en citas anteriores. Todos los especímenes de *C. longicollis* se identificaron morfológicamente, confirmándose su identidad mediante métodos moleculares, encontrando un 100% de coincidencia con secuencias de la región 28S disponibles en GenBank (AY222171). Las secuencias de la región ITS de ADNr de huevos y adultos mostraron una coincidencia entre 99,7% y 100%, y se depositaron en Genbank (XXXX, XXXX).

Los GLMM mostraron que la prevalencia general de *C. longicollis* es mayor en otoño e invierno, en comparación con primavera (P-valor<0,001), mientras que no mostró diferencias significativas entre hábitats (P-valor>0,05), posiblemente debido a los valores heterogéneos en los peces demersales y pelágicos y una única muestra bentónica. Sin embargo, la abundancia media mostraba niveles mayores en peces bentónicos en comparación con los demersales y pelágicos (LMM). Enfocándonos en el efecto del hábitat, sin tener en cuenta el efecto del pez, los peces demersales y bentónicos mostraron mayor prevalencia y abundancia media de *C. longicollis* en comparación con los pelágicos, sin diferencias entre ellos. Incluyendo datos de publicaciones previas, la abundancia media es 4 veces mayor en peces demersales, y 23 veces mayor en bentónicos que en pelágicos. Se observó una correlación positiva entre la prevalencia e intensidad media de *C. longicollis* en todas las muestras de peces.

La prevalencia de *C. longicollis* mostró un aumento progresivo hasta 93-100%, en las dos clases de tamaño más altas de *D. vulgaris*. En estas clases, la intensidad y la abundancia disminuyeron con respecto a las clases de tamaños intermedios, donde estos valores eran máximos (120 a 148mm, 33-38 metacercarias/pez), y la agregación decreció notablemente. En *D. annularis* se observó un aumento de la intensidad y abundancia alcanzando los valores máximos en las dos clases de tamaño más altas (140 a 162 mm, 73 metacercarias/pez), a la vez que disminuyó también el nivel de agregación. Los GLM mostraron que el número de metacercarias en las dos especies de peces era mayor en las clases de tamaño más altas ($P<0,01$).

Sólo se encontraron infecciones en láridos, mostrando *L. michahellis*, *L. argentatus* y *L. andouinii* valores mucho más altos que los encontrados previamente, con 66,7%, 69,2% y 50% respectivamente. No se encontró ninguna relación entre la prevalencia y la intensidad en las muestras de aves (incluyendo datos de la literatura). La prevalencia de huevos de *C. longicollis* en las muestras fecales fue mayor en primavera (42,9%), seguido de verano (35%), otoño (27,5%) e invierno (22,2%). Sin embargo, aunque las diferencias estacionales no resultaron significativas la mayor intensidad (número de huevos en una muestra) se dio en invierno (150,2 ± 534,0), seguido de primavera (71,9 ± 170,7).

0.8.4. Discusión

Para saber el grado en que un parásito influye en el ecosistema y las redes tróficas, es esencial conocer el espectro completo de hospedadores y la abundancia del parásito en ellos. En este estudio, se han investigado qué hospedadores forman parte del ciclo vital de *C. longicollis*, demostrando que afecta a un amplio espectro de segundos hospedadores intermediarios. Además, las tasas de infección en hospedadores definitivos es mayor que las descritas con anterioridad, por lo que se quiere estudiar qué factores podrían ser responsables del aumento detectado en la prevalencia de *C. longicollis*, y por lo tanto de su transmisión.

Debido a la longevidad de las metacercarias dentro del hospedador (incluso años, Chubb 1979), no se esperaba observar estacionalidad en la prevalencia, siendo consumidas por las gaviotas durante todo el año. Éstas, al ser organismos homeotérmicos, permiten el desarrollo continuo de los adultos, como demuestra la falta de cambios estacionales en la prevalencia de huevos en las muestras fecales de *L. michahellis*. La detección de una abundancia de metacercarias mayor en invierno y otoño se puede deber tanto a un

aumento de las infecciones de peces después del verano, cuando la producción de cercarias aumenta con las temperaturas altas (ver Poulin 2006), como a una mayor depredación de peces en primavera por parte de las gaviotas (Annett & Pierotti 1989), eliminando así a los peces más infectados y provocando una disminución de las tasas de infección.

El comportamiento de las cercarias determina el nicho ecológico donde los peces van a ser infectados, guiándose por la luz para elegir el microhábitat (Bartoli & Prévot 1986, Combes *y col.* 1994, Smith & Cohen 2012). Hasta lo que conocemos, esta es la primera vez que se han comparado niveles de infección de *C. longicollis* entre peces de diferentes hábitats, concluyendo que la abundancia media en peces demersales y bentónicos es entre 4 y 23 veces mayor que en pelágicos, aunque probablemente los peces bentónicos actúen de sumideros al no ser consumidos por las aves. El comportamiento de natación dirigido de las cercarias incrementa la exposición de los peces demersales, por lo que éstos de manera general, mostraron los mayores valores de infección, con hasta 100% de prevalencia de infección. El efecto acumulativo de parásitos con el tamaño del pez se ha estudiado en varias especies (Thomas *y col.* 1995, Rousset *y col.* 1996, Osset *y col.* 2005), caracterizado por un descenso de la agregación de los parásitos que podría indicar que los individuos altamente infectaos son eliminados de la población (mortalidad inducida por los parásitos) (Anderson & Gordon 1982, Rousset *y col.* 1996, Poulin 2001). Esto se observó en los peces *D. annularis* y *D. vulgaris*. La interferencia de estos parásitos en la visión y natación de los peces modifica su comportamiento y aumenta su permanencia en la superficie del agua (Osset *y col.* 2005, Bartoli & Boudouresque 2007), lo que explicaría la eliminación de los individuos más infectados.

La variación en la abundancia de los parásitos en las gaviotas podría ser una consecuencia de los hábitos alimenticios de cada especie (Roca *y col.* 1999). Por ejemplo, *L. michahellis* se alimenta sobre peces mayores que *L. audouinii* (Witt *y col.* 1981), mientras que aves más pequeñas (*Chroicocephalus ridibundus* o charranes, familia Sternidae) lo hacen sobre presas pequeñas (Oro & Ruiz 1997), por lo que la carga parasitaria que les llega es diferente según el tamaño del pez. Cabe destacar que el tamaño de presa sobre el que depredan *L. audouinii*, y sobretodo *L. michahellis*, coincide con el de los peces con mayores niveles de infección, por lo que eliminan de la población a los peces con mayor número de metacercarias, maximizando así también el número de parásitos en el hospedador final. *Larus audouinii* y *L. michahellis* tienen una plasticidad comportamental grande

dependiendo de la disponibilidad de alimento, alimentándose así de peces demersales y bentónicos de los descartes de las pesquerías (Arcos *y col.* 2001, Christel *y col.* 2012). Las altas prevalencias de *C. longicollis* en gaviotas demuestran la ingesta incrementada de hospedadores intermediarios con tasas de infección altas, es decir, peces demersales. Por lo tanto, existe una transmisión favorecida por las actividades pesqueras, que son intensas en las áreas donde se han obtenido las gaviotas; en el Delta del Ebro los descartes se estiman alrededor de un 41% de la pesca (Oro & Ruiz 1997, Coll *y col.* 2008), y mantiene poblaciones grandes de *L. audouinii, L. cachinnans*, *L. michahellis* y *C. ridibundus* (Oro & Ruiz 1997, Arcos *y col.* 2001). Además, muchas de las especies de peces hospedadoras de *C. longicollis* son también frecuentemente descartadas (Sánchez *y col.* 2004, Tzanatos *y col.* 2007), y su tamaño coincide con el tamaño de presa de las gaviotas (Oro & Ruiz 1997, Machias *y col.* 2004). Asimismo, la prevalencia de *C. longicollis* en el gasterópodo *N. reticulatus* (8,3%) en la laguna del Ebro es mayor que las citadas anteriormente, por lo que la transmisión del parásito a través de las heces de las aves que depredan sobre las explotaciones pesqueras adyacentes es exitosa. Los niveles de infección encontrados en peces (40–100%) y gaviotas (27–69%) de áreas lagunares señalan estos hábitats confinados como los más favorables para *C. longicollis*, ya que concentran a los hospedadores intermediarios y finales, facilitando la transmisión del parásito. Además, las instalaciones de acuicultura podrían actuar como potenciadores de la transmisión de *C. longicollis* por la cercanía de los hospedadores al atraer moluscos y aves piscívoras (Edgar *y col.* 2005). De hecho, en este estudio aportamos, por primera vez, datos sobre metacercarias de *C. longicollis* en *S. aurata* cultivada, con una prevalencia de 53,9%., por lo que se espera un impacto aun mayor sobre la transmisión de *C. longicollis*.

Dado el alto número de especies afectadas por *C. longicollis*, este parásito podría tener un efecto fuerte en el flujo energético de la cadena trófica, incluso en condiciones naturales. Sin embargo, la transmisión potenciada por gaviotas por causas antropogénicas (actividades pesqueras y descartes), hace que *C. longicollis* sea un candidato ideal para comparar estructuras de la cadena trófica en áreas naturales con aquellas áreas con un alto impacto pesquero.

0.9. Conclusiones

Como resultado de este estudio se obtuvieron las siguientes conclusiones:

1. Las cercarias y metacercarias que infectan a los gasterópodos *G. adansonii* y *C. neritea*, comunes en las lagunas mediterráneas, han sido descritas y comparadas con las citadas en la bibliografía, siendo identificadas como los estados larvales de *Cainocreadium labracis* (Dujardin, 1845) y *Macvicaria obovata* (Molin, 1859), ambos pertenecientes a la familia Opecoelidae. Las dos cercarias se encontraron con altas prevalencias (22.04% y 23,1%, respectivamente) en *G. adansonii*. También se encontraron metacercarias de *M. obovata* en *C. neritea* (alcanzando 100% de prevalencia), indicando una transmisión exitosa y la compleción del ciclo vital de estas especies en lagunas del Mediterráneo.

2. Las cercarias de *C. labracis* y *M. obovata* son de tipo cotilocerco y morfológicamente muy similares, sin embargo las secuencias ITS de ADNr las atribuyó a dos especies de trematodos diferentes, cuyas secuencias de ADN están publicadas y disponibles en GenBank. De este modo, el hallazgo de la cercaria de *M. obovata* ayudó a dilucidar un nuevo ciclo vital de trematodo, ya que tanto los estados larvales como el segundo hospedador intermediario eran hasta ahora desconocidos.

3. Debido al problema de comparar descripciones morfológicas de estados del ciclo vital de trematodos elaboradas por diferentes autores, frecuentemente realizadas con diferentes métodos de fijación, tinción etc., es importante subrayar la necesidad de basar la caracterización y/o identificación completa de tales estados del ciclo vital en una combinación de tanto su descripción morfológica como de la secuenciación del ADN. Esto es especialmente importante en el caso de estados morfológicamente similares, tales como las cercarias de *M. obovata* y *C. labracis*.

4. El enfoque experimental ha permitido describir los factores abióticos que tienen un impacto sobre la emergencia de las cercarias de *C. labracis* y *M. obovata* desde el caracol *G. adansonii*. Los digeneos, y en particular los estados de vida libre como las cercarias, se encuentran entre los parásitos más expuestos a las variaciones ambientales naturales; sin embargo, existe una ausencia de información sobre la adaptación de las estrategias de transmisión de los trematodos a las variaciones ambientales extremas que se dan en las lagunas. Por lo tanto, este estudio amplía nuestro conocimiento sobre los ritmos específicos de emergencia de digeneos presentes en este hábitat, y, más específicamente, en relación a cercarias con una capacidad de dispersión limitada.

5. Los patrones de emergencia larval de estas dos especies, que comparten el mismo hábitat, el mismo primer hospedador intermediario y la misma estrategia de búsqueda de segundo hospedador, contrastan entre sí, por lo que se demuestran la complejidad de los mecanismos de control exógeno y endógeno que están dirigidos a maximizar el encuentro con los hospedadores específicos. El aumento de la temperatura, el nivel de agua bajo y la oscuridad parecen desencadenar la emergencia de *M. obovata* de modo que las cercarias infecten al molusco nocturno *C. neritea*. Esto ocurre probablemente al bajar el nivel del agua y formarse pequeños charcos que concentran a las cercarias en un volumen de agua pequeño. Por el contrario, el aumento de la emergencia durante el día y principios de verano (fotoperiodo de 12 h luz: 12 h oscuridad) permite a *C. labracis* coincidir con pequeños peces bentónicos, como los góbidos, en zonas poco profundas con alta salinidad.

6. La correcta estimación de la prevalencia de infecciones larvales de trematodo es esencial para entender la estructura de la comunidad parasitaria y la dinámica de las infecciones múltiples. El diseño y la aplicación de una PCR dúplex para dos sistemas parásito-hospedador con infecciones dobles demostraron la mayor precisión de los métodos moleculares para la detección de caracoles infectados en comparación con el método clásico de observación de la emergencia de cercarias. Hasta donde llega nuestro conocimiento, esta es la primera vez que se comparan las prevalencias de infección obtenidas mediante ambas técnicas. La PCR dúplex detectó entre 0,6% y 54,2% más infecciones simples y entre 2,4% y 9,5% más infecciones dobles que la emergencia de cercarias, evidenciando así la presencia de infecciones inmaduras que no se detectan por el método clásico.

7. El diseño de la PCR dúplex ayuda, además, a la identificación de especies morfológicamente similares que co-infectan al mismo individuo, tales como *M. obovata* y *C. labracis*, así como a la resolución de infecciones inmaduras. Esto es de particular importancia en las infecciones dobles, donde la competencia interespecífica puede afectar al desarrollo de las cercarias y su liberación al medio. La prevalencia detectada por PCR dúplex sugiere que las infecciones dobles son más frecuentes de lo esperado, lo que podría apuntar a una menor competencia dentro del hospedador. Sin embargo, las posibles infecciones nuevas de una especie pueden ser eliminadas por otras especies competitivas previamente establecidas, coexistiendo por lo tanto ambas especies sólo brevemente; en este caso la PCR dúplex podría sobrestimar la frecuencia de las infecciones dobles

completamente establecidas, que conducen a la emergencia de cercarias y su transmisión, por lo que estos resultados deben ser interpretados con precaución.

8. Los resultados de esta tesis evidencian la necesidad de revisar conclusiones anteriores basadas en la emergencia de cercarias en relación a la competencia interespecífica, los parámetros poblacionales de los parásitos y las fluctuaciones estacionales de infecciones en hospedadores gasterópodos. Sin embargo, aunque en general proporciona una descripción más exacta de las tasas de infección, el análisis específico de la PCR dúplex no tiene en cuenta la madurez de las infecciones. Por lo tanto, debe ser considerado como una herramienta adicional en una aproximación combinada, tanto de la observación de la emergencia de cercarias como de análisis moleculares, que en conjunto determinarían con la máxima precisión las prevalencias y niveles de madurez de las larvas de trematodos en caracoles.

9. Las peculiaridades ecológicas, fisiológicas y biológicas del sistema parásito-hospedador *C. labracis*/*M. obovata*-*G adansonii* se examinaron más en detalle, determinando los patrones de desarrollo dentro de los esporocistos, localizados en las gónadas y la glándula digestiva del caracol. *Gibbula adansonii* se encuentra en el hábitat intermareal de la laguna del Delta del Ebro sólo de marzo a mayo, debido a la migración hacia estratos superiores relacionada con el desove y asociada a la permanencia en este hábitat intermareal lagunar con fines reproductivos. Debido a la poca movilidad de ambas cercarias, necesitan emerger del caracol en una zona próxima a sus siguientes hospedadores intermediarios. Se encontraron cercarias maduras emergiendo de manera continuada y en gran número durante los meses de primavera, mostrando una madurez de los esporocistos máxima en abril, garantizando así la compleción de ambos ciclos vitales, ya que los segundos hospedadores intermediarios de las dos especies están presentes en el hábitat intermareal durante ese momento.

10. Podría existir un vínculo entre la maduración de las larvas y la residencia del caracol en la parte más superficial del hábitat intermareal, ya que existe una sincronización espacial y temporal de la maduración del parásito y del hospedador. Además, la manipulación del comportamiento del hospedador para aumentar las probabilidades de transmisión de las larvas a los siguientes hospedadores es una adaptación extendida en los trematodos; los caracoles parasitados modifican así su distribución vertical facilitando la transmisión de las cercarias. La posible explotación de la migración relacionada con la reproducción de los caracoles por parte de las dos especies de

cercaria abre por lo tanto una puerta a futuros estudios sobre los cambios de comportamiento inducidos por parásitos como una adaptación verdadera para su transmisión.

11. El estudio a gran escala sobre el espectro de hospedadores y los niveles de infección de *C. longicollis* durante su ciclo vital, resultó en nuevas citas de primer, y especialmente, segundo hospedador intermediario, con 2 taxones de gasterópodos, 31 taxones de peces y 15 taxones de aves citados como hospedadores hasta la fecha (incluyendo los resultados actuales). *Cardiocephaloides longicollis* muestra una baja especificidad respecto a los hospedadores, especialmente en el segundo hospedador intermediario. Es la primera vez que se comparan niveles de infección de *C. longicollis* en peces de diferentes hábitats. Las tasas de infección más altas se encuentran en los peces demersales, en comparación con los peces pelágicos y bentónicos, lo que confirma que este hábitat es al que se dirigen las cercarias después de su emergencia desde el caracol.

12. La acumulación en el tiempo de metacercarias de *C. longicollis* en el cerebro de los peces se demostró en dos especies de peces espáridos. Los niveles crecientes de parásitos inducen comportamientos que hacen que los peces infectados sean más susceptibles a la depredación por el hospedador definitivo, favoreciendo así su transmisión a las gaviotas. Además, la disminución de la agregación del parásito en las clases de tamaño más grandes de peces indica una 'mortalidad inducida por parásitos', por la cual los peces más infectados son eliminados de la población.

13. La prevalencia de *C. longicollis* en caracoles y aves hospedadoras de las zonas mediterráneas, donde las actividades de pesca extractiva son comunes, fue mayor que la obtenida en cualquier estudio previo. La captura accidental y los descartes atraen carroñeros como las gaviotas, que se alimentan de peces demersales y bentónicos descartados, que de otro modo serían menos accesibles para ellos. Por lo tanto, las altas tasas de infección encontradas en las gaviotas pueden explicarse por el consumo de los descartes de peces demersales, que tienen las cargas parasitarias más altas. Esto sugiere una transmisión de *C. longicollis* facilitada por factores antropogénicos. Además, y por primera vez, se han encontrado metacercarias de *C. longicollis* en un pez de instalaciones de acuicultura, es decir, en *Sparus aurata* con una prevalencia de infección de alrededor del 54%. Por lo tanto, aunque todavía es difícil de estimar, las actividades acuícolas pueden aumentar aún más el impacto sobre el ciclo vital de *C. longicollis* aumentando su transmisión.

14. Al ser una especie generalista transmitida tróficamente con un ciclo vital complejo que afecta a un elevado número de especies hospedadoras, *C. longicollis* puede tener un gran efecto sobre el flujo de energía a través de la cadena alimenticia, incluso en condiciones naturales, siendo por lo tanto un candidato ideal para comparar futuras estructuras de la cadena trófica en comunidades naturales con aquellas afectadas por la pesquería.

~Chapter 1~

General Introduction

1.1. Parasites in the Mediterranean: An ecological view

Among metazoan parasites, trematodes (Platyhelminthes, Trematoda) are ubiquitous components in natural systems and widespread in the animal kingdom, with more than a half of all existing animal species being temporarily or permanently parasitic (Bush *et al.* 2001, Prietrock & Marcogliese 2003). Thus, parasites can be found in a wide range of conditions and habitats. Understanding their important role in natural ecosystems and the total amount of biomass they account for in relation to that of free-living organisms (Kuris *et al.* 2008), contributes to our knowledge on parasites from an ecological point of view.

Parasites affect ecosystem processes and play crucial ecological roles by regulating host populations (Hudson *et al.* 2002), affecting the structure and stability of natural communities and food webs (e.g. Mouritsen & Poulin 2002, Thompson *et al.* 2005, Lafferty *et al.* 2006, 2008). Especially parasite species that are transmitted via the trophic chain and that affect a high number of host species have a strong impact on food-web structure, affecting their stability, interaction, strength and energy flow (Thompson *et al.* 2005, Lafferty *et al.* 2008). Shelf waters constitute 20% of the Mediterranean, compared to 7.6% in the world's oceans, therefore playing an important role in this area (Bartoli & Gibson 2007, Coll *et al.* 2008). The biological production is inversely related to temperature and salinity increase, decreasing from north to south and west to east, thus the coastal and continental waters of the western Mediterranean show a high species richness, with the highest diversity of fish and invertebrates predicted to occur in coastal and shelf waters. Platyhelminths in the Mediterranean constitute 6.7% of the total species diversity; however, they are poorly known compared to other species in the Mediterranean, with less than 50% of the species described (Coll *et al.* 2008). Bartoli and Gibson (2007) contributed majorly to our knowledge of parasites in lagoons, as they described 72 nominal digenean species from lagoons of the western Mediterranean, and the life of 56 species was fully elucidated.

Trematodes are the most common parasites in intertidal soft-sediment habitats (Sousa 1991, Mouritsen & Poulin 2002, Poulin & Mouritsen, 2006) and areof major importance in lagoons (Bartoli & Boudouresque 2007). Intertidal habitats are characterized by extreme fluctuations in abiotic factors (Palacín *et al.* 1991, Koutsoubas *et al.* 2000). Small, sporadic water level changes occur (Palacín *et al.* 1991), exposing slow moving organisms such as molluscs to high temperatures and elevated salinities due to evaporation, ranging from 6°C to 30°C and 21‰ to 38 ‰, respectively (Solé *et al.* 2009), in

some Mediterranean lagoons. Due to the steep gradient in thermal and desiccation stress during low water levels, these areas have long served as "natural" experimental areas for examining changing abiotic conditions. Moreover, freshwater inflow from agricultural lands and rivers can change sediment characteristics, temperature and salinity in shallow waters markedly. Molluscs often constitute the dominant part of the benthos in coastal and estuarine waters (15-25% of benthic macrofauna, Coll *et al.* 2008), being well adapted to the fluctuating factors governing these habitats and serving as hosts for a great variety of parasites, particularly larval digenetic stages (Lauckner 1980). Lagoons often act as feeding and nursery grounds of seabirds and fish species, so that the transmission of trematode life cycle stages between different hosts (see section 1.2. for details on life cycles and hosts) may be facilitated due to their close proximity (Bartoli & Prévot 1986, Bouchereau & Guelorget 1998, Koutsoubas *et al.* 2000, Soppelsa *et al.* 2007).

The Mediterranean is a marine biodiversity hot spot, not just with regard to parasites. In the last century, fishing activities have evolved from a small scale production for local supply to an extensive and industrialized exploitation, leading to progressive degradation as a result of industrial and biological pollution. Commercial fishery is extensive in the western Mediterranean, with Spain heading the production in the European Union (about 20% of total European production, ca. 1.2 million tonnes fish in 2010; Business Association of Marine Aquaculture Producers of Spain [APROMAR], 2012). Marine habitats in the intertidal zone (coastal shelf and estuaries) have been estimated to provide approx. 42% of the global total goods, such as food (Costanza *et al.* 1997). However, coastal marine ecosystems are threatened by anthropogenic impact such as climate change (IPCC 2001), and temporal trends indicate that overexploitation and habitat loss are the main human drivers of important historical changes in biodiversity. Providing concise data on fisheries impacts may be difficult due to the pool of conditions that play a role in marine ecosystems (Tasker *et al.* 2000). At present, habitat loss and degradation, followed by fishing impacts, pollution, climate change, eutrophication, and the establishment of alien species are the most important threats to Mediterranean biodiversity and affect most taxonomic groups (Coll *et al.* 2008). Obviously, environmental factors play an important role, and climate change appears as a general cause for changes in marine ecosystems. Since extreme temperature changes have already been linked to mortality in intertidal organisms (Harley *et al.* 2006), these systems have been suggested as early warning systems for the detection of climate change as the abundance of intertidal organisms or their spatial distribution are affected (Harley *et al.* 2006, Helmuth *et al.*

2006). Since parasites are sensitive to temperature changes, they have been suggested for monitoring the ecological impact of climate change (Marcogliese 2001, Poulin & Mouritsen 2006). Similarly, fish parasites are useful as potential indicators of environmental quality in lagoons and eutrophic areas (Lafferty 1997, Sasal *et al.* 2007, Dzika & Wyzlic 2011). Generally, decreased parasite abundance and prevalence are linked to pollution since heteroxenous species are unable to complete their life cycles (Dzikowski *et al.* 2003) as free-living stages or intermediate hosts such as molluscs are greatly affected (Evans 1982). Discards from fisheries are exploited by opportunistic animals as scavengers (Bozzano & Sardà 2002, Coll *et al.* 2008), possibly perturbing faunal diversity and even favouring marine scavengers, and provide large quantities of food to seabirds, particularly demersal fish species, that would be inaccessible in other circumstances (Tasker *et al.* 2000, Oro & Ruiz 1997). Despite some knowledge of the impact of this extra food availability on host populations (e.g. increase of larids populations, Oro 1996, Oro *et al.* 1995, 1996), the effects on parasite transmission have been studied poorly. Furthermore, climate change affects the distribution and abundance of parasites directly (Marcogliese 2008, Harvell et *al.* 2009), so that the study of the factors described above, which affect parasites in marine environments and are known to be related to global changes, is primordial.

1.2. Trematode life cycle

The subclass Digenea is a large group of trematodes that comprises about 18000 nominal species (Cribb *et al.* 2001, Bartoli & Gibson 2007), representing the largest number of endoparasites found in all the classes of vertebrates (Bush *et al.* 2001). Most digenean trematodes typically have a complex life cycle involving three hosts and a number of different developmental stages (Galaktionov & Dobrovolskij 2003; Figure 1.1). Definitive hosts are usually vertebrates, such as birds or fish. Fertilized parasite eggs are released into the water, usually via the faeces. A ciliated larva, the miracidium, hatches from the egg and actively encounters and penetrates the first intermediate host, usually a gastropod. Within the gastropod, trematodes show progressive clonal reproduction of parthenitae, each developing from a single, unfertilized cell to a sack-like sporocyst or redia. Numerous sporocysts eventually replace partially or completely the snail host tissue, commonly the gonads and the digestive gland, which may lead to a partial or complete castration of the mollusc. Within the sporocysts, a high number of developmental stages mature to cercariae, i.e. dispersive offspring that is released from the first intermediate host into the

environment. Single as well as multiple infections can occur in the mollusc, often resulting in interspecific competition between parasites (Sousa 1993). After emergence, the free-living, non-feeding cercariae transmit to the second intermediate host, usually another mollusc or a fish, within a short period of time (sometimes less than 24h). Most cercariae swim freely in the water column (Figure 1.2. A, B), but some have a distinctive cup-shaped (cotylocercous) tail that permits attachment to the substrate, hence following a "sit and wait" strategy until consumed by the downstream host (Figure 1.2. C, D). After the penetration of the second intermediate host, the cercaria forms a cyst in the target tissue and becomes a metacercaria. Usually, the definitive host acquires the infective metacercariae via the trophic chain. Host specificity of trematodes in the first intermediate host is usually high when compared to the second intermediate host, and prevalence can often approach 100% in fish hosts (Sousa 1991, Combes 2001). Each parasite life cycle stage has a particular transmission strategy to successfully complete the life cycle (Combes *et al.* 2002).

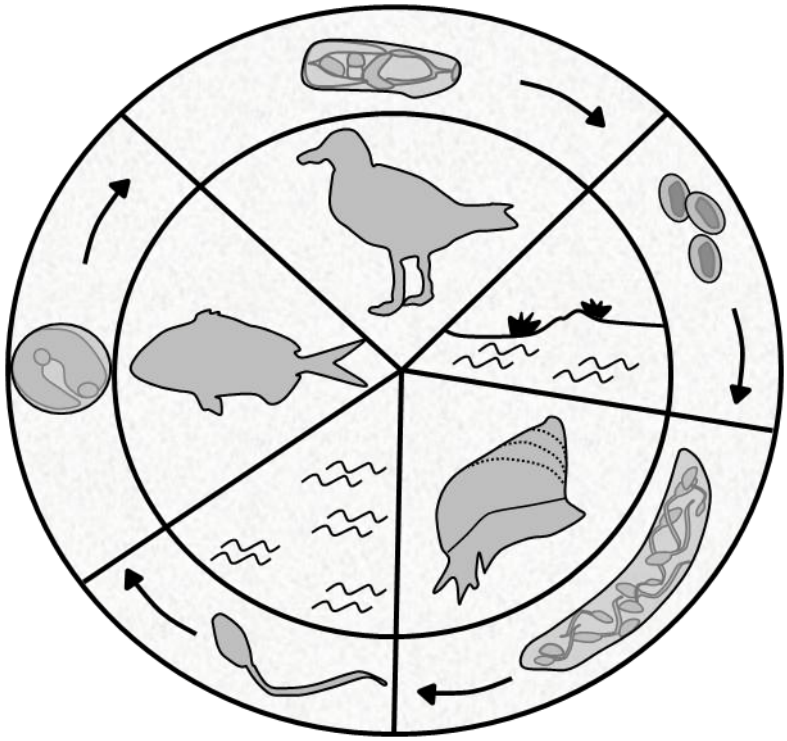


Figure 1.1. Representation of a general complex life cycle of a digenean trematode, redrawn from Lauckner (1980). Sectors show free-living and parasitic stages in their intermediate or final hosts.

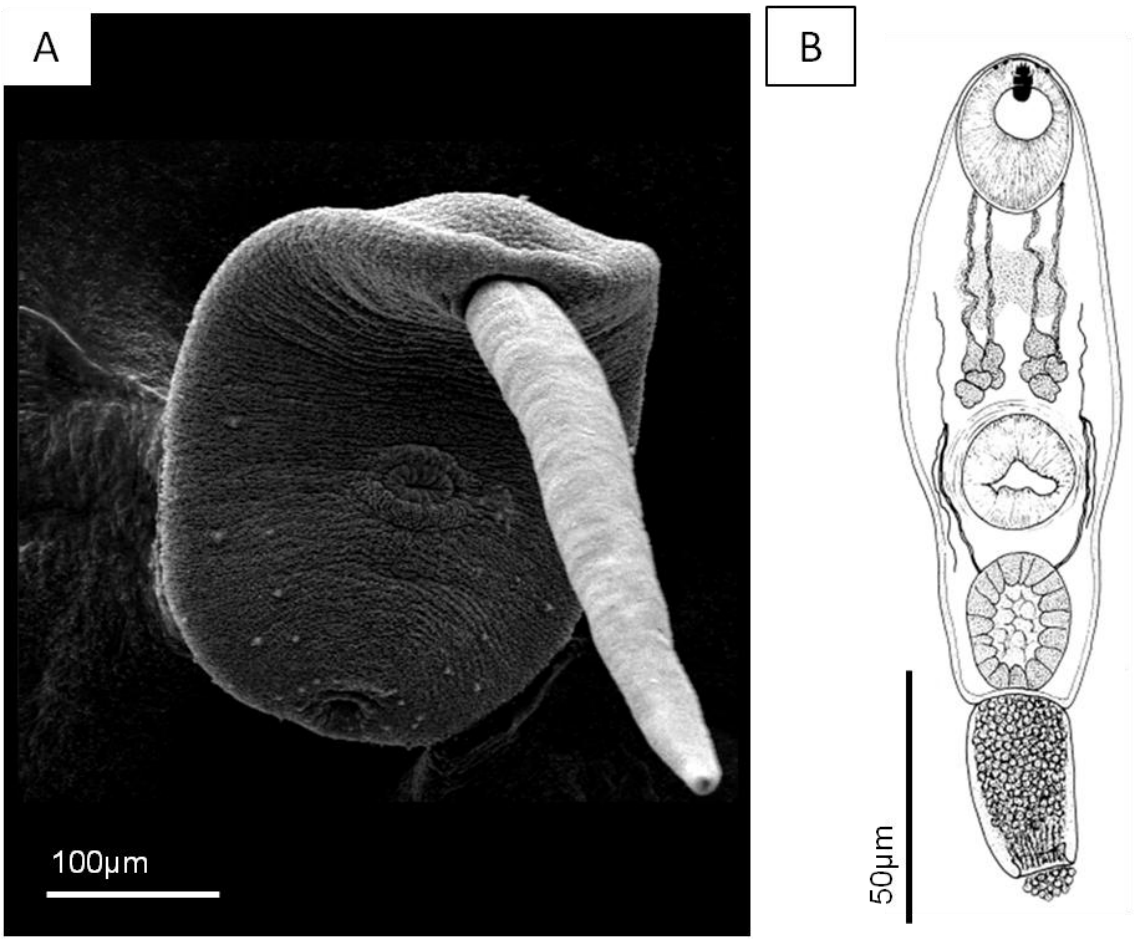


Figure 1.2. Cercariae from digenean trematodes. a) Cercariae of *Renicola* sp. (Renicolidae), with a leptocercous tail (Scanning Electron Microscopy (SEM) photograph), b) Cercariae with a cotylocercous tail (*Opecoeloides columbellae* n. comb. [syn. *Cercaria columbellae* Pagenstecher, 1863], drawing from Jousson & Bartoli 2000).

1.3. Factors affecting trematode transmission success

1.3.1. Environmental factors

Free-living stages of trematodes, eggs, miracidia and cercariae rely on their own stored energy resources and are directly exposed to environmental changes in their microhabitats, which condition the interaction between parasites and potential hosts. Cercariae are especially exposed since it is the only stage that actively searches for its next host, a crucial step for the completion of the life cycle. The transmission success of free-living larval stages is generally modulated by different abiotic and biotic environmental factors that alter their survival or infectivity (Fingerut *et al.* 2003, Pietrock & Marcogliese 2003, Thieltges *et al.* 2008). Moreover, abiotic conditions become more important in

lagoons, as they are characterized by extreme environmental changes and a high internal heterogeneity (see above). The effect of natural abiotic factors on cercarial development and emergence has been extensively studied (Thieltges 2007, see Pietrock & Marcogliese 2003 and Thieltges *et al.* 2008 for reviews). Especially temperature seems to influence the embryonic development of the intramolluscan stages (Lo & Lee 1996, Galaktionov & Dobrovolskij 2003, Pietrock & Marcogliese 2003, Poulin 2006, Thieltges & Rick 2006, Koprivnikar & Poulin 2009), but also salinity (Koprivnikar & Poulin 2009, Lei & Poulin 2011, Studer & Poulin 2012a) and other factors such as light, photoperiod, or tidal level which have only been sporadically studied (Bell *et al.* 1999, Mouritsen 2002, Fingerut *et al.* 2003, Thieltges 2007, Evans *et al.* 2011). Understanding the effects of environmental factors on cercarial emergence is important to be able to estimate the impact of changing conditions on transmission and life cycle completion, particularly in the case of an intertidal habitat, which, as pointed before, may be strongly affected by climate change.

1.3.2. Artificial/anthropogenic factors

Nowadays, natural habitats are considerably influenced by human activities, as e.g. by fishing or aquaculture, the latter having an important impact on the surrounding habitat and the associated macrobenthos (e.g. Edgar *et al.* 2005, Wood *et al.* 2010,). Furthermore, there is evidence for spreading of parasites and diseases from farmed to wild fish, and vice versa (see Grigorakis & Rigos 2011, and references therein, for a review on aquaculture impact on the environmental and public health in Mediterranean mariculture; Mladineo & Marsic-Lucic 2007, Karlsbakk *et al.* 2013,). However, the impact on the parasitic rates of the organisms in fishing areas has been extensively studied (see above, section 1.1).

Furthermore, parasite transmission can be influenced by anthropogenic actions since potential final hosts such as fish-eating birds and other wild predators are attracted by aquaculture installations (Witt *et al.* 1981, Arcos *et al.* 2001, Christel *et al.* 2012), promoting an opportunistic feeding behaviour. Since an important fraction of the total catch of Mediterranean fisheries is discarded (Sánchez *et al.* 2004, Tzanatos *et al.* 2007), a large number and diversity of fish hosts become accessible to fish-eating birds, potentially enhancing the transmission of trophically transmitted parasites to the final hosts (Osset *et al.* 2005, Bartoli & Boudouresque 2007). Aquaculture facilities and discarded organisms may attract scavengers, such as fish (Bozzano & Sarda 2002), birds (Oro & Ruiz 1997), and gastropods (Morton & Yuen 2000). Different host groups are thus concentrated in the

same area, facilitating transmission even in case of heteroxenous life cycles. The environmental impact of the increasing fishery and aquaculture installations and actions makes it primordial to study possible enhanced life cycles that may affect a wide range of host species, cultured organisms and the ecosystem as a whole.

1.3.3. Biological, physiological and behavioural factors

The intimate coexistence of two organisms, characterized by the parasite obtaining benefits (e.g. energy, space) at the expense of the host, creates a metabolic dependence of the parasite (Lauckner 1980). As a consequence, by definition, any parasite will have effects on its host, ranging from minor metabolic changes to severe tissue destruction, and often leading to a decrease in their reproductive output (see Lauckner 1980, 1983, Price 1980). Since ectothermic organisms have a limited ability to regulate their body temperature, they can be more vulnerable to changes. Hence, infected snails show a lower tolerance to physical, thermal and/or osmotic stress, conditions frequently experienced in the intertidal zone (e.g. Tallmark & Norrgren 1976, McDaniel 1969, Lauckner 1980, Fredensborg *et al.* 2005). In a similar manner, the physiology and immunology of fish, poikilothermic organisms, is strongly influenced by both abiotic and biotic factors (Rohlenová *et al.* 2011). Hormones, trophic or reproductive migrations and biotic conditions can modulate the efficiency of the immune system in fish (Bartoli *et al.* 2000, Rohlenová *et al.* 2011), but it is also impacted by increasing parasitic infection (Rohlenová *et al.* 2011). Overall, the ecological, physiological and biological peculiarities of each host-parasite system produce a feedback, influencing the infection (Gorbushin & Levakin 1999).

Host body size is an important factor for the development of a trematode, since larger host species should provide more space and thus more nutrients to the parasite (Poulin & Morand 2004). Despite the general trend that cercarial reproductive success is correlated with mollusc host size (Cort *et al.* 1954, Baudoin 1975, Gérard *et al.* 1993, Lo & Lee 1996, Poulin 2007), the resources of the parasite within the mollusc are limited, so that a trade-off between number of emerging cercariae and their size should exist (Loker 1983, McCarthy *et al.* 2002). In fish and snail hosts it is commonly observed that trematode prevalence increases with age, i.e. host size (e.g. Hughes & Answer 1982, McCurdy *et al.* 2000, McCarthy *et al.* 2004, Karvonen *et al.* 2006), the most simple explanation being the prolonged period of time that hosts have been exposed to trematodes. However, other cues such as different microhabitats or diets with increasing age or the reproductive stage

can play a role in infection rates (e.g. Annett & Pierotti 1989, Rodríguez-Ruiz *et al.* 2002). Nevertheless, the accumulation of stages over time, usually metacercariae, has been often observed, and a cumulative effect of parasites with host size has been studied in several species (e.g. Thomas *et al.* 1995, Karvonen *et al.* 2003, Osset *et al.* 2005). High numbers of parasites may lead to parasite-induced mortality, by which the most infected hosts, are eliminated from the population (Rousset *et al.* 1996, Poulin 2001), which has been observed both in invertebrates (e.g. Thomas *et al.* 1995, Latham & Poulin 2002) as well as in fish (e.g. Sandland & Goater 2001, Seppälä *et al.* 2004). The intensity-dependent removal of parasites thereby also serves the regulation of parasite populations (Poulin 2007).

Given the short life-span of cercariae, emergence timing has to be optimized to enhance the transmission to the next intermediate host (Combes *et al.* 1994) and a combination of a variety of factors may be important to achieve this. For example, light is important for cercariae using phototaxis to settle in microhabitats frequented by potential hosts, to optimize their contact (Combes *et al.* 1994, Bartoli & Boudouresque 2007, Smith & Cohen 2012). At the same time, the distribution of hosts is important for successful transmission, since a de-synchronization between infected definitive hosts and available intermediate hosts, as well as with parasite maturity will lead to the disruption of the parasite's life cycle (Marcogliese 2001). Some parasites manipulate the behaviour of their hosts to increase the probability to reach the downstream host (Poulin 2007), thus facilitating host finding and enhancing their transmission (Curtis 1993, Thomas & Poulin 1998, Karvonen *et al.* 2003, Seppälä *et al.* 2004). For example, the metacercariae of *C. longicollis*, once encysted in the optic lobes of the fish brain, manipulate the behaviour of the fish by causing them to swim in surface waters where they are more visible to definitive bird hosts (Prévot & Bartoli 1980, Bartoli & Boudouresque 2007). This condition facilitates transmission of the parasite to the avian definitive host. In any particular case, a combination of numerous factors may have an impact on parasite emergence, transmission and life cycle completion, and this interdependence has to be considered when a host-parasite system is studied.

1.4. Detection and experimental methods

Studies in soft-sediment habitats often require repeated sampling under different conditions (e.g. different temperatures) thus requiring the collection of a larger number of animals. To avoid this, experimental approaches commonly use the same individuals for

testing the effects of various factors, thereby controlling for the stability of the conditions that are not tested. The interpretation of these results, however, has to be done in relation to other conditions that may vary in the ecosystem where the host comes from.

For the collection of endoparasites, necropsy of the animal is required, although faecal samples can sometimes be examined, e.g. for the presence of eggs in the definitive host. In the case of mollusc first intermediate hosts, the most common method for the identification of infected specimens is dissection or testing for emergence of mature cercariae. Both methods have been criticized as inaccurate since trematode infections can be overlooked, especially in case of immature or mixed infections (DeCoursey & Vernberg 1974, Curtis & Hubbard 1990, Mouritsen & Poulin 2002). Thus, molecular analyses have recently become an important tool for the identification of infected snails (Cucher *et al.* 2006, Martínez-Ibeas *et al.* 2011). Furthermore, the molecular characterisation of larval stages improves their correct identification and aids the elucidation of life cycles. The use of molecular tools is thus encouraged in experimental procedures; however, their combination with classical methodologies allowing direct observation of parasitic processes as well as morphological features allows for the most holistic view of the host-parasite system.

~Chapter 2~

Aims and Objectives

AIMS

The aims of the present study are four-fold. First, to describe a snail-parasite system that is common in Mediterranean lagoons and specifically mirrors and adjusts to the conditions in the intertidal habitat, second to increase the knowledge of the impact of the environmental factors that play a role in the emergence, transmission and development of different trematode larval stages in lagoons. Third, to provide new evidence on the higher accuracy of molecular methods to detect intramolluscan infections, keeping in mind their disadvantages. Fourth, to expand our knowledge of the host range and transmission of the life cycle stages of *C. longicollis,* a parasite that occurs in high numbers in the Mediterranean and that is firmly embedded in its food webs.

OBJECTIVES

In order to achieve these aims, the following objectives were undertaken:

I.- Morphological and molecular identification of different morphotypes of cercariae released by the snail *Gibbula adansonii,* which is common in the intertidal habitat of the Ebro Delta lagoon, determination of infection rates as well as phylogenetic relationships with related trematode species.

II.- Experimental assessment of the effect of changes in temperature, salinity, photoperiod and water level on the larval emergence of *Cainocreadium labracis* and *Macvicaria obovata* from their first intermediate host, i.e. *G. adansonii*, by using an *in vitro* approach and in the context of their next host-finding strategy.

III.- Comparison of traditional emergence methods and molecular tools, using a specifically designed duplex PCR assay for the detection of intramolluscan stages in single and double infections in two host-parasite systems in the intertidal habitat: i) Two co-occuring trematode species, *C. labracis* and *M. obovata* infecting *G. adansonii* as sporocysts, which within-host interactions have never been studied, and ii) Two co-occurring species, *Maritrema novaezealandensis* and *Philophthalmus* sp. infecting the snail *Zeacumantus subcarinatus*, as sporocysts and rediae, respectively, which interspecific competition is known to occur.

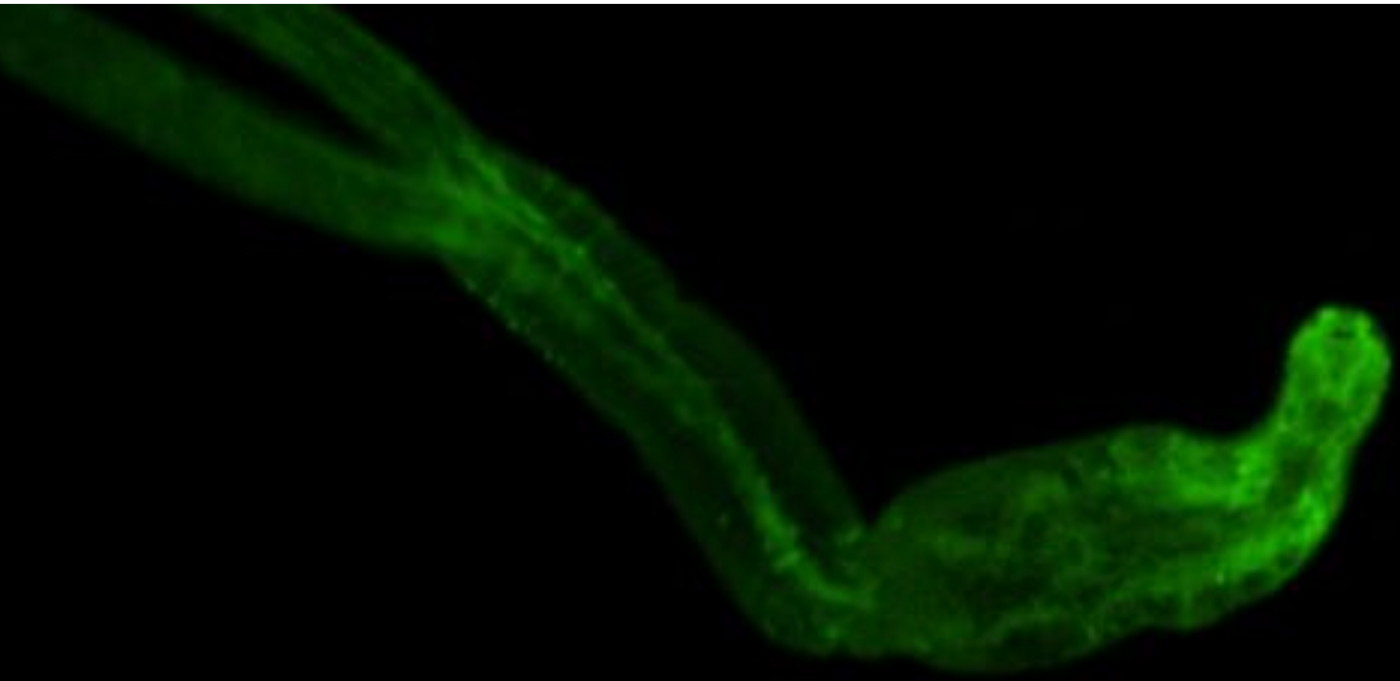
IV.- Morphological differentiation and quantification of parasite stages in sporocysts and subsequent description of developmental patterns inside sporocysts of *C. labracis* and *M. obovata* in *G. adansonii* during the spring months, when this snail is found in the intertidal habitat of the lagoon, and determine whether a link exists between larval maturation and snail migration.

V.- Large scale screening of potential mollusc, fish and bird hosts to document the host-spectrum and parasite load of *Cardiocephaloides longicollis* in the Mediterranean, also determining the marine microhabitat targeted by its cercariae by comparing infection levels in fish species from different habitats.

VI. –Quantification of metacercariae of *C. longicollis* in the brains of sparids to analyze the effect of fish size on their accumulation over time and to explore the role of anthropogenic factors such as fisheries and aquaculture that may enhance the transmission of these metacercariae to the final host.

~Chapter 3~

General Materials and Methods



This chapter provides an overview of the general sampling methodology, the sites, and the number and taxonomic summary of the host species analysed for trematode infections. It also explains general microscopic and molecular techniques applied, the setup and design of experimental trials and provides basic information on the statistical analyses performed. A more detailed account of the materials and methods related to each study is given in the relevant chapters (Chapters 4 to 8).

3.1. Trematode host collection

Snail, fish and bird hosts of trematodes were collected from a number of sites along the Spanish Mediterranean coast (Fig. 3.1). During a study visit at the University of Otago (New Zealand), snails were also collected at Lower Portobello Bay and Oyster Bay (Fig. 3.1.). For the study on *C. longicollis*, data obtained in the course of this thesis was combined with infection data provided by two collaborators, J. Culurgioni (data from Italy) and A. Pérez-del-Olmo (additional sites along the Spanish coast), for details see Chapter 8.

3.1.1. Snail hosts

A total of 6170 snail hosts belonging to 29 species were sampled at four different sites (Appendix-Table 1).

The largest number of samples was obtained from two sites, 1. The "Els Alfacs" lagoon (Ebro Delta, Spain) (Figures 3.1, 3.2.A), and 2. the Lower Portobello Bay and the Oyster Bay (Otago Habour, New Zealand) (Figures 3.1., 3.3. A). These sites were chosen because they are inhabited by large numbers of snails, known to harbour both, high infection levels and mixed infections with different trematode species, as determined in preliminary (Ebro Delta) and previous studies (Otago Harbour, Martorelli *et al.* 2004, Keeney *et al.* 2008, Studer & Poulin 2012b). They were furthermore selected because they represent lagoonal habitats with special physiological characteristics, such as a wide range of temperatures and salinities, to study the impact of these factors on cercarial release from the snails.

Figure 3.1. Map of the Mediterranean coast (Spain, Europe) and Dunedin (New Zealand) showing the collection sites of snail, fish and bird hosts.

The shallow lagoon formed by the sediments deposited on the side of the mouth of the river Ebro (Fig. 3.2.A) has muddy soft sediment and a patchy cover of dwarf eelgrass *Zostera noltii* Hornemann, 1832 (Figure 3.2. C), on which *G. adansonii* (Payraudeau, 1826) (Prosobranchia, Trochidae) grazes in large numbers. The fine muddy sediments are furthermore covered by large numbers of *C. neritea* (L.) and *N. reticulatus* (L.) (Prosobranchia, Nassaridae) (Figure 3.2.D). Due to the open connection of this lagoon to the Mediterranean and the freshwater input from rainfall and from the Ebro River via rice field drainage (Figure 3.2.E)(Camp & Delgado 1987, Palacín *et al.* 1991), the Els Alfacs lagoon (Ebro Delta, Spain) is characterized by strong daily and seasonal changes in temperature and salinity. Salinity ranges from 39 to 44‰ and temperature from 13 °C to 34 °C (measurements taken during sampling). Diurnal water level changes cause snails to be uncovered by water at times. The Ebro Delta is near a protected area and supports permanent and temporary bird communities (Figure 3.2. E). The two sampling areas in New Zealand, Portobello Bay and Oyster Bay (Figure 3.3. A) are similar intertidal marine ecosystems, also subjected to extreme fluctuations in abiotic factors. Temperature and salinity range from 16°C to 30°C temperature and 0 psu to 38 psu salinity (Studer *et al.*

2010, Lei & Poulin 2011), which increases when the water level decreases and tidal pools are formed, where individuals of *Zeacumantus subcarinatus* (Sowerby, 1855) (Prosobranchia: Batillariidae) are extremely common and easy to collect in amongst *Ulva* sp.-covered areas (Figure 3.3. B, C).

Snails were transported to the laboratory in aerated seawater taken from the locality and left to settle for 24h in aquaria (Figure 3.3 D, Figure 3.4. A, B). To determine the infection prevalence by cercarial emission or to obtain living cercariae for *in vivo* studies, the snails were acclimatised to laboratory conditions. Thereafter, they were screened for infections by incubating them individually in cell well plates containing 3ml seawater, using a 14-h light: 10-h dark photoperiod for *G. adansonii* to mimic the natural conditions of the habitat snails were taken from, whereas 3h constant illumination for *Z. subcarinatus* was enough for cercarial emergence. Incubation was generally carried out at 25°C as warmer temperatures promote cercarial emission (Thieltges & Rick 2006, Koprivnikar & Poulin 2009, Studer *et al.* 2010). After the incubation, individual wells were examined for the presence of cercariae under a dissecting microscope, to ascertain infection status and identification of the emitted cercariae. When necessary, snails were crushed and the digestive gland as well as the gonads were examined for the presence of sporocysts or rediae. Snails were measured (shell height (apex to aperture)) to the nearest 0.1 mm. Snails were used for a study describing their infections (Chapter 4), determining cercarial emission rates under varying environmental conditions mimicked experimentally in the laboratory (Chapter 5), to compare classical methods of determining infection levels (emission of cercariae) with molecular detection using specific PCRs (Chapter 6) and to determine the development of infections and production rates of cercariae in the snail hosts (Chapter 7). Apart from the Ebro Delta, two further Mediterranean sites were sampled for snails to determine if *C. longicollis* infections are present in a variety of snail species common in the Mediterranean (Chapter 8).

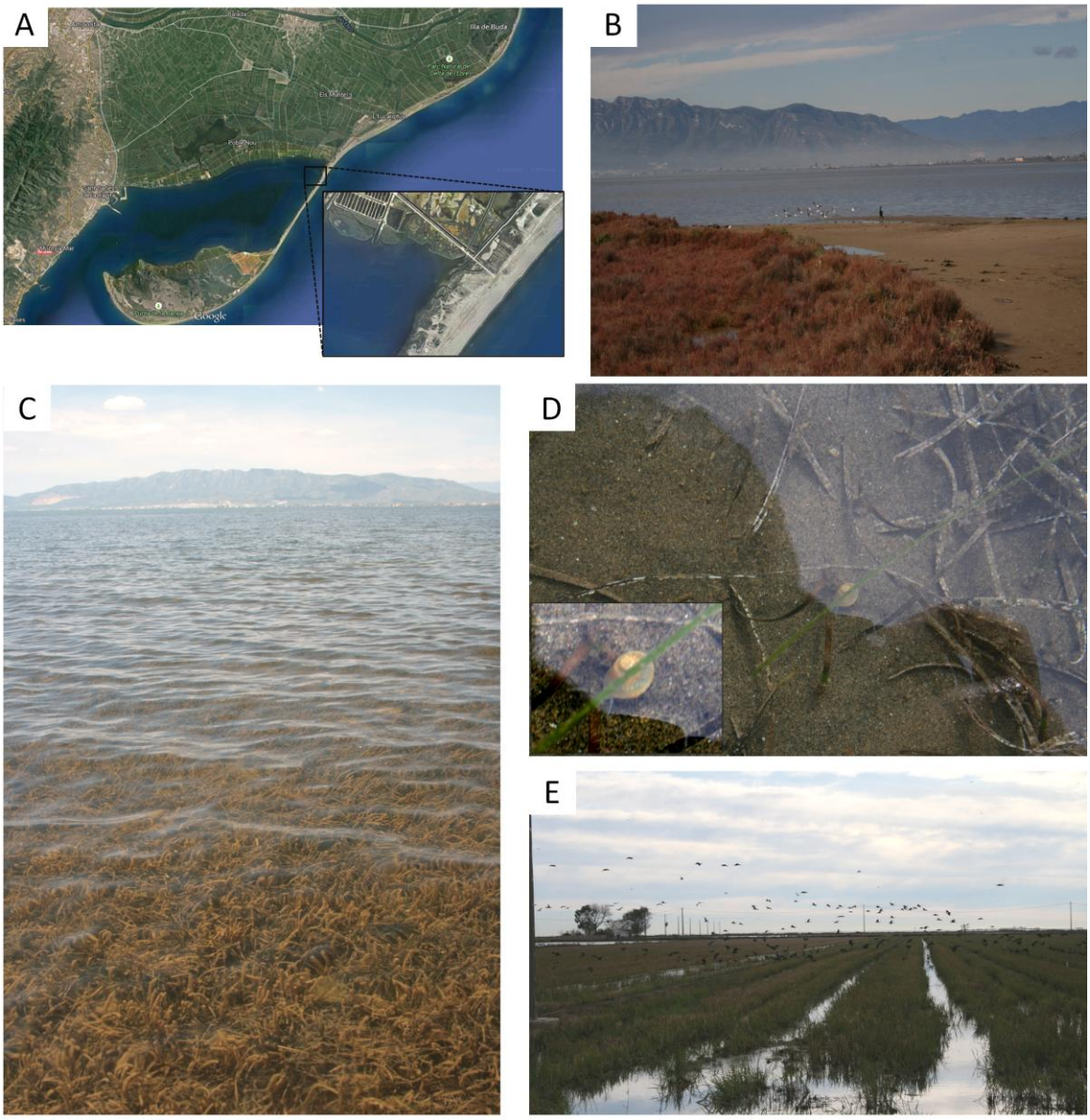


Figure 3.2. Snail sampling site "Els Alfacs" lagoon and the Ebro Delta (Spain). a) Aerial image of the lagoon showing the location of the sampling site (Google), b) Lagoon at low tide, c) Exposure of the dwarf eelgrass *Z. noltii*, on which snails graze, at falling tide, d) Detail of *Z. noltii* leaves, with the snail *C. neritea* in detail, e) Rice fields near the Ebro River with local bird populations.

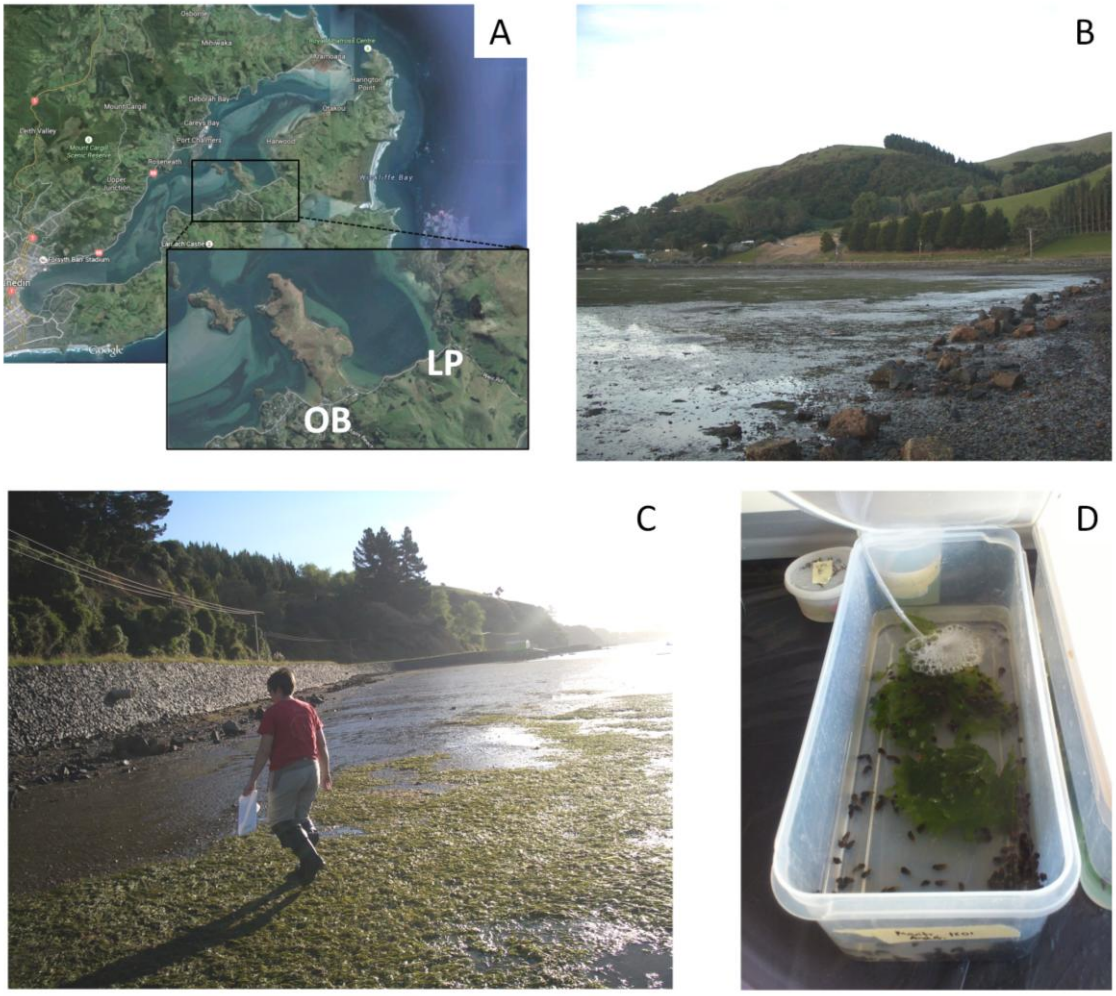


Figure 3.3. Snail sampling site Oyster Bay (Otago, New Zealand). A) Aerial image of the Lower Portobello Bay (LP) and Oyster Bay (OB) sampling sites (Google), b, c) Low tide exposing the *Ulva* sp. beds populated by the snail *Z. subcarinatus,* d) Snails kept in aerated seawater until their examination.

3.1.2. Fish and bird hosts

Fish and birds specimens were obtained opportunistically and examined in the search for different life cycle stages of the trematode *C. longicollis* (Chapter 8). This included 1130 fish specimens belonging to 24 species, obtained from 9 different localities (Appendix-Table 2), and 154 birds belonging to 17 species, obtained from two bird sanctuaries (Appendix-Table 3). Fish were captured, trapped or obtained from local fishermen, fish markets and aquaculture sites and were analyzed within 48 hours after their arrival at the laboratory. Two bird sanctuaries "Centro de Recuperación de Fauna La Granja" (Valencia, Spain) and "Centre de Recuperació de Fauna Salvatge de Torreferrussa" (Barcelona, Spain) provided birds recovered from different localities, so that their origin corresponds to the

surrounding geographic areas. Given the opportunistic nature of the samples, sample sizes of some species are low. Fish brains and bird alimentary tracts (from oesophagus to the cloacae of bird) were removed (Figure 3.4. C-F) and examined for metacercariae and adult specimens of *C. longicollis*, respectively. Total fish length and weight were recorded before dissection. Bird's carcasses were frozen at -20°C until necropsy. In order to determine the presence of trematode eggs in seagull faeces, fresh faecal samples of *Larus michahellis* J.F. Naumann, 1840 were obtained at Benidorm Island (Spain), in collaboration with the staff of the Natural Park Serra Gelada, collected from the floor with a pipette and using filtered seawater (5µm filter). Individual faeces were stored in individual containers (Figure 3.4. G, H) until examination under the stereomicroscope, where the number of *C. longicollis* eggs was quantified.

Since no formal approval or ethic statement is required for research on gastropods under the New Zealand and the Spanish legislation, a waiver was granted from the University of Otago and the University of Valencia Animal Ethics Committees. Fish obtained from traditional or extensive fishing were obtained freshly sacrificed on ice. Life fish caught by trapping or line fishing were euthanized by an overdose of MS222 or clove oil, followed by spinal pitching.

Particular details of the sampling methodology or additional localities and analyses of each group of hosts and parasites are indicated in their respective chapters.

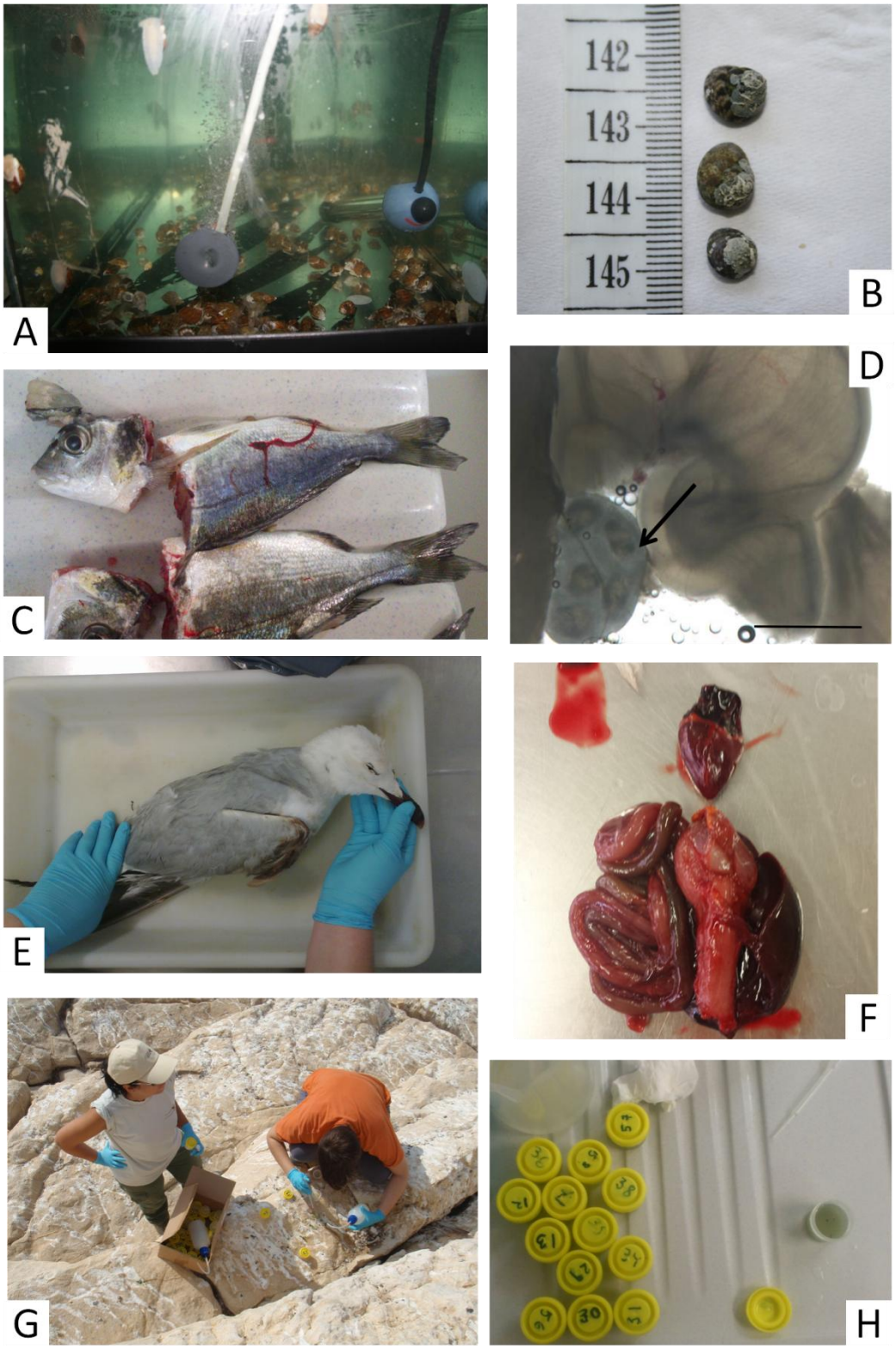


Figure 3.4. Different images taken during sampling and examination of trematode hosts. a) Snails *N. reticulatus* kept in aerated seawater until their examination, b) Specimens of *G. adansonii*, c) *Sparus aurata* L. after brain removal, d) Five encysted metacercariae of *C. longicollis* (arrow) detected during examination of a fish brain (scale-bar), e) *Larus audouinii* Payraudeau, 1826 before necropsy, and f) its alimentary tract isolated for examination, g) Collection of faecal samples of *L. michahellis* at Benidorm Island (Spain), with h) faeces individually stored until examination for the presence of eggs.

3.2. Morphological data, microphotographs and SEM images

The morphology of sporocysts, cercariae, metacercariae, adults and eggs was first studied on live material when available, using light microscopy for identification and detailed morphological examination (Leica DMR microscope, magnification up to 400X). Thereafter, cercariae, metacercariae and adult worms were fixed in 70% ethanol, and stained with aluminium carmine or with iron acetocarmine, dehydrated in an ethanol series (Georgiev *et al.* 1986), cleared in dimethyl phthalate and mounted in Canada balsam. Digital photographs were taken with a Leica DC300 or a Leica DFC295 camera on a Leica DMR microscope. The program ImageJ (v 1.44, National Institutes of Health, USA) was used to obtain morphometric data from the images, using an image of a microscope standard graticule as reference.

Cercarial samples used for scanning electron microscopy (SEM), i.e. *C. labracis*, *M. obovata* (Opecoelidae) and *C. longicollis* (Strigedae), were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffered saline (PBS, pH 7.4) or 70% ethanol. Thereafter, specimens fixed in glutaraldehyde where washed in 0.1M phosphate buffer (pH 7.4), post fixed in 2% osmium tetroxide, washed in 0.1M phosphate buffer and dehydrated in an acetone series. Specimens fixed in ethanol were dehydrated in an ethanol series, transferred to 100% acetone after a graded series of absolute ethanol/absolute acetone. After dehydration, all specimens were critical point-dried with liquid CO_2 and sputter-coated with gold for their observation by SEM (see chapter 4). Samples were examined using a JEOL JSM-7401F at an accelerating voltage of 4 kV. Images were taken on a FeG-SEM hitachi S4100.

3.3. Molecular analyses

Samples used for molecular studies, whether snail tissues or trematode specimens, were first fixed in 100% ethanol. For DNA extraction, the ethanol was decanted carefully and the last drop was evaporated on a heat block at 55°C, for 30 min. Thereafter, the tissue samples/trematodes were dissolved and preserved in TNES-urea buffer (Asahida *et al.* 1996). DNA was extracted after digestion of cells with 100 $\mu g mL^{-1}$ Proteinase K overnight at 55°C, using a simple phenol-chloroform extraction protocol (Holzer *et al.* 2004). Briefly, after the addition of phenol and chloroform and overend turning, the phases were separated by centrifugation and the aqueous part was removed to a fresh tube for DNA precipitation with 92% ice cold ethanol. After pelleting by centrifugation, the DNA was washed with 70% ethanol and dried. Extracted DNA was resuspended in RNAse/DNAse-

free water and left to dissolve overnight in the fridge. This method was used for all samples but cercariae sequenced in chapter 6, i.e. *M. novaezealandensis* and *Philophthalmus* sp. For the latter, a different DNA extraction method was used, which consisted of placing ethanol-dried samples into 5% Chelex containing 100 μgmL^{-1} Proteinase K, incubating at 60°C overnight, boiling at 90°C for 8 min and centrifuging at 15,000g for 10 min. The supernatant was used for PCR.

ITS and/or 28S rDNA sequences were produced, depending on species, using two to four individual specimens of each trematode species or cercarial morphotype. The ribosomal RNA gene is appropriate to address systematic questions at different taxonomic levels due to the different rates of evolution of its regions, as for example the highly conserved 28S and the highly variable ITS rDNA fragments (Hillis & Dixon 1991). The larger and more conserved 28S region permits studying relationships among genera as well as related families, while ITS rDNA has a faster evolutionary rate and is comparable only within a species or genus. Therefore ITS rDNA is suitable to distinguish between closely related trematode species and thus for specific primers design, with the ITS 1 region often providing a greater resolution than ITS 2 (Nolan & Cribb 2005, for an extensive review).

Polymerase chain reaction amplifications (PCRs) were performed with a programmable thermal cycler (Techne TC- 512 or Mastercycler ep gradient S, Eppendorf) in a final volume of 10 to 30µL, containing ~0.5 units of polymerase (ThermoPrime Plus DNA or MyTaqRed DNA Polymerase, Bioline) and the related 5x buffer (containing 15mM $MgCl_2$ and 5mM dNTPs), 0.5µM of each primer and approximately 100ng of template DNA. The general thermocycling profile consisted of initial denaturation (94°C-95°C 3 min) followed by 35 cycles of amplification (94°C for 50s, specific annealing temperature (50s-1min) and 72°C (1 min to 1min 20s)) and 4min final extension at 72 °C(see chapters 4, 6 and 8 for details on primers and annealing temperatures). After checking for the presence of PCR amplicons in a 1% agarose gel in sodium borate buffer (running voltage 90-100V), PCR amplicons were excised and purified using the Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare UK Ltd.), cycle-sequenced using the BIG Dye Terminator v 3.1 Ready Sequencing Kit on a ABI 3730 sequencer (Applied Biosystems) (SCSIE, University of Valencia). Partial sequences were assembled and edited using Bioedit v7.0.5 (Hall 1999). All obtained sequences obtained were submitted to the Basic Local Alignment Search Tool (BLAST) on GenBank™. New sequences were submitted to GenBank™ and accession numbers were obtained (Chapters 4, 6 and 8).

To estimate the phylogenetic position of some sequences obtained in this study in relation to closely related species, taxa were aligned using Mafft v5.531 (Katoh *et al.* 2002). In order to estimate phylogenetic relationships between species likelihood-based and parsimony-based approaches were used (for details see Chapter 4).

Specific primer pairs were designed with the Primer3 program (Rozen & Skaletsky 2000), choosing variable regions of the rDNA gene and considering physical and structural properties of the oligonucleotides and the potential formation of self- and hetero-dimers. Using DNA extractions from both, single cercariae and an artificial mix of two co-ocurring species, primers for duplex assays were tested for their optimal joint annealing temperature. This resulted in the design of a single-step duplex PCR in which co-infecting species were differentiated by amplicon size after gel electrophoresis, thus detecting single and double infections.

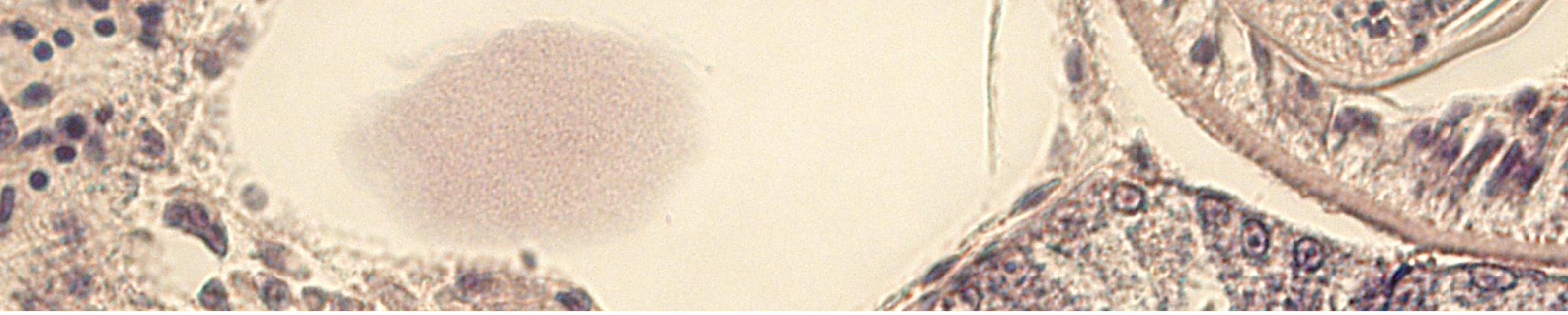
3.4. Experimental design of cercarial emission from snails study

In Chapter 5, the effect of exogenous factors that are known to exhibit marked fluctuations in lagoons on cercarial emergence from snail hosts was evaluated. Specimens of *G. adansonii* collected at "Els Alfacs" lagoon were acclimatized and screened for infections with *C. labracis* and *M. obovata* as described before (see 3.1.1). For each trematode species, infected snails were randomly assigned to the different experimental procedures. Four experiments were conducted, estimating the effect of changing temperatures, salinities, photoperiod and water level, with the range of factors mimicking the natural conditions that can occur in the "Els Alfacs" lagoon (reference values measured during sampling dates together with data consulted in the literature): 1. Temperatures were decreased and thereafter increased in 5 degree steps, from 25 to 10 °C and vice versa. 2. Salinity was decreased from 45 to 25 practical salinity units (psu), water level (below top of the snail shell or covering it completely), and with two different photoperiods (12-h light: 12-h dark and 15-h light: 9-h dark). Daily cercarial counts were done in 6-15 snail replicates maintained individually in cell-well plates, and statistically compared to assess differences between treatments.

3.5. Infection rates, related terminology and statistical analyses

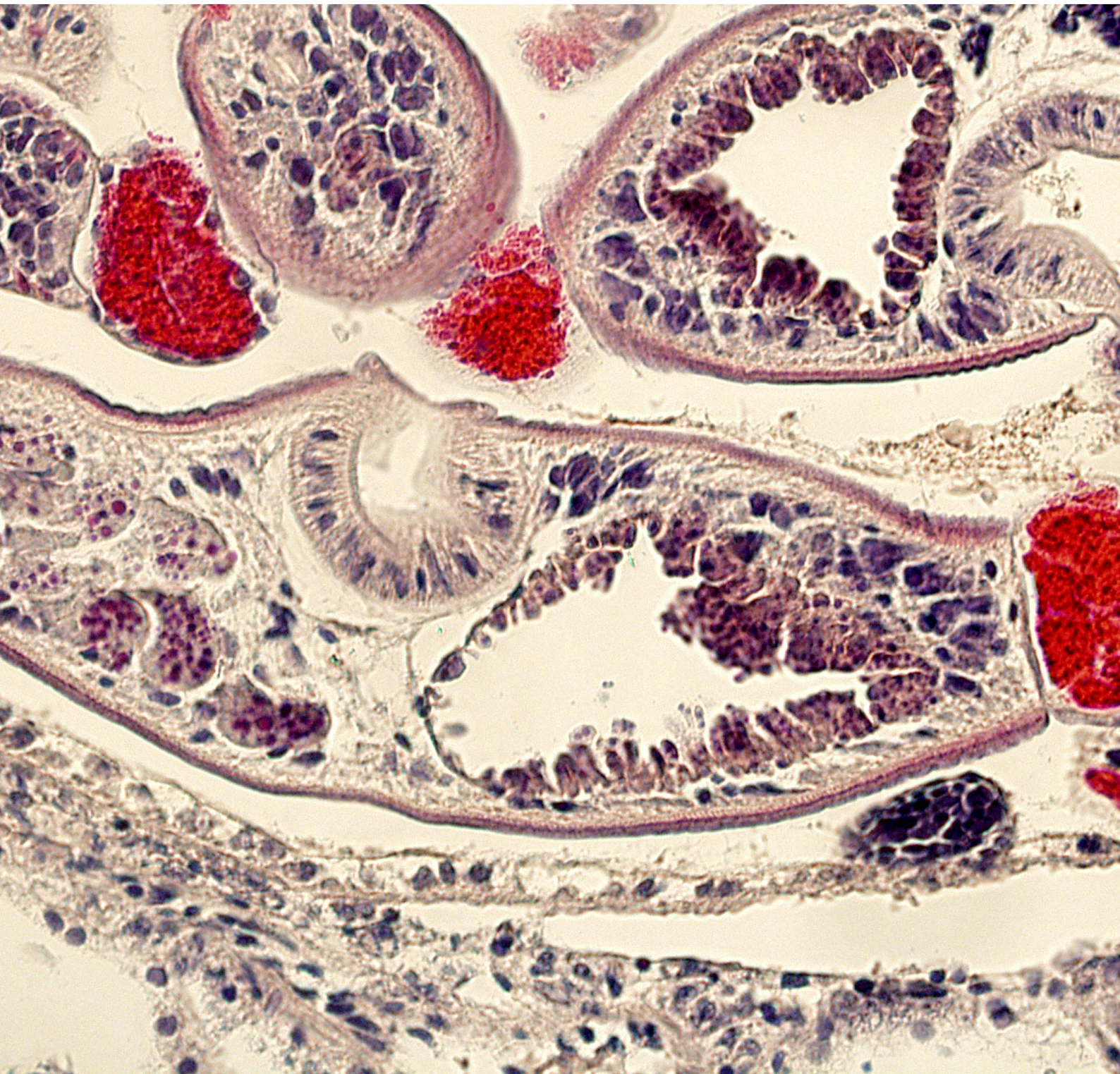
Ecological terms follow Bush *et al.* (1997). 'Prevalence' relates to the percentage of hosts in a sample that is infected by a helminth species; 'intensity' is the number of individuals of a helminth species in an infected host, and 'mean abundance' is the average number of individuals of a helminth species in a population or sample of hosts, regardless of whether they are infected or not.

Most statistical analyses were conducted using the software R (R Development Core Team, version 3.0.1). For the analysis of different data sets generated in the present thesis, Generalized Linear Mixed Models (GLMMs), Generalized Linear Models (GLMs), Linear Mixed Models (LMMs) and Linear Models (LMs) were estimated within the lme4 package (Bates *et al.* 2014) or the MASS package; and multiple comparisons were performed with the multcomp package (Hothorn *et al.* 2008). In the study on cercarial emission with changing conditions, the program STATISTICA 7.0 (StatSoft, Inc. 1984–2004) was used. Statistical differences between the prevalences obtained by classical emission and the duplex PCR detection method were evaluated by McNemar's Chi-squared test for paired proportions (stats package, Agresti 1990) and Cohen`s Kappa Statistic (fmsb package, Nakazawa 2013), to assess the extent of agreement between both methods. The details of the specific analyses performed in individual studies are detailed in the relevant chapters (Chapters 5 to 8). Statistical significance was set at $P < 0.05$, unless otherwise stated.



~Chapter 4~

Molecular and morphological identification of larval opecoelids (Digenea: Opecoelidae) parasitising prosobranch snails in a Western Mediterranean lagoon



~Chapter 4~

Molecular and morphological identification of larval opecoelids (Digenea: Opecoelidae) parasitising prosobranch snails in a Western Mediterranean lagoon

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Molecular and morphological identification of larval opecoelids (Digenea: Opecoelidae) parasitising prosobranch snails in a Western Mediterranean lagoon

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Western Mediterranean

ABSTRACT

In a study of the digeneans parasitising molluscs in the Els Alfacs lagoon (Ebro Delta, Western Mediterranean) we found heavy infections with sporocysts emitting two types of cotylocercous cercariae in the prosobranch trochid gastropod *Gibbula adansonii* and with metacercariae in the prosobranch nassariid gastropod *Cyclope neritea*. A comparative analysis using ITS ribosomal DNA sequences from these larval stages and published sequences of 17 larval and adult opecoelid stages allowed us to elucidate the life-cycle of *Macvicaria obovata* and to confirm the identification of *Cainocreadium labracis* based on cercarial morphology. We provide molecular evidence for the identification and the first detailed morphological descriptions of the intramolluscan larval stages of the two opecoelid species as well as partial 28S rDNA sequences to aid future studies on systematic relationships within the Opecoelidae.

1. Introduction

Shallow inshore habitats of the Mediterranean coastal lagoons provide nursery and feeding grounds for many coastal fish species. The emergent vegetation after low tides provides habitat and refuge for many small fishes such as gobies, and the submerged vegetation cover has also a positive effect on the nursery role of coastal lagoons [1–4]. Many of these shallow habitats support diverse and abundant fish assemblages, and sustain significant populations of juveniles of many commercially important fish species [3].

Trematodes form an important part of marine communities in intertidal habitats, representing the most common parasites of intertidal animals [5]. Due to their shallow and relatively confined nature, lagoons provide excellent conditions for the transmission of the digenean trematodes that require two or more intermediate hosts for the completion of the complex life-cycles [6]. The greater accessibility of the lagoons also provides better opportunities for investigations on the ecology of trematode transmission compared with open-sea ecosystems. Studies carried out in the lagoons along the French and Italian coasts have resulted in elucidation of a substantial number of digenean life-cycles (56 species; of these 32 use fish as definitive hosts [6]). As suggested by Bartoli & Gibson [6] in their recent synopsis of elucidated digenean life-cycles in the lagoons of the northern coast of the Western Mediterranean, the fauna of these lagoons represents one of the best understood digenean marine faunas in the world. However, almost all the cited references by Bartoli & Gibson [6] relate to ecological studies on life-cycles rather than to systematics and few provide morphological data that would help larval identification in future studies.

The Opecoelidae Ozaki, 1925 is a large, cosmopolitan family of digeneans, represented in the Western Mediterranean by at least 25 named species parasitising marine fishes [7]. Cercariae develop in sporocysts and possess a stylet and characteristic cotylocercous tail; these in combination with a smooth tegument represent distinguishing features at the family level. However, species identification of opecoelid cercariae using morphological characters was deemed impossible [8] and demonstrating the links to the successive larval stages in intermediate hosts and the adult stages in definitive hosts requires experimental infections. To date the life-cycles of four opecoelid species have been elucidated, i.e. those of *Cainoceadium labracis* (Dujardin, 1845) [9–11]; *Helicometra fasciata* (Rudolphi, 1819) [8,12–15]; *Helicometra gobii* (Stossich, 1883) [12–15]; and *Podocotyle scorpaenae* (Rudolphi, 1819) [16]. Notably, links between larval and adult stages for two of these species have been established with the aid of molecular tools [8,16].

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In a study of the digeneans parasitising molluscs in the Els Alfacs lagoon (Ebro Delta, Spain) we found heavy infections with sporocysts emitting cotylocercous cercariae of two morphotypes in *Gibbula adansonii* (Payraudeau, 1826) (Prosobranchia, Trochidae) and with metacercariae in *Cyclope neritea* (L., 1758) (Prosobranchia, Nassariidae). We generated ITS ribosomal DNA sequences from these larval stages and carried out a comparative analysis using the sequences of opecoelid larval and adult stages reported by Jousson et al. [8,17] and Jousson & Bartoli [18]. This allowed us to elucidate the life-cycle of *Macvicaria obovata* (Molin, 1859) and to confirm the identification of *Cainocreadium labracis* (Dujardin, 1845) based on cercarial morphology. In this paper we provide molecular evidence for the identification and the first detailed morphological descriptions of the intramolluscan larval stages of these two opecoelid species.

2. Materials and methods

2.1. Sample collection

A total of 514 *G. adansonii* and 129 *C. neritea* was collected at the "Beach of the Eucalyptus" (40°37′35.06″N 0°44′30.52″E) in Els Alfacs lagoon (Ebro Delta, Spain) between March and July of two subsequent years, 2010 and 2011. Due to the open connection to the sea, salinity in the lagoon ranged from 39 to 44‰ and temperature varied from 13 °C to 34 °C (measurements taken at sampling dates). The lagoon has muddy soft sediment with a patchy cover formed by the dwarf eelgrass *Zostera noltii* Hornemann, 1832. In addition to *G. adansonii*, this site was inhabited predominantly by two other gastropods, the nassariids *Nassarius reticulatus* (L., 1758) and *C. neritea*. Snails were collected manually from the surface of the leaves of *Z. noltii* and of the muddy substrate and transported to the laboratory in aerated seawater taken from the same locality.

2.2. Molecular data

For DNA extraction, two replicates of three to four cercariae of each morphotype ex *G. adansonii* and two metacercariae ex *C. neritea* fixed in 100% ethanol were dissolved in 300 μL of TNES-urea extraction and preservation buffer [19]. The samples were digested with 100 μg mL^{-1} Proteinase K overnight at 55 °C. Following a simple phenol-chloroform protocol for DNA extraction [20], the DNA was resuspended in 40 μL of RNAse/DNAse-free water and left to dissolve overnight in a fridge. Polymerase chain reaction amplifications (PCRs) were performed with a programmable thermal cycler (Techne TC-512) in a final volume of 30 μL containing ~0.5 units of ThermoPrime Plus DNA polymerase and the related 10× buffer containing 1.5 mM $MgCl_2$ (ABgene), 0.2 mM of each dNTP, 0.5 μM of each primer and approximately 100 ng of template DNA. Two fragments of the ribosomal DNA were amplified: the internal transcribed spacer region (ITS1 + 5.8S + ITS2) and partial 28S rDNA (domains D1–D2). Complete ITS rDNA sequences were amplified using primers S18 (5′-TAACAGGTCTGTGATGCC-3′) and L3T (5′-CAACTTTCCCTCACGGTACTTG-3′; [8]) and partial 28S rDNA sequences were amplified using primers U178F (5′-GCACCCGCTGAAYTTAAG-3′) and L1642R (5′-CCAGCGCCATCCATTTTCA-3′; [21]). The following thermocycling profile was used for amplification of both gene regions: denaturation (94 °C for 3 min); 35 cycles of amplification (94 °C for 50 s, 55 °C for 1 min and 72 °C for 1 min); and 4 min final extension at 72 °C. PCR amplicons were gel-excised and purified using illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare UK Ltd.), cycle-sequenced from

Table 1
List of species used in the molecular study, their hosts, stage of the life-cycle (isolate where available) and accession numbers of the ITS sequences and 28S rRNA sequences generated in the present study. Authors' original identifications of isolates as submitted to Genbank is listed first; those resulting from the cited or subsequent studies are given in parentheses.

Species	Host (parasite stage)	GenBank accession number	Source
Cainocreadium labracis	*Gibbula adansonii* (cercaria)	JQ694148 (ITS) JQ694144 (28S)	Present study
Macvicaria obovata	*Gibbula adansonii* (cercaria)	JQ694145 (ITS) JQ694146 (28S)	Present study
Macvicaria obovata	*Cyclope neritea* (metacercaria)	JQ694149 (ITS) JQ694147 (28S)	Present study
Cainocreadium labracis (described as *Cainocreadium dentecis* n. sp. by Jousson & Bartoli, 2001)	*Dentex dentex* (adult)	AJ241795	Jousson et al. (1999); Jousson & Bartoli (2001)
Cainocreadium labracis (described as *Cainocreadium dentecis* n. sp. by Jousson & Bartoli, 2001)	*Haliotis tuberculata* (cercaria; isolate C2)	AJ241806	Jousson et al. (1999); Jousson & Bartoli (2001)
Opecoelidae gen. sp. (identified as *Cainocreadium labracis* by Jousson & Bartoli, 2001)	*Calliostoma striatum* (cercaria; isolate C4)	AJ241808	Jousson et al. (1999); Jousson & Bartoli (2001)
Opecoelidae gen. sp. (identified as *Cainocreadium labracis*? by Jousson et al., 1999)	*Gibbula adansonii* (cercaria; isolate C3)	AJ241807	Jousson et al. (1999)
Gaevskayatrema perezi	*Symphodus roissali* (adult)	AJ241800	Jousson et al. (1999)
Helicometra fasciata	*Symphodus rostratus* (adult)	AJ241793	Jousson et al. (1999)
Macvicaria alacris	*Labrus merula* (adult)	AJ241801	Jousson et al. (1999)
Macvicaria crassigula	*Diplodus annularis* (adult)	AJ277372	Jousson et al. (2000)
Opecoelidae gen. sp. (identified as *Macvicaria crassigula* by Jousson et al., 1999)	*Paracentrotus lividus* (metacercaria; isolate M1)	AJ241814	Jousson et al. (1999)
Macvicaria mormyri	*Lithognathus mormyrus* (adult)	AJ241802	Jousson et al. (1999; 2000)
Opecoelidae gen. sp. (identified as *Macvicaria obovata* by Jousson et al., 1999)	*Tricolia speciosa* (metacercaria; isolate M3)	AJ241816	Jousson et al. (1999)
Opecoelidae gen. sp.	*Hippolyte inermis* (metacercaria; isolate M4)	AJ241817	Jousson et al. (1999)
Pachycreadium carnosum	*Dentex dentex* (adult)	AJ241799	Jousson et al. (1999)
Peracreadium characis	*Diplodus puntazzo* (adult)	AJ241796	Jousson et al. (1999)
Podocotyle scorpaenae	*Scorpaena scrofa* (adult)	AJ241794	Jousson et al. (1999)
Pseudopycnadena fischthali	*Diplodus vulgaris* (adult)	AJ241798	Jousson et al. (1999)
Pycnadenoides senegalensis	*Sparus aurata* (adult)	AJ241797	Jousson et al. (1999)

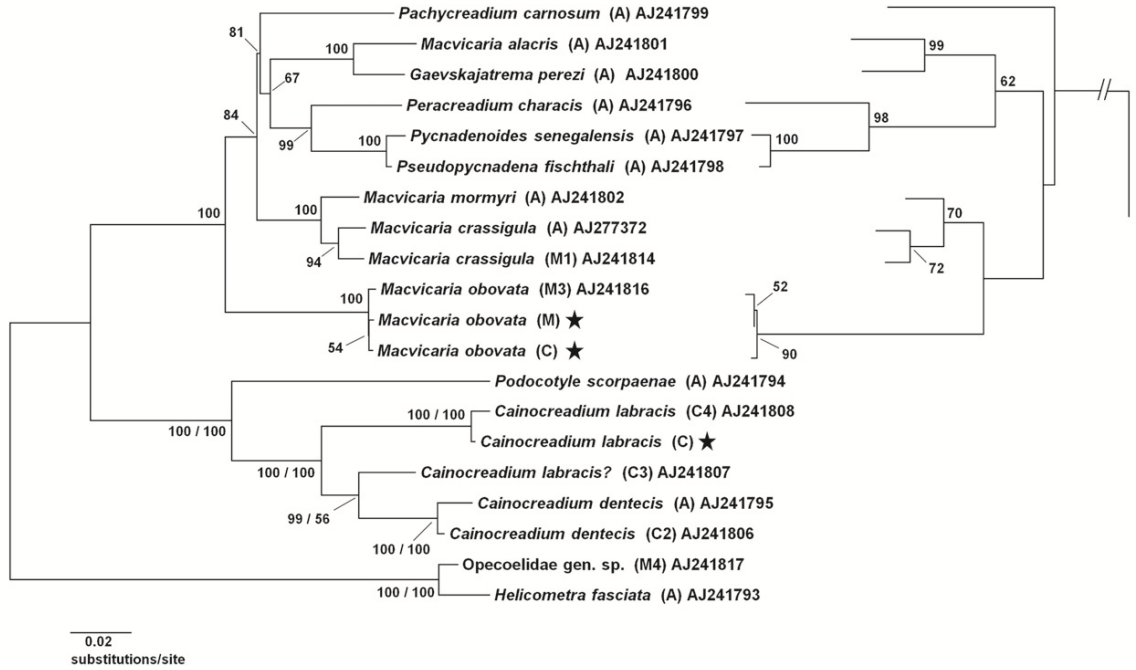

Fig. 1. Phylogenetic trees resulting from the neighbour-joining (NJ, left) and maximum-likelihood analysis (ML, right; part showing different topologies only) using ITS ribosomal DNA sequences (ITS1, 5.8S, ITS2) for the larval isolates ex *Gibbula adansonii* and *Cyclope neritea* and published sequences for 17 larval and adult opecoelid stages. The newly generated sequences are marked with a star. Regions of ambiguous alignment were removed. Combined nodal support (values >50%, 500 replicates) for the same topology given as NJ/ML. Scale bar indicates substitutions/site. Life-cycle stage indicated by letters: C, cercariae; M, metacercariae; A, adult. Numbers indicate isolates studied by Jousson et al. (1999).

both strands using ABI BigDye™ Terminator v3.1 Ready Sequencing Kit, alcohol-precipitated, and run on an ABI 3730 sequencer (Applied Biosystems). The PCR primers and, in the case of 28S rDNA products, the internal primer LSU1200R (5′-GCATAGTTCACCATCTTTCGG-3′; [21]) were used for cycle sequencing. Contiguous sequences were assembled and edited using Bioedit v7.0.5. (©1997–2005; [22]).

The newly generated ITS sequences were aligned with sequences of species of the family Opecoelidae available on GenBank (detailed in Table 1) using Mafft v5.531 [23], with the default gap parameters (gap opening penalty of 1.53 and gap extension penalty of 0.0) and using L-INS-i accuracy-oriented strategy as an iterative refinement method for incorporating local pairwise alignment information. Thereafter, with the aid of Gblocks v 2.0 [24] regions of ambiguous alignment were detected and removed from the analyses using the following parameter set: "Minimum Number of Sequences for a Conserved Position" set to 11; "Minimum Number of Sequences for a Flank Position" set to 12; "Maximum Number of Contiguous Non-conserved Positions" set to 20; "Minimum Length of a Block" set to

Table 2
Pairwise nucleotide sequence comparisons between *Macvicaria* spp. and *Cainocreadium* spp., calculated as percentage of nucleotide differences (gaps treated as missing data) for the aligned ITS sequences ($n = 2181$ bps). Newly generated sequences marked with a star. Life-cycle stage indicated by letters in parentheses: C, cercariae; M, metacercariae; A, adult. Numbers indicate isolates studied by Jousson et al. (1999).

No.		1	2	3	4	5	6
	Macvicaria spp.						
1	*M. crassigula* (A) AJ277372						
2	*M. crassigula* (M1) AJ241814	1.5					
3	*M. alacris* (A) AJ241801	9.7	10.0				
4	*M. obovata* (C)*	8.0	8.3	11.7			
5	*M. obovata* (M)*	7.9	8.2	11.6	0.3		
6	*M. obovata* (M3) AJ241816	8.0	8.0	11.9	0.5	0.2	
7	*M. mormyri* (A) AJ241802	3.3	2.8	9.4	7.6	7.6	7.3
	Cainocreadium spp.						
1	*C. labracis* (C4) AJ241808						
2	*C. labracis* (C)*	0.7					
3	*C. dentecis* (C2) AJ241806	8.6	8.3				
4	*C. labracis*? (C3) AJ241807	8.1	7.8	4.4			
5	*C. dentecis* (A) AJ241795	9.4	9.0	1.3	5.3		

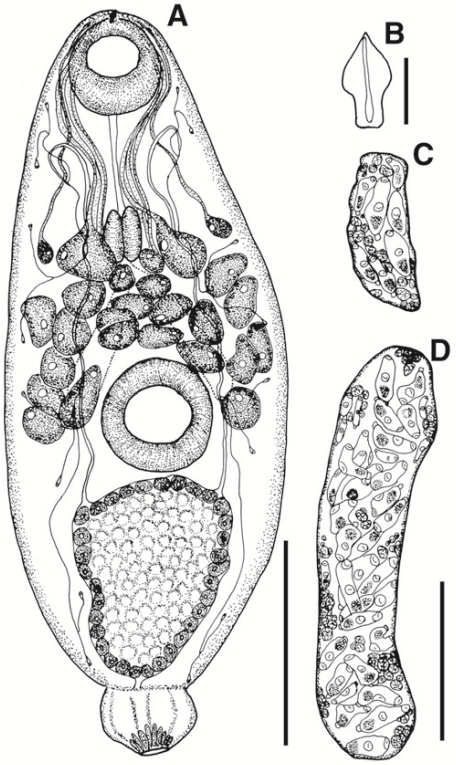

Fig. 2. Larval stages of *Cainocreadium labracis* ex *Gibbula adansonii*. A. Naturally emitted cercaria, examined live after staining with neutral red; B. Stylet; C. Mother sporocyst; D. Daughter sporocyst. Scale-bars: A, 100 μm; B, 10 μm; C–D, 500 μm.

10; and "Allowed Gap Positions" set to "With Half". Due to the large ITS sequence divergence between more distantly related opecoelid genera [25], an outgroup was not ascribed in phylogenetic analyses.

In order to estimate the position of the newly generated opecoelid sequences in relation to members of the family Opecoelidae, the ITS dataset (with regions of ambiguous alignment removed) was analysed using two different methods. First, neighbour-joining (NJ) analysis [26] applied to distances corrected for multiple hits, and for unequal transition and transversion rates using Kimura's two-parameter model [27] was carried out in MEGA5 [28]. Nodal support was estimated from 500 bootstrap replicates. Distance matrices (p-distance model, i.e. percentage of pairwise character differences with pairwise deletion of gaps) were also calculated with MEGA5 based on complete alignment (2181 bp). In addition to the distance-based method, maximum likelihood (ML) phylogenetic inference was performed with RAxML [29] under the general time-reversible model with an estimate of gamma-distributed among-site rate variation (GTR + Γ). Nodal support was estimated from 500 bootstrap re-samplings. FigTree v1.3.1 [30] was used to visualize the trees.

2.3. Morphological data

In the laboratory, individual *G. adansonii* were isolated in well cell plates containing 3 ml of filtered seawater and maintained for 24 h at 20 °C and a light:dark cycle of 14:10 h, without providing any food. Thereafter, the wells were checked for spontaneous cercarial emission. Cercarial morphology was initially studied on live material under the light microscope, using a vital stain (neutral red). Samples fixed in 70% ethanol were stained with aluminium carmine or with iron-acetocarmine, dehydrated in an ethanol series, cleared in dimethyl phthalate and mounted in Canada balsam. Body organs and tissues of *C. neritea* were examined and encysted metacercariae isolated and studied under light microscope. Digital photographs were taken with a Leica DC300 camera on a Leica DMR microscope. Cercarial samples used for scanning electron microscopy (SEM) were fixed in 2.5% glutaraldehyde and 70% ethanol. Specimens fixed in glutaraldehyde were washed three times (15 min) in 0.1 M phosphate buffer (pH 7.4); post fixed in 2% osmium tetroxide for 2 h; washed in 0.1 M phosphate buffer (three changes, 15 min each); and dehydrated in an acetone series. Specimens

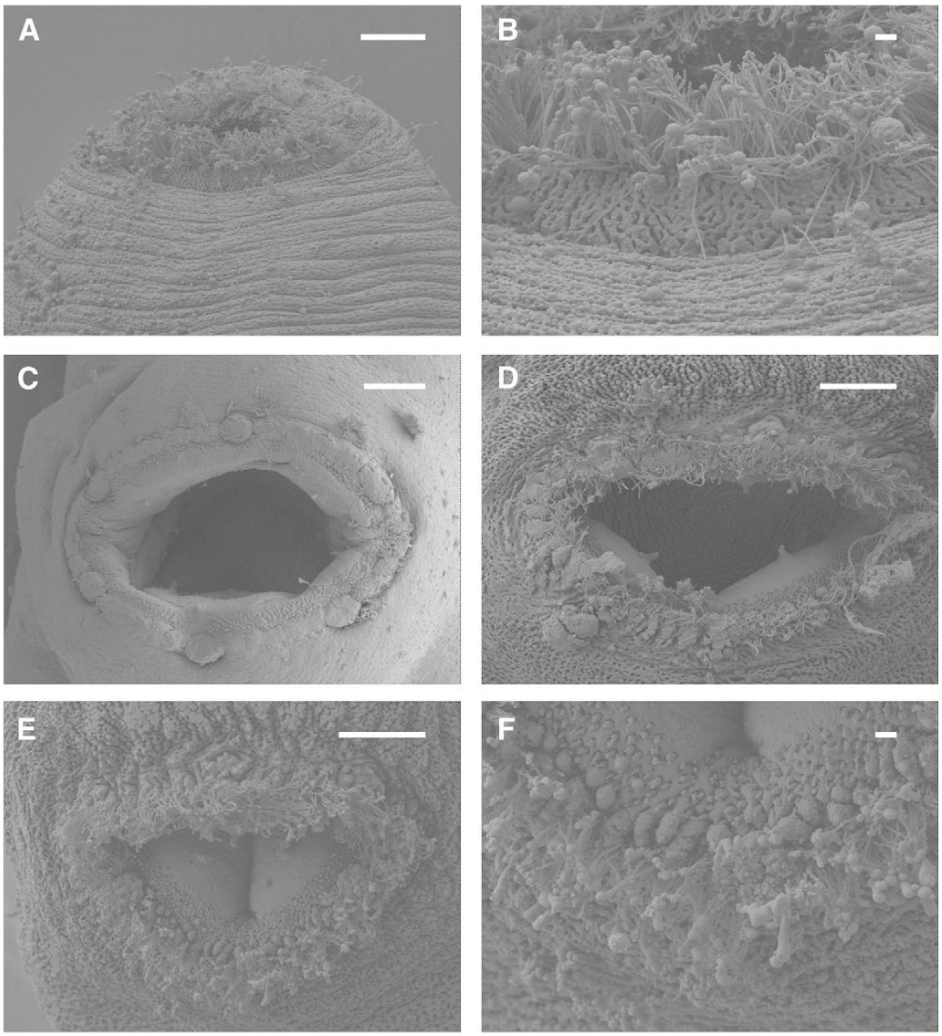

Fig. 3. SEM microphotographs of cercariae of *Cainocreadium labracis*. A. Anterior extremity of the body showing a circle of filiform microtriches around the opening of the oral sucker; B. Detail of A at higher magnification showing the area with smooth tegument between the ring of microthiches and the tegumental fold; C. Ventral sucker bearing six large dome-shaped papillae; D. Ventral sucker with filiform microtriches and three sensilla of the innermost circle; E. Ventral sucker with ring of dense filiform microtriches, followed by two alternating rows of papilliform structures and an inner area covered by 5–7 alternating rows of spines; F. Detail of E, showing the inner surface of the posterior margin of ventral sucker. All specimens were fixed in glutaraldehyde, except the one illustrated in C (fixed in 70% ethanol). Scale-bars: A, C–E, 10 μm; B, F, 1 μm.

fixed in ethanol were dehydrated in an ethanol series; transferred to 100% acetone using graded series of absolute ethanol/absolute acetone. After dehydration all specimens were critical point-dried with liquid CO and sputter-coated with gold. Samples were examined using a JEOL JSM 7401-F at an accelerating voltage of 4 kV. All measurements in the text are in micrometres.

3. Results

3.1. Molecular identification

G. adansonii emitted cercariae that we initially assigned to two morphotypes based on slight differences in their size, hereafter referred to as 'small morph' and 'large morph' for simplicity. We generated complete ITS rDNA sequences of 1739 bp (JQ694145) and 1618 bp (JQ694148) for the cercarial isolates of the two morphotypes and of 1732 bp (JQ694149) for the metacercariae and partial 28S rDNA sequences of 1458 bp (JQ694146), 1562 bp (JQ694144) and 1599 bp (JQ694147), respectively. The two replicate sequences per morphotype were identical for both gene regions. The alignment of a total of 20 opecoelid ITS sequences (Table 1) incorporated 2181 characters (bp and gaps); of these 1297 unambiguously aligned positions were selected for phylogenetic analyses according to the results of Gblocks analysis. Neighbour-joining (NJ) and maximum-likelihood (ML) analyses resulted in trees with similar topologies (Fig. 1, local differences shown by providing topology and branching patterns for both methods). Moreover, both analyses revealed the same relationship between the newly generated sequences and their closest relatives.

The sequences of the 'small morph' and the metacercarial isolate ex *C. neritea* formed a highly supported clade (bootstrap NJ/ML: 100/90) with *Macvicaria obovata* in both analyses. Sequence divergence within this clade was much lower than the intraspecific value for *Macvicaria crassigula* (Linton, 1910) (0.2–0.5% *vs* 1.5%) and well below the levels of interspecific divergence among known *Macvicaria* spp. (2.8–11.9%, Table 2). Notably, all *Macvicaria* spp. isolates formed a strongly supported clade in the ML analysis, except for *Macvicaria alacris* (Looss, 1901) that clustered with *Gaevskajatrema perezi* (Mathias, 1926) (Fig. 1); a similar relationship was depicted by Jousson et al. [8]. The sequence of the isolate of *M. alacris* ex *Labrus merula* L. provided by these authors also differed from that of *G. perezi* by 4.1% (compared to 9.4–11.9% divergence in relation to sequences for the remaining *Macvicaria* spp. isolates). Removal of this isolate resulted in a lower range of interspecific divergence within *Macvicaria* (2.8–10.0%, Table 2).

The isolates representing species of *Cainocreadium* also formed a highly supported monophyletic clade (bootstrap NJ/ML: 100/100). The sequence of the 'large morph' clustered (bootstrap NJ/ML: 100/100) with the sequence of cercarial isolate C4 ex *Calliostoma striatum* (L., 1758) identified as *C. labracis* by Jousson & Bartoli [18] (Fig. 1, Table 1). These two sequences differed by 0.7%, a value much lower than the known range for interspecific divergence within the genus *Cainocreadium* (5.3–9.4%, Table 2); this value was also lower than the intraspecific divergence for *C. dentecis* (1.3%). The two isolates of *C. dentecis* clustered together joined by an isolate (C3 ex *G. adansonii*) provisionally identified as *Cainocreadium labracis*? by Jousson et al. [8]. However, this isolate is separated from both *C. labracis* and *C. dentecis* (4.4–8.1%); these values are well above the values for intraspecific divergence within the genus *Cainocreadium* (Table 2).

Analyses of molecular data therefore suggest that the newly obtained cercarial isolates of the 'small morph' ex *G. adansonii* and the metacercarial isolate ex *C. neritea* can be confidently identified as *M. obovata* whereas the cercarial isolates of the 'large morph' are conspecific with *C. labracis*.

3.2. Taxonomic summary

3.2.1. Cainocreadium labracis (Dujardin, 1845) Nicoll, 1909

First intermediate host: *Gibbula adansonii* (Payraudeau, 1826) (Prosobranchia, Trochidae).

Locality: Lagoon Els Alfacs, Ebro Delta (Spain).

Prevalence: 22.04% (overall, 2010); 20.9%; (March, 2011); 17.6% (April, 2011); 30.8% (May, 2011).

3.2.1.1. Sporocysts (Fig. 2C,D). [Measurements based on 96 unflattened specimens *in vivo*.] Located in snail gonad and hepatopancreas. Mother sporocysts small, 257–900×157–247, motionless, pale orange, cylindrical with rounded extremities, filled with germinal balls, occasionally contain immature cercariae. Daughter sporocysts large, 900–2377×225–389, cylindrical, bright orange with darker spots, very mobile, contain many fully developed cercariae, some immature cercariae and germinal balls. Birth pore terminal.

3.2.1.2. Naturally emitted cercariae (Figs. 2A,B,3–4). [Measurements (means in parentheses) in description based on 10 specimens stained with neutral red and examined live under slight coverslip pressure; measurements of fixed cercariae taken from temporary (in marine water) and permanent (in Canada balsam) mounts are provided in Table 3.] Body elongate-oval, wider at level of ventral sucker, 208–402×74–133 (294×109). Forebody 114–226 (155) long, 46–63% (53%) of body length. Tegument smooth, with fine striations, 1–3 (2) thick. Tail very short, 23–41×31–52 (31×40), cup-shaped (cotylocercous), with six longitudinal groups of well-defined muscle fibres forming 'ridges'. Tegument with fine annulations and smooth

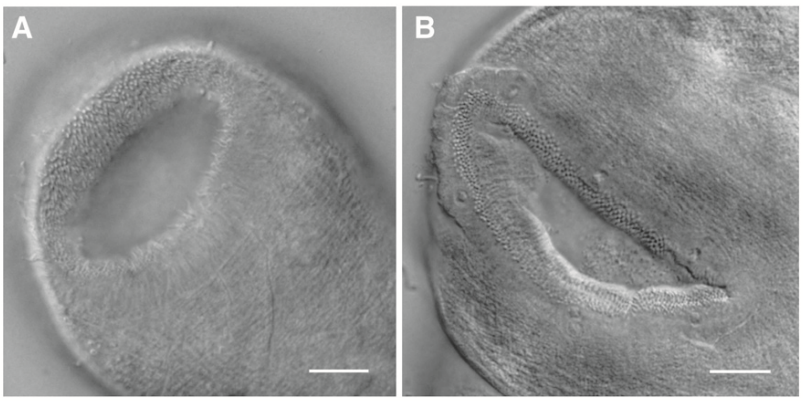

Fig. 4. Light-microscopy microphotographs of cercariae of *Cainocreadium labracis*. A. Oral sucker, with a circle of filiform microtriches; B. Ventral sucker opening surrounded by a narrow fleshly fold bearing six large papillae and an inner circle of filiform microtriches. Scale-bars: 10 μm.

Table 3
Comparative metrical data expressed as range (mean ± standard deviation) for alcohol-fixed cercariae of *Cainocreadium labracis* and *Macvicaria obovata* obtained from temporary mounts under slight coverslip pressure (samples A) and from permanent mounts in Canada balsam (samples B).

Species	*Cainocreadium labracis*		*Macvicaria obovata*	
Sample	Sample A	Sample B	Sample A	Sample B
	($n=10$)	($n=25$)	($n=10$)	($n=32$)
Body length	433–505	312–530 (409 ± 53)	304–345	195–343 (251 ± 30)
Body width	70–109	50–100 (77 ± 11)	102–116	46–90 (64 ± 7)
Tail length	27–39	22–35 (30 ± 3)	26–31	12–40 (21 ± 6)
Tail width	40–47	45–22 (34 ± 5)	38–43	28–37 (32 ± 2)
Forebody length	222–279	121–241 (194 ± 39)[a]	132–156	88–128 (106 ± 14)[a]
Oral sucker length	55–65	39–57 (48 ± 6)	54–59	29–57 (36 ± 6)
Oral sucker width	51–55	35–53 (42 ± 4)	43–54	30–49 (40 ± 4)
Stylet length	12–13	11–14 (12 ± 1)	9–10	9–10 (9 ± 1)
Stylet maximum width	4–6	4–6 (5 ± 1)	4–5	4–5 (4 ± 1)
Stylet width at base	3–4	–	2–3	–
Prepharynx length	44–53	23–59 (44 ± 12)	19–32	18–27 (18 ± 6)
Pharynx length	22–26	22–28 (23 ± 3)	19–20	19–26 (15 ± 4)
Pharynx width	8–17	9–11 (10 ± 1)	8–15	7–16 (12 ± 3)
Ventral sucker length	77–82	48–64 (56 ± 5)	54–63	34–52 (48 ± 4)
Ventral sucker width	51–75	41–65 (51 ± 5)	62–75	36–47 (42 ± 3)
Excretory vesicle length	102–176	50–155 (94 ± 25)	102–116	54–122 (83 ± 15)
Excretory vesicle maximum width	59–82	36–82 (56 ± 11)	50–91	40–64 (50 ± 7)

[a] ($n=10$).

posterior margin. Posterior half of the tail protrusible (up to 7–26; mean 15) comprised of tubular glandular structures probably involved in adhesion to substrate.

Oral sucker subterminal, muscular, spherical, 33–47 × 33–45 (40 × 40). Anterior margin encircled by an area (3–5 wide) of dense filiform microtriches (5–6 long) (Fig. 3A,B) also visible under light-microscope (Fig. 4A). Narrow area of smooth tegument with a somewhat different appearance located posterior to microtriches region and delimited from the remaining body tegument by a faint fold (Fig. 3B). Inner surface of oral sucker covered with 5–8 alternating rows of small spines. Ventral sucker large, subglobular, 32–57 × 42–59 (47 × 50), opening surrounded by a narrow fleshy fold (Fig. 4B) bearing six large papilla (Fig. 3C; 4B) encircling three inner regions: a ring (3–5 wide) with dense filiform microtriches (4–5 long) (Fig. 3D–F) also visible under light-microscope (Fig. 4B); a narrow ring (2 alternating rows) of papilliform structures and an inner area covered by 5–7 alternating rows of spines (Fig. 3E,F). Sucker length ratio 1:0.86–1.32 (1:1.19); width ratio 1:1.19–1.40 (1:1.28).

Stylet in a depression just anterior to anterior margin of oral sucker, very small, 11.5–12.5 long and 3–4 wide at base; with a single sharp point and lateral thickenings (1.5–2 wide), located at the middle third of stylet (Fig. 2B); maximum width at this level 4–5. Cystogenous gland-cells numerous, widespread throughout body. Two types of penetration gland-cells present in forebody (Fig. 2A): one pair of small more intensely stained gland-cells, 6–13 × 3–7 (9 × 4) with thin ducts opening laterally at mid-level of oral sucker; 24–25 larger gland-cells, 10–21 × 12–33 (15 × 19), comprising one anterior pair at level of pharynx, seven lateral pairs between pharynx and mid-level of ventral sucker and eight-nine median gland-cells above ventral sucker. Gland-cell ducts in two groups, open close to anterior margin of oral sucker on both sides of stylet. Prepharynx long, 22–61 (42). Pharynx elongate, 12–31 × 10–21 (20 × 16). Caeca short. Genital primordium dorsal to ventral sucker, visible in iron acetocarmine-stained material only. Excretory vesicle saccular, wider anteriorly, 58–118 × 48–85 (88 × 71), thick-walled, 7–10, lined by almost round-shaped cells, 7–10 × 7–9. Excretory pore at body-tail junction. Flame-cell formula: 2 [(2 + 2) + (2 + 2)] = 16.

3.2.1.3. Behaviour and patterns of sensory receptors. Cercariae of *C. labracis* attached to the substrate upon emergence and the body moved only in lateral directions in a reverse pendulum-swing motion; these movements became more vigorous under light regime. The relatively sedentary cercariae possessed a well developed system of sensory receptors represented by three types of sensilla (*sensu* Bogéa & Caira [31]): receptors with a high tegumentary collar and a short cilium-like structure and with a low or moderately high tegumentary collar and no cilium-like structures. The basic chaetotaxy reconstructed from SEM examination of the cercariae agreed well with that described for *C. labracis* by Bayssade-Dufour & Maillard [32] especially concerning the cephalic region (identical circles C_I and C_{II}: $1C_IV$-$4C_IL$-$1C_ID$; $1C_{II}0$-$1C_{II}1$-$1C_{II}2$-$1C_{II}3$-$1C_{II}4$). However, due to the treatment of the material for SEM relatively few sensilla were detected in circles C_{III} and C_{IV} and on the lateral and dorsal body regions and those of the stylet region appeared to be invaginated within the stylet depression thus preventing exact counts. Nevertheless, SEM study provided a better resolution for the pattern of sensory receptors of the ventral sucker comprising an inner circle of three sensilla (Fig. 3D), an outer circle of six large dome-shaped papillae (Fig. 3C), and an intermediate circle of eight sensilla (i.e. $3S_I$-$8S_{II}$-$6S_{III}$).

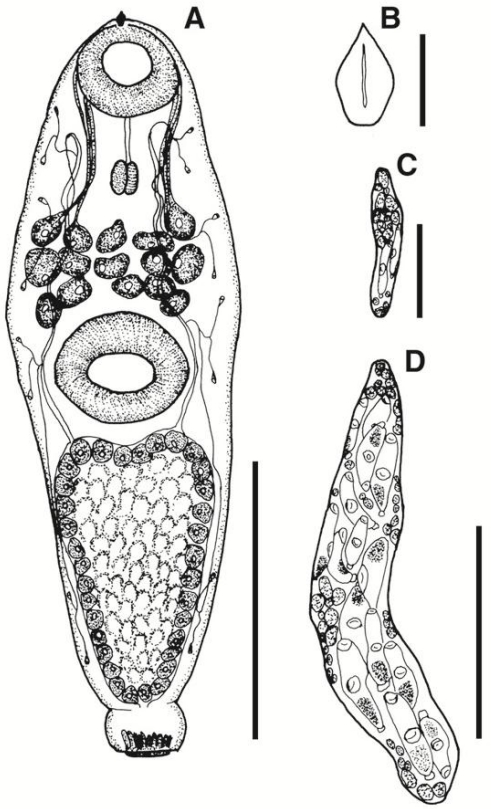

Fig. 5. Larval stages of *Macvicaria obovata* ex *Gibbula adansonii*. A. Naturally emitted cercaria, examined live after staining with neutral red; B. stylet; C. Mother sporocyst; D. Daughter sporocyst. Scale-bars: A, 100 μm; B, 10 μm; C, 400 μm; D, 500 μm.

3.2.2. Macvicaria obovata (Molin, 1859)

First intermediate host: *Gibbula adansonii* (Payraudeau, 1826) (Prosobranchia, Trochidae).

Second intermediate host: *Cyclope neritea* (L., 1758) (Prosobranchia, Nassariidae).

Locality: Lagoon Els Alfacs, Ebro Delta (Spain).

Prevalence: Cercariae: 23.1% (overall, 2010); 0.9% (March, 2011); 3.3% (April, 2011); 7.7% (May, 2011). Metacercariae: 100% (June, 2011; $n=69$); 96.7% (July, 2011; $n=60$).

3.2.2.1. Sporocysts (Fig. 5C,D). [Measurements based on 176 unflattened specimens *in vivo*.] Located in snail gonad and hepatopancreas. Mother sporocysts very small, 340–600×119–258, motionless, whitish, elongate-oval to cylindrical, filled with germinal balls. Daughter sporocysts larger, 600–1650×177–339, cylindrical, colourless, mobile, contain fully developed cercariae and some immature cercariae. Birth pore terminal.

3.2.2.2. Naturally emitted cercariae (Figs. 5A,B; 6–7). [Measurements (means in parentheses) in description based on 10 specimens stained with neutral red and examined live under slight coverslip pressure; measurements of fixed cercariae taken from temporary (in marine water) and permanents (in Canada balsam) mounts are provided in Table 3.] Body elongate-oval, small, 197–296×62–88 (248×80), with maximum width at level just anterior to ventral sucker. Forebody 76–138 (109) long, 38–48% (44%) of body length. Tegument 1–4 (3) thick, smooth. Tail cup-shaped (cotylocercous), very short, 13–18×19–28 (16×23). Posterior half of the tail protrusible (up to 2–17; mean 9) comprised of glandular structures probably involved in adhesion to substrate.

Oral sucker subterminal, globular, 30–40×32–39 (34×35). Anterior margin encircled by an area (3–5 wide) of dense filiform microtriches (3–4 long) (Fig. 6A,B). Posterior to this region is a narrow

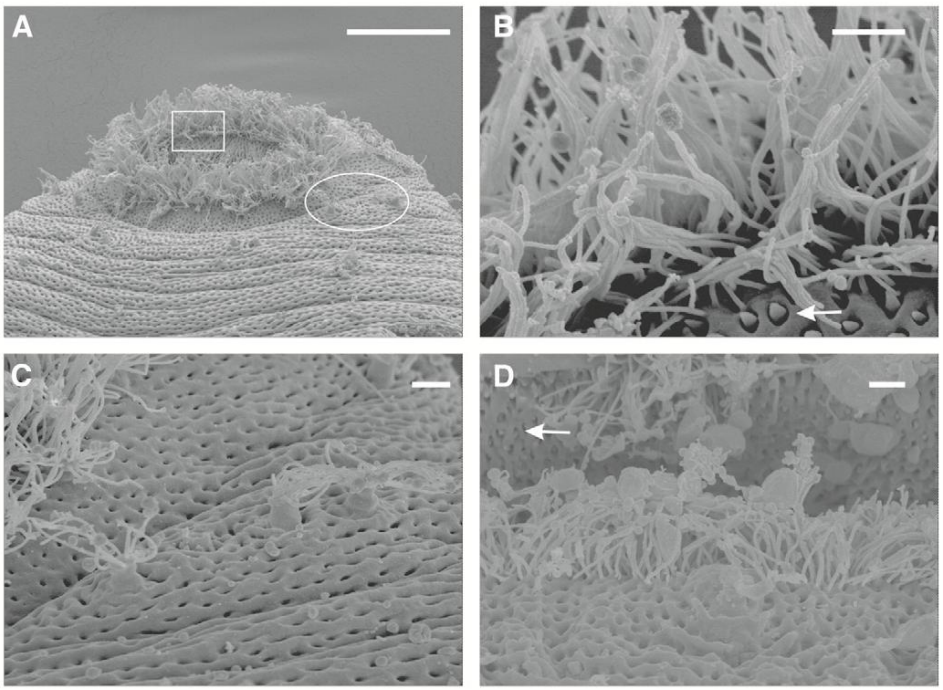

Fig. 6. SEM microphotographs of cercariae of *Macvicaria obovata*. A. Anterior extremity of the body showing a circle of dense filiform microtriches around the opening of the oral sucker, followed by a narrow smooth area, delimited from the remaining body by a faint fold. B. Detail of A (indicated by a square) at higher magnification with an arrow indicating one of the spines on the inner surface of the oral sucker; C. Detail of A (indicated by an ellipse) at higher magnification, showing the three sensilla of circle C_{III} with multiple long cilium-like structures; D. Detail of the ventral sucker with a circle of dense filiform microtriches and rows of spines covering the inner surface (arrow). All specimens were fixed in glutaraldehyde. Scale-bars: A, 10 μm; B–D, 1 μm.

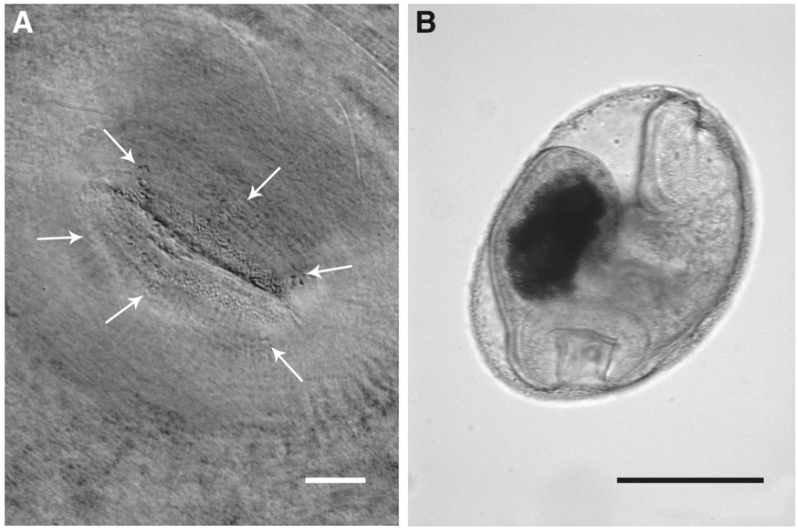

Fig. 7. Light-microscopy microphotographs of *Macvicaria obovata*. A. Ventral sucker of the cercaria showing the opening surrounded by a ring of filiform microtriches and six large papilla (arrows); B. Encysted metacercaria. Scale-bars: A, 10 μm; B, 100 μm.

smooth area delimited from the remaining body tegument by a faint fold (Fig. 6A). Inner surface of oral sucker covered with 6–7 alternating rows of small spines (Fig. 6B). Ventral sucker located at mid-body, 36–49×37–48 (41×44), opening surrounded by six large papilla (Fig. 7A) encircling two inner regions: a ring (2–3 wide) with filiform microtriches (3–4 long) (Fig. 6D) also visible under light-microscope (Fig. 7A); and an inner area covered by 7–8 alternating rows of spines (Fig. 6D). Sucker length ratio 1:1.04–1.35 (1:1.19); width ratio 1:1.15–1.35 (1:1.25).

Stylet in a depression just anterior to anterior margin of oral sucker, very small, 8.5–9 long and 3 wide at base; with a single sharp point and lateral thickenings (2 wide), located at the middle of stylet (Fig. 5B); maximum width at this level 5. Cystogenous gland-cells numerous, widespread throughout body. Penetration gland-cells total 14–15, relatively small, poorly stained; between pharynx and ventral sucker; comprise three lateral pairs plus two–three median gland-cells (Fig. 5A). Gland-cell ducts in two groups, open close to anterior margin of oral sucker on both sides of stylet. Prepharynx short, 15–35 (25). Pharynx subglobular, 9–15×8–14 (12×12). Two patches of dark granules seen anterior to pharynx in ethanol-fixed specimens. Caeca not seen. Genital primordium visible in iron acetocarmine-stained material only, dorsal to ventral sucker. Excretory vesicle broader anteriorly, heart-shaped, 77–102×40–65 (88×52), thick-walled, 5–7, lined by round to subtriangular cells, 4–8×7–10. Excretory pore at body-tail junction. Flame-cell formula: 2 [(2+2)+(2+2)]=16.

3.2.2.3. Metacercariae (Fig. 7B). [Measurements (means in parenthesis) in description based on nine specimens examined live under slight coverslip pressure.] Metacercariae in thin-walled (2–4), elongate-oval cysts, 227–248×169–231 (235×214), located in snail foot, hepatopancreas and gonad. Body robust, oval, with maximum width at level of ventral sucker, 227–339×138–209 (276×180). Forebody 97–160 (128) long, 42–51% (46%) of body length. Tegument thick, smooth, with fine striations. Oral sucker subterminal, strongly muscular, transversely elongate, 34–71×53–106 (55×73). Inner surface of oral sucker covered with small spines. Ventral sucker massive, located on distinct eminence in middle body third, transversely elongate, 61–91×63–105 (78×91). Sucker length ratio 1:1.05–1.77 (1:1.36); width ratio 1:0.99–1.71 (1:1.23). No stylet seen. Prepharynx short; pharynx massive, elongate-oval, 19–38×7–15 (27×12). Oesophagus very short, bifurcation just anterior to ventral sucker; caeca reach to about midlevel of excretory vesicle in hindbody. Excretory vesicle large, saccular to heart-shaped, wider anteriorly, 58–91×90–162 (74×126); excretory pore terminal.

3.2.2.4. Behaviour and patterns of cercarial sensory receptors. Upon emergence cercariae of *M. obovata* attached to the substrate. Movements of the so 'fixed' cercariae involved contractions so that the body formed an immobile sphere close to the substrate, and vertical expansions of the body in reaction to light. Only during light periods cercariae sometimes showed weak pendulum-swing motions. Cercariae of *M. obovata* attached more frequently to the walls of the wells than cercariae of *C. labracis*. Three types of sensory receptors (*sensu* Bogéa & Caira [31]) were observed: with a very low tegumentary collar and a short cilium-like structure (circle C_I); with a moderately high tegumentary collar and a short cilium-like structure (circle C_{III}); and with a moderately high tegumentary collar and multiple (6–7) long cilium-like structure (second group of circle C_{III}; see Fig. 6A,C). The chaetotaxy patterns reconstructed from SEM examination revealed the number of sensilla in circles C_I ($1C_IV$-$3C_IL$-$1C_ID$) and C_{III} ($2C_{III}1$-$3C_{III}2$-$1C_{III}3$; ventral aspect only), the presence of seven sensilla in the group St_2 ($3+4\,St_2$) and two lateral sensilla of circle C_{IV}.

4. Discussion

Elucidation of digenean life-cycles in the marine environment has proven to be a difficult task, most of research in the past being focused on the more accessible coastal lagoons (reviewed by Bartoli & Gibson [6]). Because digenean identification is based on the morphology of the adult stages, establishment of links between the larval and adult stages invariably required experimental completion of the life-cycles until recently. Molecular approaches to species identification resulting in rapid accumulation of digenean sequence data, especially of the ITS region of rRNA gene, have provided sound basis for examination *via* primary sequence comparisons of digenean species complexes, identification of cryptic species and life-cycle elucidation (reviewed by Nolan & Cribb [25]). Regarding the marine representatives of the large and diverse family Opecoelidae, Jousson and colleagues [8,16–18,33] have reported sequences of the entire ITS from 17 adult opecoelid species from marine fishes in the Mediterranean, belonging to 13 genera, and thus have established a molecular framework for identification of marine larval opecoelid stages.

Our analysis of the newly generated sequences within this framework and the detailed morphological examination of the larval stages of the two sympatric opecoelids in *G. adansonii* from the Western Mediterranean has allowed their identification, thus elucidating the life-cycle of *M. obovata* and providing molecular evidence for a second snail species (i.e. *G. adansonii*) acting as first intermediate host in the life-cycle of *C. labracis*. Furthermore, we provided representative 28S rDNA sequences and ample morphological descriptions of the intramolluscan larval stages of both species that will enable their identification in future studies and aid establishment of relationships at the suprraspecific level.

Comparative sequence analysis revealed conspecificity with *M. obovata* of the 'small morph' opecoelid cercariae and the metacercariae ex *C. neritea* and the 'large morph' cercariae were found to correspond to *C. labracis*. In both cases ITS sequences exhibited notably low intraspecific (0.2–0.7%) compared with interspecific (2.8–10.0%) variation. These results, combined with previous knowledge, reveal that in the Mediterranean the life-cycle of *M. obovata* is completed with *G. adansonii* as the first intermediate host, *Tricolia speciosa* (von Mühlfeld, 1824), *C. neritea* as the second intermediate hosts, and *Oblada melanura* (L.), *Sparus aurata* L., and *Blennius* spp. as the definitive hosts [7,8,34,35]. The incongruent position and close relationship with *G. perezi* of the isolate identified by Jousson et al. [8] as *M. alacris*, the type-species of the genus *Macvicaria* deserves a comment. Both *M. alacris* and *G. perezi* are parasites of labrid fishes being recorded in a wide range of species with overlapping feeding ecologies (*Symphodus cinereus* (Bonnaterre), *S. ocellatus* (L.), *S. rostratus* (Bloch), *S. tinca* (L.) and *S. cinereus*, *S. melops* (L.), *S. roissali* (Risso), *S. tinca* and *L. merula*, respectively) [7,36]. It is therefore possible, that the position of the isolate of *M. alacris* ex *L. merula* indicates that a species of *Gaevskajatrema* has been misidentified by Jousson et al. [8].

The life-cycle of *C. labracis* was described based on an experimental study by Maillard [9]. He infected *G. adansonii* with miracidia developed from eggs from adult worms from *Dicentrarchus labrax* (L.) (type-host of *C. labracis*) and *Gobius niger* L. (second intermediate host) with cercariae emitted from the experimentally infected snails. Maillard [9] used the metacercariae from gobiids to 'close' the life-cycle by infecting young *D. labrax* and briefly described all stages of *C. labracis*. Jousson et al. [8] compared sequences of the entire ITS from 16 adult stages of opecoelids and from unidentified cercarial isolates collected from gastropod species of *Tricolia*, *Haliotis*, *Clanculus* and *Gibbula*. They suggested that a cercarial isolate ex *Haliotis tuberculata* L. 1758 (C2, see Table 1) corresponds to *C. labracis* ex *Dentex dentex* (L.) but could not identify two other isolates branching close to *C. labracis* (C3 ex *G. adansonii* and C4 ex *C. striatum*; Table 1). Based on the knowledge of the life-cycle of *C. labracis* elucidated by Maillard [9], Jousson et al. [8] hypothesised that the sequences in their '*C. labracis* clade' may correspond to two cryptic species: one using *D. labrax* and *G. adansonii* and one using *D. dentex* and *H. tuberculata* as definitive and first intermediate hosts, respectively. They could not test this hypothesis at the time due to the lack of sequences from adult worms from *D. labrax*.

Jousson & Bartoli [18] partially confirmed this hypothesis i.e. that sequences of two cryptic species were present in '*C. labracis*' clade of Jousson et al. [8]. However, *C. labracis* was represented by their newly obtained sequences from three adult specimens ex *D. labrax* and that from the cercarial isolate C4 ex *C. striatum*. The second species was represented by three adult specimens ex *D. dentex* and the cercarial isolate C2 ex *H. tuberculata*. Based on the evidence that the specimens isolated from *D. labrax* and *D. dentex* represent clearly distinct entities from molecular, morphological and statistical points of view, Jousson & Bartoli [18] described the form infecting *D. dentex* as a new species, *C. dentecis*. However, these authors did not attempt

Table 4
Comparative data (ranges observed on live specimens) for the cotylocercous cercariae with a single-pointed stylet reported from the Mediterranean and North East Atlantic coasts of Europe.

Species	*Macvicaria obovata*	*Cainocreadium labracis*	*Cainocreadium labracis* (syn. *Cercaria cotylura* Pagenstecher, 1862)	*Cercaria linearis* Lespès, 1857	*Cercaria ruvida* Palombi, 1938	*Cercaria pisaniae* Palombi, 1938
Locality	Ebro Delta (Spain)	Ebro Delta (Spain)	Thau Lagoon (France)	Off Naples (Italy); off Arcachon, Roscoff (France)[a]; Budle Bay, Millport (UK)[a]	Off Naples (Italy)	Off Naples; Pozzuoli Gulf (Italy)
Host	*Gibbula adansonii*	*Gibbula adansonii*	*Gibbula adansonii*	*Littorina littorea*; *Gibbula umbilicalis*; *Tricolia speciosa*	*Calliostoma conulus*; *Jujubinus striatus*	*Pisania maculosa*
Source	Present study	Present study	Maillard (1971)	Lespès (1857); Lebour (1911); Palombi (1938)	Palombi (1938)	Palombi (1938)
Sporocyst						
Length	340–600/600–1650[b]	257–900/900–2377[b]	>1500	1000–2600	1000	750
Width	119–258/177–339[b]	157–247/225–389[b]	—	400–600	200	180
Cercaria						
Body length	197–296	208–402	450–500	165–400	230	200
Body width	62–88	74–133	—	80–120	67	45
Tail length	13–18	23–41	—	15–50	31	66
Tail width	19–28	31–52	—	15–50	27	14
Stylet points	1 (lateral thickening)	1 (lateral thickening)	1	1 (lateral thickening)	1	1
Stylet length	9–10	12–13	—	16–18	10	—
Stylet maximum width	4–5	4–5	—	—	—	—
Penetration gland-cells (groups×no. of gland-cells)	2×6+3 median (14–15 in total)	2×9+8–9 median (26–27 in total)	2×12 (20–26 in total)	2×7 (14 in total)	2×4	2×2
Oral sucker width	32–39	33–45	—	35–75	46	25
Ventral sucker width	37–48	42–59	—	40–90	51	22
Pharynx length	9–15	12–31	—	20–24	—	Not evident
Pharynx width	8–14	10–21	—	12–16	—	Not evident
Excretory vesicle	Saccular, heart-shaped, lined by large cells	Saccular, wider anteriorly, lined by large cells	Elongate-saccular	Elongate-saccular, lined by large cells, not reaching ventral sucker	Elliptic, not reaching ventral sucker	Elongate
Flame-cell formula	2[(2+2)+(2+2)]	2[(2+2)+(2+2)]	2[(2+2)+(2+2)]	2[(2+2)+(2+2)]	—	—

[a] Localities in the North East Atlantic.
[b] Mother/daughter sporocysts.

to resolve the status of the cercarial isolate C3 ex *G. adansonii* provisionally identified as "*C. labracis*?" which they expected to represent a larval stage of *C. labracis*.

Our study provided molecular evidence that *G. adansonii* does act as the first intermediate host of *C. labracis* and thus adds to the few examples (e.g. [8,37,38]) of relaxed digenean host specificity towards the first snail intermediate hosts i.e. that two trochid snails (*C. striatum* and *G. adansonii*) serve as intermediate hosts of *C. labracis* in the Western Mediterranean. Furthermore, both comparative sequence and ML/NJ analyses indicate that the isolate C3 ex *G. adansonii* (*C. labracis*? of Jousson et al. [8]) represents another yet unidentified cryptic species of *Cainocreadium*.

The possibility of much higher opecoelid diversity and the virtual lack of modern descriptions of larval opecoelids prompted us to examine in detail the morphology of the cercariae of the two species. Examination of old literature sources revealed that some morphological data, however scarce, exist for nine species infecting gastropods along the Mediterranean and North East Atlantic coasts of Europe. Of these, five species differ consistently from the cercariae studied by us in the shape of the stylet: *Cercaria brachyura* Lespès, 1857 (syn. *Cercaria pachycerca* Diesing, 1858) possesses five-pointed stylet [39–43]; *Cercaria stunkardi* Palombi, 1934, *Cercaria buccini* Lebour, 1911 and *Opecoeloides columbellae* (Pagenstecher, 1863) (syn. *Cercaria columbellae* Pagenstecher, 1863) possess four-pointed stylet [33,41,42,44,45] and *Cercaria tridentata* Palombi, 1938 has a three-pointed stylet [43].

Both opecoelid cercariae studied by us belong to the group of cotylocercous cercariae with single-pointed stylet (Table 4). The cercaria of *C. labracis* differs from the known opecoelid cercariae with a single-pointed stylet in the much larger number of penetration glands (Table 4). Its morphology and the basic chaetotaxy pattern agree well with the descriptions by Maillard [9] and Bayssade-Dufour & Maillard [32] and this confirms its identification based on morphology alone. *Cercaria linearis* Lespès, 1857 shares the number of penetration glands with the cercaria of *M. obovata* but exhibits a higher upper limit of variation for most metrical data. The question of the conspecificity of these two forms can be resolved by the use of molecular data. Overall, the two species studied by us can be confidently distinguished from each other and from the remaining unidentified cercariae in the group using the number and pattern of the penetration glands in combination with the metrical data.

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~Chapter 5~

Same host, same lagoon, different transmission pathways: effects of exogenous factors on larval emergence in two marine digenean parasites

~Chapter 5~

Same host, same lagoon, different transmission pathways: effects of exogenous factors on larval emergence in two marine digenean parasites

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ORIGINAL PAPER

Same host, same lagoon, different transmission pathways: effects of exogenous factors on larval emergence in two marine digenean parasites

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Abstract Due to their shallow and confined nature, lagoons provide excellent conditions for the transmission of digenean trematode parasites that require two or more intermediate hosts for the completion of their complex life cycles. However, these unstable environments are characterised by an internal heterogeneity and a large variation of a range of abiotic variables. We conducted a series of experiments in a comparative framework to assess the effect of a number of exogenous factors known to exhibit marked fluctuations in the lagoonal environment, i.e. temperature, salinity, water level and photoperiod, on larval emergence of two sympatric parasites, *Cainocreadium labracis* and *Macvicaria obovata*, which share the snail intermediate host, *Gibbula adansonii*, and a sit-and-wait downstream host-finding strategy. Our results demonstrated contrasting patterns and rates of larval emergence indicating an overall differential response of the two species to the variation of the environmental factors. Cercariae of *M. obovata* exhibited increased emergence rates at elevated temperature (with a sharp increase at 15 °C), decreased salinity (35–25 practical salinity units (psu)) and low water levels, whereas larval emergence of *C. labracis* was unaffected by the experimental variation in temperature and water level and showed decreased rates at decreased salinity levels (35–25 psu). The differential impact of the variable environmental conditions indicates the complexity of the patterns of exogenous control modifying parasite transmission and abundance in the lagoonal environment. Most importantly, the contrasting rhythms of larval emergence indicate endogenous control associated with the different transmission pathways of the two opecoelid digeneans.

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Introduction

Lagoons represent important ecotones between terrestrial, freshwater and marine ecosystems with key ecosystem services (Basset et al. 2006). They are characteristic of coastal areas with low tidal excursion (micro- or non-tidal; Barbone et al. 2012) and thus particularly numerous in the Mediterranean basin. Due to their shallow and confined nature, lagoons provide excellent conditions for the transmission of digenean trematode parasites that require two or more intermediate hosts for the completion of their complex life cycles. For example, 72 nominal species of digeneans have been recorded in the lagoons along the northern coast of the Western Mediterranean, corresponding to c. 12 % of the 584 'marine' digenean species reported for Europe (Bartoli and Gibson 2007). Of these, 56 species complete their life cycles in the lagoons.

However, lagoons are unstable environments characterised by an internal heterogeneity and a large variation of a range of abiotic variables. Digenean parasites are among the organisms that are most exposed to the natural variability patterns in lagoons because their life cycles involve a sequence of at least two host species and two transmission events. The transmission between the first intermediate host (typically a mollusc) and the second intermediate host (downstream host) involves short-lived, non-feeding, free-living stages, cercariae, which emerge from the molluscs and are particularly sensitive to the

action of both endogenous (e.g. depletion of their limited energy reserves) and exogenous factors such as environmental conditions and predation. The former are associated with the development of diversified transmission strategies (e.g. cercarial emergence rhythms and dispersal patterns) that ensure coincidence in both time and space with the downstream hosts thus increasing the probability for a successful completion of the digenean life cycle (Combes et al. 1994, 2002; Pietrock and Marcogliese 2003). On the other hand, the influence of exogenous environmental factors on the patterns and rates of cercarial emergence may have direct effects on transmission success and hence the levels of parasitism in lagoonal ecosystems.

The important role of digenean productivity and especially of the output of free-living digenean transmission stages for both energy flow and infectious processes in the marine ecosystems has recently been elucidated (Kuris et al. 2008; Thieltges et al. 2008). However, relatively few studies have focused on the role of abiotic factors on the rates of cercarial emergence in the marine environment. Studies applying experimental approaches with statistical assessment of the changes in larval output predominantly focused on variations induced by temperature and/or salinity and are based on a limited number of marine digenean species, i.e. the microphallids *Maritrema subdolum* (see Mouritsen and Jensen 1997; Mouritsen 2002a, b) and *Maritema novaezealandensis* (see Koprivnikar and Poulin 2009; Studer et al. 2010; Studer and Poulin 2012, 2013), the renicolid *Renicola roscovita* (see Thieltges and Rick 2006); the philophthalmids *Philophthalmus* sp. (Koprivnikar and Poulin 2009; Lei and Poulin 2011) and *Parorchis acanthus* (see Prinz et al. 2011), the heterophyid *Euhaplorchis californiensis* and the echinostomatid *Acanthoparyphium spinulosum* (Koprivnikar et al. 2010). Notably, although digeneans are among the parasites most exposed to the natural environmental variability in lagoons due to their life cycles, all systems studied to date originate from intertidal habitats (bays, mudflats), and no data exist on digeneans inhabiting lagoons.

Here we employ experimental approaches to examine the response of larval production and emergence to changing environmental factors using two related digenean species that utilise the same first intermediate host, the prosobranch trochid gastropod *Gibbula adansonii* (Gastropoda: Trochidae), in a lagoon in the Western Mediterranean. These are the opecoelids *Cainocreadium labracis* (Dujardin, 1845) and *Macvicaria obovata* (Molin, 1859) which occur sympatrically in the Els Alfacs lagoon (Ebro Delta, Western Mediterranean) (Born-Torrijos et al. 2012). The dispersal stages emerging from the snail intermediate host of both species are cotylocercous cercariae that posses very short cup-like tails, modified from a conventional swimming tail and serving for adhesion to the substrate (Cribb 1985). Because of their limited movement abilities, cercariae utilise the sit-and-wait strategy for finding the next hosts in the life cycle, gobiid fish (*Gobius niger* L.; see Maillard 1971) for *C. labracis* and snails (*Cyclope neritea* (L.); see Born-Torrijos et al. 2012) for *M. obovata*.

We conducted a series of experiments in a comparative framework to assess the effect of a number of exogenous factors known to exhibit marked fluctuations in the lagoonal environment, i.e. temperature, salinity, water level and photoperiod, on larval emergence of the two sympatric lagoonal parasites. In particular, we hypothesised that the two species sharing the habitat, the intermediate snail host and the downstream host-finding strategy would exhibit similarities in the patterns and rates of cercarial emergence in response to the variation of the exogenous environmental factors. Alternatively, differences would indicate the influence of endogenous factors associated with the different transmission pathways of the model parasites.

Materials and methods

Study site, sampling and screening for digenean infections

A total of 514 *G. adansonii* was collected in spring 2010 from the shallow littoral zone of Els Alfacs lagoon (Ebro Delta, Spain) along the 'Beach of the Eucalyptus' (40°37′35.06″N, 0°44′30.52″E). The lagoon has an area of 50 km^2, with an average depth of 4 m (littoral platform 0–2 m deep) and a total volume of c. 200 million cubic metres water (Camp and Delgado 1987; Palacín et al. 1991), and an open connection to the Mediterranean Sea (mouth 3 km in width). Freshwater input (<1 % of the total capacity of the bay) is derived from rainfall and the River Ebro through rice field drainage outlet channels, which are open from April to October (Palacín et al. 1991). The hydrographical structure of this semi-enclosed lagoon is characterised by permanently stratified conditions. The surface and shallow areas in particular are subjected to daily and seasonal variations in temperature and salinity that are stronger than in the adjacent Mediterranean Sea (Camp and Delgado 1987; Palacín et al. 1991). Because of the shallow nature of the lagoon, salinity changes along its margins are further enhanced by evaporation during the long periods of sunshine and the strong winds registered in the area; nevertheless, salinity is lower than that of the sea (Camp and Delgado 1987). The stratified environment of the Els Alfacs lagoon, associated with freshwater inputs, has been shown to correlate with spatial variations in the composition and abundance of the marine benthic infaunal communities (Palacín et al. 1991).

Snails were collected haphazardly by hand from the muddy sediment and from the surface of the leaves of the dwarf eelgrass *Zostera noltii* Hornemann, 1832 that occurs in numerous small patches in the lagoon. Thereafter, they were

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transported to the laboratory in aerated seawater taken from the locality and were left to settle for 24 h in aquaria containing seawater from the sampling site before experimental work was conducted.

Snails were measured (shell height (apex to aperture)) to the nearest 0.1 mm, placed individually in 24-well cell culture plates containing 3 ml of seawater and maintained for 24 h at 20 °C under a 14-h light:10-h dark photocycle. The wells were checked for the presence of emerged opecoelid cercariae (Fig. 1) which we identified as *C. labracis* and *M. obovata* (see Born-Torrijos et al. 2012). Snails which emitted more than 10 cercariae during the screening were chosen for experiments. For each trematode species, 20 infected *G. adansonii* were randomly assigned to the different experimental settings. Infected snails showing cercarial emergence during the screening but not during the first experimental day were excluded from the experiments. This resulted in 6–15 *G. adansonii* used in the experiments with different sets of individual snails being assigned to each experiment (see Table 1 for a breakdown of replicate snails).

Cercarial emergence was quantified every 24 h by examination of the individual wells after moving the snails to new incubation plates. Cercarial counts were carried out twice for each snail under a stereomicroscope, and the average was calculated. When the two counts differed by more than 20 cercariae, a third count was obtained and a mean was calculated. The basic conditions in all experiments were 20 °C, high water level (i.e. 6 ml, covering the shell completely) and a 14-h light:10-h dark photocycle. Only conditions for a single factor were varied in each experiment. The water used in all experiments except for that on the effect of salinity was filtered (a 5-μm filter) seawater taken from the sampling site and maintained with aeration at 20 °C.

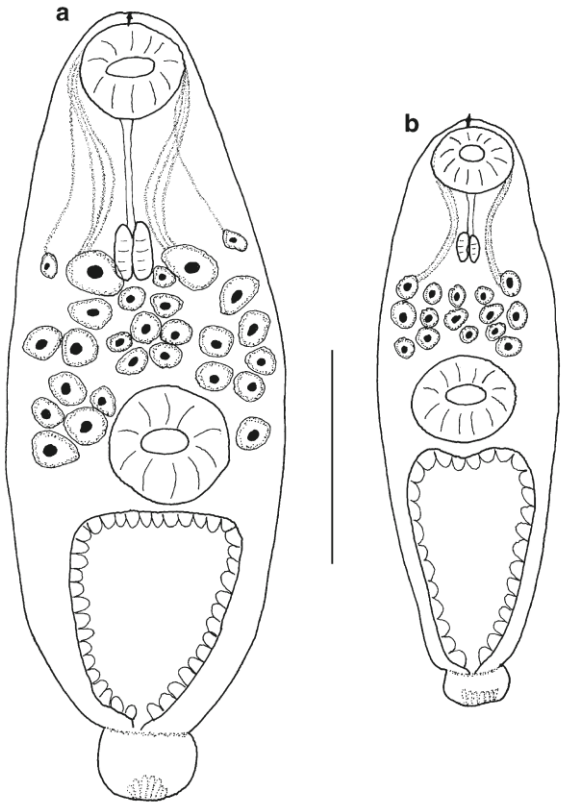

Fig. 1 Line drawings of the cercariae of *C. labracis* (**a**) and *M. obovata* (**b**) ex *Gibbula adansonii*. Scale-bar: 100 μm

Experimental design

Four main experiments were conducted to investigate the effect of environmental factors with reported measurable importance for the emergence of the marine cercariae. The treatments within each factor were designed to mimic the changing conditions in the lagoon. The first experiment was designed to assess the effect of temperature on cercarial emergence and carried out in an environmental chamber (Matek, Model Grow, temperature control ± 0.1 °C). The selected factor levels (treatments) were chosen within the temperature range at the sampling site (10–29 °C, Camp and Delgado 1987; Palacín et al. 1991; 15–29 °C, our measurements during field sampling in March–June 2010). First, a 4-day time series (96 h) was conducted in which the snails were exposed to a gradient of decreasing temperature (25 to 10 °C) with a decrease of 5 °C every 24 h. Thereafter, the same snails were exposed to a gradient of increasing temperature (10 to 25 °C) with an increase of 5 °C every 24 h (i.e. second 4 day-time series, 96 h). In addition to daily emergence rates, we calculated the values for the temperature coefficient Q_{10} for each species and for two different temperature ranges, 10–20 °C and 15–25 °C using the formula $Q_{10} = (C_2/C_1)^{10/(T_2-T_1)}$, where C_1 and C_2 are cercarial emergence rates at temperatures T_1 and T_2, respectively. Q_{10} represents the factor by which the rate of cercarial emergence increases for every 10 °C rise in the temperature (see e.g. Poulin 2006).

In the second experiment we investigated the effect of salinity on cercarial emergence in a decreasing salinity gradient, from 45 to 35 practical salinity units (psu) and thereafter to 25 psu, changing the salinity every 24 h. The gradient was chosen on the basis of the salinity range (32–37 psu) reported by Camp and Delgado (1987) and Palacín et al. (1991) in the lagoon and the values we measured (40–46 psu) in the sampling area between March and June 2010. The third level (25 psu) was selected to represent an extreme situation that can be encountered locally close to the irrigation outlet channels in the northern part of the lagoon. Solutions of defined salinities were made with Reef Crystals Salt (Instant Ocean, Blacksburg, VA, USA) and distilled water.

Table 1 Changes in cercarial emergence rates of *C. labracis* and *M. obovata* in response to the different treatments of the experimental factors: temperature, water level, salinity (daily emergence rates i.e. number of emerged cercariae per snail per day) and photocycle (hourly emergence rates i.e. number of emerged cercariae per snail per hour). Non-transformed data given as the mean ± SD, standard deviation

Experiment	Treatment/factor level	Species			
		C. labracis		*M. obovata*	
		No. of snails	Mean ± SD	No. of snails	Mean ± SD
Temperature	Decreasing gradient				
	25 °C	12	237±167	9	438±224
	20 °C	12	128±227	9	320±252
	15 °C	12	40±58	9	99±125
	10 °C	12	16±38	9	13±15
	Increasing gradient				
	10 °C	9	35±51	6	14±29
	15 °C	9	34±57	6	196±242
	20 °C	9	67±111	6	402±561
	25 °C	9	47±65	6	235±268
Salinity	45 ‰	15	187±131	9	215±151
	35 ‰	15	91±79	9	198±123
	25 ‰	15	45±44	9	230±198
Water level	Low	14	Day 1: 148±157	11	Day 1: 275±242
			Day 2: 234±202		Day 2: 170±108
	High	14	Day 1: 151±156	11	Day 1: 104±161
			Day 2: 291±264		Day 2: 99±87
Photocycle	12-h light:12-h dark cycle				
	Light period	11	17±11	13	1±2
	Dark period	11	7±12	13	9±9
	15-h light:9-h dark cycle				
	Light period	11	11±10	13	4±5
	Dark period	11	5±9	13	8±8

In the third experiment, we studied the effect of water level on cercarial emergence. Snails were held for 48 h at low water level conditions (3 ml, below top of shell) and transferred thereafter to high water level conditions (6 ml, covering shell completely) for 48 h, imitating the microtides in the Els Alfacs lagoon.

Finally, to assess the effect of photoperiodicity on cercarial emergence, snails were held for the first 24 h at a 12-h light: 12-h dark photocycle and then transferred to a 15-h light: 9-h dark photocycle. Cercarial counts were made separately for the light and dark cycle. The light:dark cycles were set up in an incubator with artificial light. The selected photocycles correspond to the natural light:dark cycles in the field during early spring and late summer, respectively.

Statistical analyses

We carried out a comparative statistical assessment of cercarial emergence on the data for cercarial counts per snail per 24 h (i.e. number of emerged cercariae per snail per day, further referred to as daily emergence rates) in all experiments except for the photoperiod experiment. In the latter, emerged cercariae were counted for each light and dark periods separately and calculated as number of emerged cercariae per snail per hour (further referred to as hourly emergence rates). The data on emergence rates were ln $(x+1)$-transformed prior to analyses. There were no significant differences in shell height among snails infected with *C. labracis* and *M. obovata* in each experimental setup (t-test, decreasing temperature $t=-0.29$, $p=0.78$; increasing temperature $t=0.17$, $p=0.87$; salinity $t=-1.35$, $p=0.19$; water level $t=0.97$, $p=0.34$; photocycle $t=0.77$, $p=0.45$). Linear regressions carried out separately for each experiment revealed no significant associations between cercarial emergence rates and snail shell height (ln-transformed) (all $p>0.05$).

We used a general linear model (GLM) repeated measures ANOVA (RM-ANOVA) to assess the effect of the different treatments/conditions on the daily (hourly) emission rates. First, to determine if there are interspecific differences in

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emergence rates, we developed general models with 'species' as the between-subjects factor and the specific treatments as within-subjects factors and time (water level experiment only). Second, after examination of the main effects and interactions in these more complex models, we developed individual models for each species with the same within-subject factors. There were four (temperature), three (salinity), two × two (water level × time) and two × two (photoperiod × light:dark) repeated measure variables, respectively. All analyses were carried out using STATISTICA 7.0 (StatSoft, Inc. 1984–2004).

Results

Effects of temperature

Daily cercarial emergence of *C. labracis* and *M. obovata* reached maxima of 318 and 1,145 cercariae per snail per day, respectively, at 20 °C. The repeated measures ANOVA (GLM) with 'species' as between-subjects factor indicated no marked differences between *C. labracis* and *M. obovata* in the dynamics of daily cercarial emergence rates in response to both temperature gradients (decreasing temperature gradient RM-ANOVA $F_{(1,19)}=2.96$, $p=0.101$; increasing temperature gradient RM-ANOVA $F_{(1,13)}=2.21$, $p=0.161$); however, there was a significant interaction between the factors 'species' and 'temperature' in the experiment with increasing temperature gradient. Temperature had a significant effect on cercarial emission in both 4-day time series with mean rates being typically higher within the upper temperature range (reaching up to 438 and 237 cercariae per snail per day at 25 °C, see Table 1). However, the pattern of changes in cercarial emergence in response to the direction of temperature change differed.

The decrease of the experimental temperature resulted in a steady decrease of cercarial output in both species in the first 4-day time series (RM-ANOVA $F_{(3,57)}=90.03$, $p=0.001$) (Fig. 2; see Table 1 for non-transformed mean rates). There was a nearly eight-fold (*C. labracis*) and a 24-fold (*M. obovata*) decrease in the daily emergence rates within the range 20–10 °C (≈15 °C). Q_{10} values for the range 25–15 °C (≈20 °C) were similar for *C. labracis* (nearly six-fold decrease) and much lower for *M. obovata* (4.4-fold decrease). On the other hand, although there was a significant effect of temperature on the rates of cercarial emergence (RM-ANOVA $F_{(3,39)}=5.07$, $p=0.005$) in the second 4-day time series, the response of the two species differed as indicated by the significant interaction between the factors 'temperature' and 'species' (RM-ANOVA $F_{(3,39)}=4.140$, $p=0.012$) (Fig. 3). There was no significant change in cercarial emergence rates of *C. labracis* (RM-ANOVA $F_{(3,24)}=0.119$, $p=0.948$; Q_{10} values smaller than 2, see Table 2), whereas those of *M. obovata* exhibited a sharp increase at 15 °C (Q_{10} of 28.0 for the range 10–20 (≈15 °C)) followed by a plateau at 20–25 °C (RM-ANOVA $F_{(3,15)}=4.153$, $p=0.026$) (Fig. 3; Table 1). Emergence rates of *M. obovata* at this upper temperature range were on average five- to six-fold higher than those of *C. labracis* (Table 1).

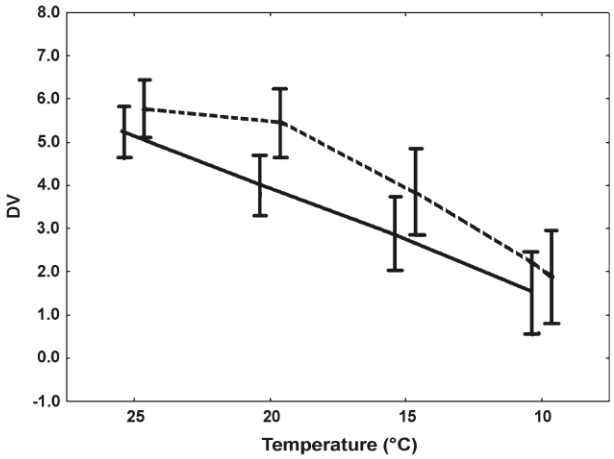

Fig. 2 Effect of decreasing temperature on emergence of cercariae of *C. labracis* (*solid lines*) and *M. obovata* (*dashed lines*). *DV* daily emergence rate (transformed dependent variable)

Effects of salinity

Daily cercarial emergence of *C. labracis* reached a maximum of 506 cercariae per snail per day at the highest salinity level (45 psu) and that of *M. obovata* reached a maximum of 660 cercariae per snail per day at the lowest salinity level (25 psu). There was a significant effect of both factors, 'species' (RM-ANOVA $F_{(1,22)} = 5.379$, $p=0.030$) and 'salinity' (RM-ANOVA $F_{(2,44)}=5.325$, $p=0.008$) on cercarial output and a significant interaction between the two factors (RM-ANOVA $F_{(2,44)}=5.761$, $p=0.006$) (Fig. 4). Both species had similar emergence rates at elevated salinity levels (45 psu) but

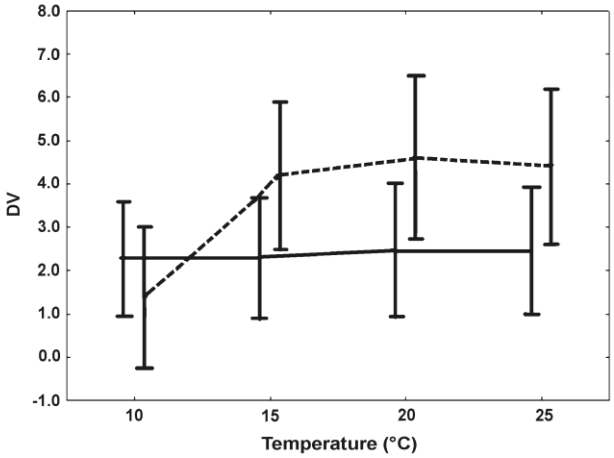

Fig. 3 Effect of increasing temperature on emergence of cercariae of *C. labracis* (*solid lines*) and *M. obovata* (*dashed lines*). *DV* daily emergence rate (transformed dependent variable)

Table 2 Q_{10} values, i.e. change in emergence rates when temperature is changed by 10 °C, for *C. labracis* and *M. obovata* within two 10 °C temperature ranges in the experiments with decreasing and increasing temperature

Temperature range	Gradient	*C. labracis*	*M. obovata*
10–20 °C (≈15 °C)	Decreasing temperature	7.8	24.0
10–20 °C (≈15 °C)	Increasing temperature	1.9	28.0
15–25 °C (≈20 °C)	Decreasing temperature	5.9	4.4
15–25 °C (≈20 °C)	Increasing temperature	1.4	1.2

showed a differential response to the salinity gradient. The models developed for each species demonstrated a rapid decrease of cercarial emergence for *C. labracis* at lower salinity levels (on average two- and four-fold decrease of emergence rates at 35 and 25 psu, respectively) (RM-ANOVA $F_{(2,28)}$=28.123, p=0.001), whereas no significant effect was observed for the cercarial emergence of *M. obovata* (RM-ANOVA $F_{(2,16)}$=0.049, p=0.952) (Fig. 4; Table 1).

Effects of water level

There was somewhat higher variation in the rates of cercarial emergence during the two days of experiment at both low and high water levels for *C. labracis* (reaching a maximum of 765 cercariae per snail per day at high water levels) and at low levels for *M. obovata* (reaching a maximum of 832 cercariae per snail per day) (Table 1). However, the repeated measures factor 'time' had no significant effect on emergence rates for either species (*C. labracis*: RM-ANOVA $F_{(1,13)}$=3.272, p=0.094; *M. obovata*: RM-ANOVA $F_{(1,10)}$=0.259, p=0.622). Rates of cercarial emergence of the two species exhibited a differential response to water level with no significant changes for *C. labracis* (RM-ANOVA $F_{(1,13)}$=0.167, p=0.690; Fig. 5a; Table 1) and significantly higher rates at low water levels for *M. obovata* (RM-ANOVA $F_{(1,10)}$=10.132, p=0.010; Fig. 5b; Table 1).

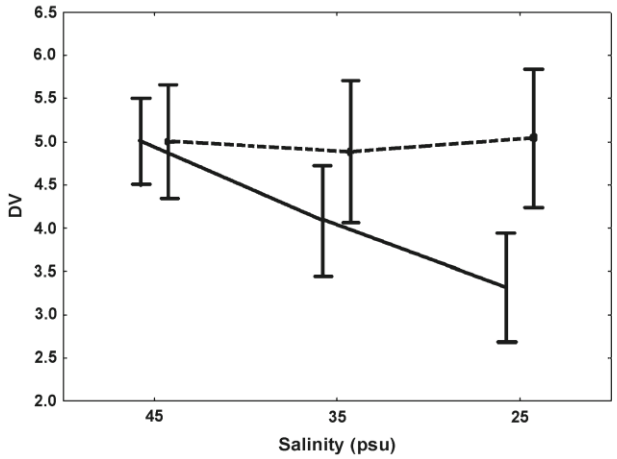

Fig. 4 Effect of salinity on emergence of cercariae of *C. labracis* (*solid lines*) and *M. obovata* (*dashed lines*). *DV* daily emergence rate (transformed dependent variable)

Effects of photocycle

The repeated measures model used to assess the effect of 'species' (between-subjects factor) and two within-subjects factors ('photocycle' and 'light:dark conditions') on the hourly cercarial emergence rates of the two species studied indicated a lack of overall significant differences between species (RM-ANOVA $F_{(1,22)}$=0.857, p=0.365; $F_{(1,22)}$=0.105, p=0.748 and $F_{(1,22)}$=1.485, p=0.236, respectively). However, there were significant interactions between the factor 'species' and the within-subjects factors ('photocycle': RM-ANOVA $F_{(1,22)}$=10.617, p=0.004; 'light:dark conditions': RM-ANOVA $F_{(1,22)}$=35.498, p=0.0001). Emergence rates of *C. labracis* were on average higher than those of *M. obovata* at 12-h light:12-h dark photocycle (Fig. 6a) and also during the light period of the cycle (Fig. 6b). Therefore, we assessed the variations in emergence rates of the two species in separate repeated measures ANOVAs. The model developed for *C. labracis* revealed a significant effect of both the photocycle and light–dark conditions (RM-ANOVA $F_{(1,10)}$=14.167, p=0.004 and $F_{(1,10)}$=16.849, p=0.002, respectively), emergence rates being higher during the 12-h light:12-h dark photocycle and during the light periods. On the other hand, cercarial emergence rates of *M. obovata* did not show differences between the two photocycles (RM-ANOVA $F_{(1,12)}$=3.162, p=0.101) but were distinctly higher during the dark periods (RM-ANOVA $F_{(1,12)}$=18.630, p=0.001) (Table 1).

Discussion

To the best of our knowledge, this study is the first to document experimentally the effects of factors characteristic for the variable environmental conditions in lagoons on parasite larval emergence and the first to examine digenean species with complex life cycles but with limited larval dispersal abilities. The two digenean systems studied are united by a number of features such as the development in the same intermediate snail host, sympatric occurrence in the lagoon, phylogenetic relatedness and morphological similarity of the free-living dispersal stages which also utilise a common sit-and-wait host-finding strategy (Born-Torrijos et al. 2012). However, in contrast with the initial expectations based on these similarities, our results show an overall differential response of the two species to the variation of the environmental factors.

Temperature is perhaps the most important factor influencing the functional activity and embryonic development within the intramolluscan larval stages and, ultimately, the rates of cercarial emergence and transmission success (Galaktionov

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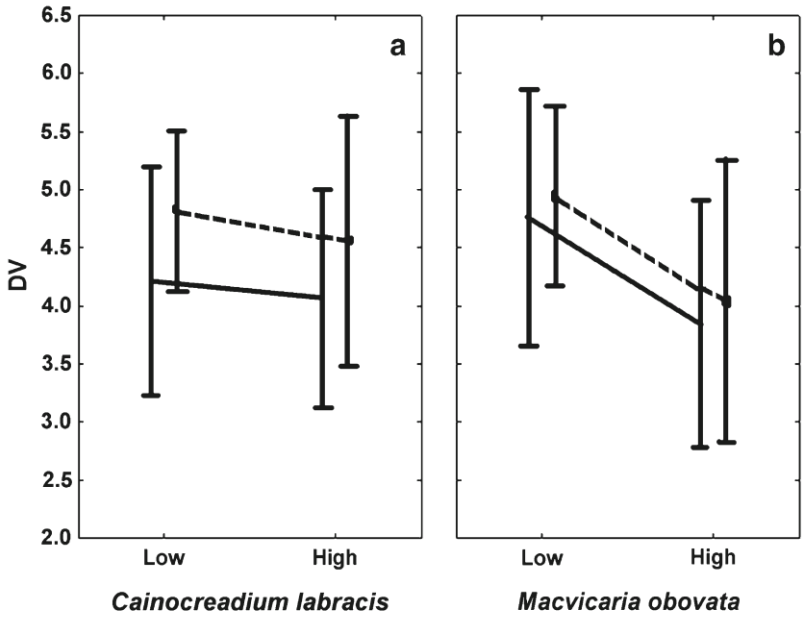

Fig. 5 Interaction plot for the emergence of cercariae of *C. labracis* (**a**) and *M. obovata* (**b**) at low and high water levels during day 1 (*solid lines*) and day 2 (*dashed lines*) of the experiment. *DV* daily emergence rate (transformed dependent variable)

and Dobrovolskij 2003; see also Poulin 2006 for a recent review). Elevated temperatures trigger these processes so that an increase in temperature is typically coupled with an increase in cercarial output in the habitat (e.g. Poulin 2006 and references therein; but see Koprivnikar and Poulin 2009). An opposite pattern, i.e. arrested development of the intramolluscan larval stages and decrease in cercarial emergence, is expected as temperatures decrease; although observations in natural conditions exist (see examples in Galaktionov and Dobrovolskij 2003), this has not been tested yet experimentally. Our experimental design therefore aimed at examination of the effects of both decrease and increase in temperature. Although adaptation to variability in temperature is one of the niche-filtering traits that can lead to the selection of similar species in lagoons (the second being salinity; see Basset et al. 2006), we observed interspecific differences in the response of the two opecoelid digeneans to the direction of the temperature gradient. Whereas the cercarial emergence rates of both species exhibited a similar trend of substantial decrease in the gradient of decreasing temperature, the response to the rapid temperature increase in the following 4-day time series differed. An exogenous effect of increased temperature was detected in *M. obovata* as documented in a number of marine species studied to date (Mouritsen and Jensen 1997; Mouritsen 2002a; Thieltges and Rick 2006; Koprivnikar and Poulin 2009; Studer et al. 2010; Prinz et al. 2011). However, no significant effect on cercarial output of *C. labracis* was observed.

A common feature in the species studied is that cercarial emergence still occurred at the lowest experimental temperature that represents the lower limit of the temperature variation at the lagoon (10–29 °C, Camp and Delgado 1987; Palacín et al. 1991). This suggests that transmission of both species perhaps occurs throughout the year in Els Alfacs lagoon. Although low temperature thresholds for cercarial emergence have not been a subject of experimental investigation, cercarial emission at 10 °C has been observed in two marine digenean species, *P. acanthus* (see Prinz et al. 2011) and *R. roscovita* (see Thieltges and Rick 2006). Observations of larval parasites in populations of *Hydrobia* spp. and *Littorina saxatilis* from the Barents and White seas by Galaktionov and Dobrovolskij (2003) indicate that these thresholds are much lower in some marine environments. These authors reported reduced cercarial emergence for *Cryptocotyle lingua* and *Cryptocotyle concavum* at 2–3 °C and cessation of emergence for *Himasthla elongata* only when water temperature drops below 1 °C.

The atypical results observed in the gradient of increasing temperature deserve further exploration. Although the trend of increase of the rates of larval emergence with increase in temperature did not mirror closely the response of *M. obovata* to the gradient of decreasing temperature, Q_{10} values depicted a common pattern: 24-fold decrease and 28-fold increase in daily emergence rates at≈15 °C which was in contrast to the low variation in both directions at≈20 °C. These data indicate a major effect of temperature on larval emergence of *M. obovata* within the lower temperature range. Overall, the rates of cercarial emergence in response to rapid temperature changes of this species appear to be under exogenous control, whereas cercarial emergence in *C. labracis* was almost unaffected in the gradient of increasing temperature. We believe that our experimental design helped to pinpoint intrinsic differences between the two opecoelid species. Cercariae of *M. obovata* are smaller than those of *C. labracis* (see Born-

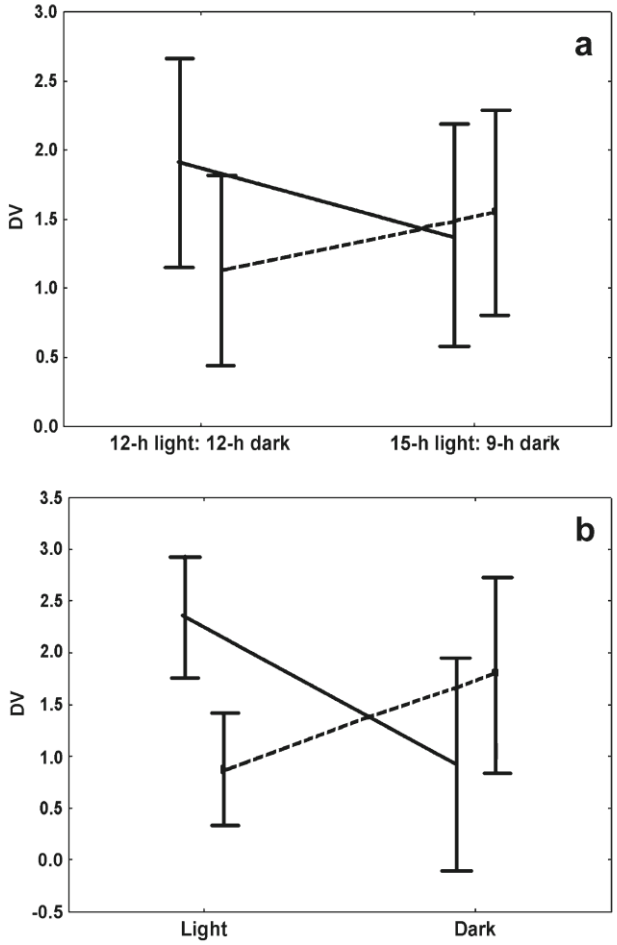

Fig. 6 Effect of the photocycle (**a**) and light–dark conditions (**b**) on emergence of cercariae of *C. labracis* (*solid lines*) and *M. obovata* (*dashed lines*). *DV* hourly emergence rate (transformed dependent variable)

Torrijos et al. 2012). All conditions being equal and in the lack of effect of snail size on emergence rates, our results indicate that a trade-off between numbers of emerging cercariae and size (Loker 1983; McCarthy et al. 2002) may be the cause of differential response to increased temperature. Therefore, it is possible that the pool of mature cercariae ready to emerge has become exhausted faster in *C. labracis* than in *M. obovata* during the second 4-day series (i.e. gradient of increasing temperature).

The effect of salinity on cercarial emergence has been occasionally studied, and a general trend of increased larval emergence at increasing salinity gradients has been depicted within a range of values characteristic for marine coastal habitats (Rees 1948; Lei and Poulin 2011; Studer and Poulin 2012). Exceptions have also been detected, i.e. increased cercarial emergence at lower salinity in two marine species, *M. novaezealandensis* and *Philophthalmus* sp. (Koprivnikar and Poulin 2009); this has been attributed to a short-term stress effect (Lei and Poulin 2011). Previous studies have also shown fairly high tolerance levels of marine cercariae to a wide range of salinities (Stunkard and Shaw 1931; Prokofiev 1999; Mouritsen 2002a), maybe due to the high variation that occurs in the intertidal zones; the latter author also revealed that the association between cercarial emergence rates and salinity is temperature-dependent. Our initial expectations for a similar response of larval emergence rates of the two species due to the wide range of salinity fluctuations in Els Alfacs lagoon were not met. There were clear interspecific differences, with a significant decrease in larval emergence rates of *C. labracis* at decreased salinity levels indicating exogenous control and adaptation to elevated salinities, characteristic for the southern coasts of the lagoon. In contrast, the lack of significant effect of salinity observed in *M. obovata* indicates that larval emergence in this species is better adapted to the varying salinity in the lagoon and especially to the low salinities along the northern coasts due to freshwater inflow *via* irrigation canals of the Ebro Delta open for 7–8 months per year (April to November).

Although a very important habitat component for free-living intertidal organisms, tidal levels seem to be of minor importance for the transmission of trematodes (Thieltges 2007). This should apply to lagoons since because of the low variations in water levels caused by the infrequent, small, barometric tides, snail hosts do not spend time out of the water. Furthermore, the cercariae of both species studied here attach to the substrate immediately after emergence and utilise the sit-and-wait strategy of downstream host finding, so their dispersal is not dependent on changes of water level. Previous experimental results have depicted variations in the effect of water level on cercarial emergence, i.e. emergence occurs only if hosts are totally submerged (Fingerut et al. 2003; Koprivnikar and Poulin 2009), partially submerged (Koprivnikar and Poulin 2009) or no effect (Mouritsen 2002a). Our results also revealed significant interspecific differences. *Cainocreadium labracis* showed no change in cercarial emergence rates and thus appears well adapted to water level changes in the lagoon, whereas there was a significant increase in larval emergence at low water levels in *M. obovata*. These responses to water level may be associated with the different transmission pathways of the two species. The downstream hosts of *C. labracis* are small benthic fishes (i.e. gobiids) that remain in the sampling area independent from the tides, and thus, synchronisation between cercarial emergence and host availability may not be necessary for successful transmission. In contrast, the downstream hosts of *M. obovata* are snails (*C. neritea* (L., 1758) and *Tricolia speciosa* (von Mühlfeld, 1824) (see Born-Torrijos et al. 2012) so that the concentration of cercariae in a smaller water volume enhances the encounter because only snails remain in the microhabitat at low water levels. Moreover, since the cercariae of *M. obovata* have the ability to encyst

in the same first intermediate host (Born-Torrijos, unpublished observations), emergence at low water levels permits to follow the life cycle sequence and extends the transmission window, as observed in other species (Prinz et al. 2011).

One important result of our experiments indicating specific adaptive benefits is the demonstration of different cercarial emergence rhythms in *C. labracis* and *M. obovata* associated with photoperiodism and therefore under endogenous control. Both species exhibited a circadian rhythm (i.e. the interval between larval emergence peaks was 24 h; see Galaktionov and Dobrovolskij 2003), but the different emergence rates under light and dark conditions indicated a diurnal rhythm in *C. labracis* (i.e. greatest emergence during the light period) and a nocturnal rhythm in *M. obovata* (i.e. greatest emergence during the dark period). The adaptive nature of daily emergence rhythms has been questioned, and Shostak and Esch (1990) suggested three alternative variants of the hypothesis that transmission success varies with time of cercarial emergence: (i) periodicity enhances dispersal *via* upstream host activity; (ii) periodicity reduces mortality due to unfavourable physical conditions and predation; and (iii) periodicity increases the chance of finding the downstream host. Hypotheses (i) and (ii) do not appear applicable to the host–parasite system studied as evidenced by the contrasting rhythms of the two digenean species with limited dispersal abilities. First, even if the upstream intermediate host, *G. adansonii*, exhibits periodic activity (diurnal or nocturnal; evidence exists that a congeneric species exhibits nocturnal activity; see Evans et al. 2011), the rhythms of both digenean species should have been synchronised with this exogenous factor enhancing dispersal and thus transmission. Secondly, death due to exogenous mortality factors (environmental and/or predation) with presumably diel variation also cannot explain the contrasting rhythms of the two digeneans both possessing short-lived and susceptible to predation non-motile cercariae. We believe that hypothesis (iii), namely endogenous synchronisation of the cercarial emergence rhythms with target "host–time" *sensu* Combes et al. (1994) that increases the chances of transmission to the downstream host, may be applicable to the host–parasite systems studied by us. The downstream host of *M. obovata*, the small burrowing nassariid *C. neritea*, usually emerges from the sediment only at night (Southward et al. 1997). Therefore, the nocturnal rhythm of larval emergence in *M. obovata* coincides with the time of activity peaks of the downstream host. Similarly, the diurnal rhythm of cercarial emergence in *C. labracis* coincides with the diurnal activity of its downstream host, the opportunistic burrow dwelling black goby, *G. niger*, (see Hesthagen 1976; Nash 1982; this behaviour may be typical of gobies in general; del Norte-Campos and Temming 1994). Similar differences in the circadian emergence patterns associated with the behaviour of the piscine downstream intermediate hosts were shown for strigeid cercariae of *Ichthyocotylurus* spp. and *Apatemon gracilis* (Bell et al. 1999).

To summarise, our study has demonstrated contrasting patterns and rates of larval emergence in two digenean parasites sharing the habitat, snail intermediate host and downstream host-finding strategy in response to variations in exogenous factors simulating environmental variability in lagoons. Cercariae of *M. obovata* exhibited increased emergence rates at elevated temperature, decreased salinity and low water levels, whereas larval emergence of *C. labracis* was unaffected by the experimental variation in temperature and water level and showed decreased rates at low salinity levels. The differential impact of the variable environmental conditions indicates the complexity of the patterns of exogenous control modifying parasite transmission and abundance in the lagoonal environment. Most importantly, the contrasting rhythms of larval emergence indicate endogenous control associated with the different transmission pathways of the two opecoelids.

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~Chapter 6~

Estimating trematode prevalence in snail hosts using a single-step duplex PCR: how badly does cercarial shedding underestimate infection rates?

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Estimating trematode prevalence in snail hosts using a single-step duplex PCR: how badly does cercarial shedding underestimate infection rates?

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Parasites & Vectors

RESEARCH Open Access

Estimating trematode prevalence in snail hosts using a single-step duplex PCR: how badly does cercarial shedding underestimate infection rates?

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Abstract

Background: Trematode communities often consist of different species exploiting the same host population, with two or more trematodes sometimes co-occuring in the same host. A commonly used diagnostic method to detect larval trematode infections in snails has been based on cercarial shedding, though it is often criticized as inaccurate. In the present study we compare infection prevalences determined by cercarial emission with those determined, for the first time, by molecular methods, allowing us to quantify the underestimation of single and double infections based on cercarial emission. We thus developed a duplex PCR for two host-parasite systems, to specifically differentiate between single and double infections. The Ebro samples include two morphologically similar opecoelids, whereas the Otago samples include two morphologically different larval trematodes.

Methods: Snails were screened for infections by incubating them individually to induce cercarial emission, thus determining infection following the "classical" detection method. Snail tissue was then removed and fixed for the duplex PCR. After obtaining ITS rDNA sequences, four species-specific primers were designed for each snail-trematode system, and duplex PCR prevalence was determined for each sample. Results from both methods were statistically compared using the McNemar's Chi-squared test and Cohen's Kappa Statistic for agreement between outcomes.

Results: Overall infection prevalences determined by duplex PCR were consistently and substantially higher than those based on cercarial shedding: among Ebro samples, between 17.9% and 60.1% more snails were found infected using the molecular method, whereas in the Otago samples, the difference was between 9.9% and 20.6%. Kappa values generally indicated a fair to substantial agreement between both detection methods, showing a lower agreement for the Ebro samples.

Conclusions: We demonstrate that molecular detection of single and double infections by duplex PCR strongly outcompetes the classical method. Detection failure is most likely due to immature and covert infections, however, the higher incidence of misidentified double infections in the Ebro samples arises from morphological similarity of closely-related species. The higher accuracy of the duplex PCR method also adds to our understanding of community structure of larval trematodes in snail hosts, by providing a clearer assessment of the importance of interspecific interactions within the host.

Keywords: Prevalence, Detection, Snail host, Double infection, Single-step duplex PCR

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Background

Trematode communities often comprise several different species that exploit a single snail host population, with two or more trematode species sometimes co-occurring in the same individual host [1,2]. The prevalence of double or multiple infections is usually very low, suggesting that interspecific competition or some other form of negative interaction greatly limits the co-occurrence of more than one species per individual host [1,3,4]. Other processes can affect the prevalence of double infections [2]. Sewell [5] proposed that parasitized snails lose their chemical attractiveness to other searching parasites, with the original parasite perhaps altering the snail's physiology to impede or prevent the development of later infections. This might prevent certain combinations of co-occurring species and allow others [6]. Also, infection by a second species could be facilitated due to the suppression of the snail's resistance mechanism [7]; infection with one species of trematode may then predispose some molluscs to infections with another species [8]. Overall, the generally low frequency of double infections suggests that antagonistic relationships between trematodes play important roles. However, any inference regarding interspecific interactions or the structure of trematode communities in snail hosts depends on accurate methods to detect infections in snails.

One commonly used diagnostic method for the detection of larval trematode infections in snail intermediate hosts has been based on cercarial shedding. Usually, snails are individually isolated in small containers and incubated for some time under constant illumination and temperature. After this, emerged cercariae are identified under the stereomicroscope and their prevalence is recorded. In order to increase the accuracy of this non-destructive method of detection and also detect latent or covert infections with immature parasites, many researchers use either (i) multiple sequential sheddings over a period of days or weeks, thereby allowing cercariae time to mature [9], or (ii) subsequent dissection of snails [9,10]. In studies where live snails are not needed, only dissection may be used [11,12]. Other methods are rarely used as they are time-consuming, for example the enzymatic electrophoresis of snail digestive glands which allows detection and identification of immature infections ([13], see references in [14]).

Not surprisingly, cercarial release as a detection method has been criticised as inaccurate by several authors [9,15-19]. In studies where prevalence estimates obtained from both cercarial release and snail dissection were compared, the prevalence was higher with the latter method [15-17,20], including the detection rate of multiple infections [17,21]. Moreover, in some cases snails containing mature cercariae did not shed any [22].

Furthermore, it was shown by Curtis and Hubbard [17] that screening for cercarial release in snails with mixed infections is a conservative approach that only identifies mature infections and thus underestimates the true prevalence [9,17]. The detection of double infections is also more difficult as the simultaneous production of cercariae by two species in the same snail is lower than what they achieve in single infections [23-25], possibly due to competition for host resources.

To quantify the underestimation of trematode prevalences based on cercarial emission, in the present study we investigated two host-parasite systems with double infections by comparing infection prevalences determined by emission with those determined, for the first time, by molecular methods. Several earlier studies [15-17,20] have already compared the results obtained by cercarial emission with those obtained by dissection, and found significant differences. Our goal was to compare the results obtained by emission with those of a method even more powerful than dissection, to ascertain the 'true' number of infections that are missed by relying on cercarial emission alone. Despite their use for differentiation between species, molecular methods have so far only been used for comparison of the 'true' infection prevalences with those obtained with the 'classical' cercarial shedding method, in single infections [19]. Molecular methods are yet to be applied to the detection of mixed infections, where immature and covert infections may be more common.

Caron *et al.* [14], in a review of the techniques used for investigating infection levels in snails already highlighted the importance of PCR-based techniques. We thus developed a duplex PCR assay for two host-parasite systems, capable of specifically amplifying differentially sized segments of the internal transcribed spacer region of ribosomal DNA (ITS rDNA) of each larval trematode species infecting the same snail host (in the digestive glands or gonads), and differentiating between single and double infections. The two systems include i) two co-occuring trematode species both with sporocysts as their intramolluscan stages, and ii) two co-occurring species, one with rediae and one with sporocysts. The latter combination commonly shows stronger interspecific antagonism [1,7,26,27]. The two host-parasite systems are:

(i) The Ebro samples: The snail *Gibbula adansonii* (Payraudeau, 1826) (Prosobranchia, Trochidae) occurs in the Western Mediterranean, and acts as first intermediate host of the sympatric species *Cainocreadium labracis* (Dujardin, 1845) (Opecoelidae) and *Macvicaria obovata* (Molin, 1859) (Opecoelidae) [28]. Sporocysts of both *C. labracis* and *M. obovata* infect the snail's gonad and digestive gland. The prevalence of *C. labracis* in the Ebro Delta varies

from 17.6% to 30.8% [28], while for *M. obovata* the prevalence varies from 0.9% to 23.1% [28].

(ii) The Otago samples: The snail *Zeacumantus subcarinatus* (Sowerby, 1855) (Prosobranchia: Batillariidae) is highly abundant in New Zealand in soft-sediment intertidal areas as well as sheltered rocky shores, and acts as first intermediate host of, among others [29], *Philophthalmus* sp. (probably *P. burrili* Howell & Bearup) (Philophthalmidae) and *Maritrema novaezealandensis* Martorelli, Fredensborg, Mouritsen and Poulin, 2004 (Microphallidae) [30]. Rediae of *Philophthalmus* sp. occur in the digestive gland and gonad. Depending on the site of collection, its prevalence varies from less than 5% up to 30% [9]. Sporocysts of *M. novaezealandensis* can be found in the gonad, with a prevalence that varies among sites from less than 5% to over 80% [9,30,31]. The prevalence of double infections is generally very low, i.e. <2% [9].

Methods

Study design

For the study of the two parasite-snail systems, (i) *Gibbula adansonii* were collected by hand on seven different occasions at the "Beach of the Eucalyptus" (40°37′35″N, 0°44′31″E) in Els Alfacs lagoon (Ebro Delta, Spain) between March and May 2011, December 2011, and between March and May 2013, and (ii) individuals of *Zeacumantus subcarinatus* were collected by hand on four different occasions in Lower Portobello Bay (45°49′56″S, 170°40′22″E) and Oyster Bay (45°50′21″S, 170°38′33″E) (Otago Habour, New Zealand) between December 2012 and February 2013.

After two days of acclimatisation to laboratory conditions, the snails were screened for infections by incubating them individually in cell wells containing 3 ml seawater, at 25°C with illumination for (i) 14 h followed by a dark period of 10 h in the case of *G. adansonii*, and for (ii) 3 h in the case of *Z. subcarinatus*, thereafter checking for the presence of emerged cercariae. The illumination times differed because one species of cercariae shed from *G. adansonii* emerges more during dark periods [32], whereas larval trematodes infecting *Z. subcarinatus* emerge within 2-6 h of constant illumination [9,27,33].

Incubation was carried out at 25°C as warmer temperatures promote cercarial emission, a general phenomenon also documented for our study species [32-38]. After the incubation, individual wells were examined under a dissecting microscope for the presence of cercariae, to ascertain infection status. The few snails infected by other trematode species were discarded, while all remaining snails, whether or not they shed cercariae of our focal species, were then also used for the second detection method, the 'duplex PCR method'. A total of 257 *G. adansonii* and 287 *Z. subcarinatus* were used for the study. The prevalence based on either the 'classical' detection method or the duplex PCR method was calculated as the number of infected snails divided by the total number of snails examined. For the duplex PCR, the digestive gland and gonads of all snails were removed and fixed in 100% ethanol.

For the study, a waiver was granted from the University of Otago and the University of Valencia Animal Ethics Committees, since no formal approval or ethic statement is required for research on gastropods under the New Zealand and the Spanish legislation.

ITS sequences

The ITS rDNA sequences of the trematodes *C. labracis* [Genbank JQ694148] and *M. obovata* [GenBank JQ694145] published in Born-Torrijos *et al.* [28] were used for the design of specific primers for samples obtained from the snail *G. adansonii*.

New ITS rDNA sequences of the trematodes *M. novaezealandensis* and *Philophthalmus* sp. had to be produced, using two individual specimens for each species. DNA extraction consisted of placing ethanol-dried samples into 300 μl of 5% Chelex containing 100 μgmL^{-1} proteinase K, incubating at 60°C overnight, boiling at 90°C for 8 min and centrifuging at 15,000 g for 10 min. Polymerase chain reaction amplifications (PCRs) were performed with a programmable thermal cycler (Mastercycler ep gradient S, Eppendorf) in a final volume of 20 μl containing ~0.5 units of MyTaqRed DNA Polymerase (Bioline) and the related 5x buffer (MyTaq Red Reaction Buffer system, which includes 15 mM $MgCl_2$ and 5 mM dNTPs), 0.5 μM of each primer and approximately 100 ng of template DNA. ITS2 rDNA sequences were amplified using primers 3S (forward 5′-GGT ACC GGT GGA TCA CGT GGC TAG TG-3′) (middle of 5.8S rDNA) [39] and ITS2.2 (reverse 5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′) (5′end of 28S rDNA) [40]. The following thermocycling profile was used for amplification of the gene region: denaturation (95°C for 3 min); 35 cycles of amplification (94°C for 50 s, 54°C for 50 s and 72°C for 1 min 20 s); and 4 min final extension at 72°C. Two PCR amplicons per species were gel-excised and purified using Ultra-Sep Gel Extraction Kit (Omega Bio-Tek), cycle-sequenced from both strands using ABI BigDye™Terminator v3.1 Ready Sequencing Kit, alcohol-precipitated, and run on an ABI 3730 sequencer (Applied Biosystems). The PCR primers were used for cycle sequencing, and contiguous sequences were assembled and edited using Bioedit v7.0.5 (©1997–2005) [41]. The sequences were given a GenBank Accession Number [*M. novaezealandensis* KJ540203 and *Philophthalmus* sp. KJ540204].

Specific primer design and duplex PCR

For the primer design, an alignment of 17 trematode taxa [GenBank: AJ277372.1, AJ241814.1, AJ241802.1, AJ241817.1, AJ241793.1, AJ241808.1, AJ241807.1, AJ241795.1, AJ241816.1, AJ241798.1, AJ241797.1, AJ241796.1, AJ241800.1, AJ241801.1, AJ241799.1, AJ241794.1, AJ241806.1] for the Ebro samples, and an alignment of 22 trematode taxa [GenBank: JN621323.1, GQ463127.1, GQ463124.1, AJ564384.1, AF336234.1, GQ463138.1, GQ463132.1, AJ564383.1, AF067850.1, HQ650132.1, HM584170.1, HM584172.1, HM584175.1, HM584183.1, HM584198.1, HM584181.1, HM584196.1, HM584190.1, HM584180.1, HM584171.1, FJ211246.1, JF784190.1] the Otago samples, were produced with the newly generated sequences. Variable regions of the ITS rDNA were detected from those alignments. Other trematode species different from our focal species infect the snail *Z. subcarinatus* in the Otago samples, but sequences of *Microphallus* sp., *Acanthoparyphium* sp. and *Galactosomum* sp. could not be included in the alignment due to the large percentage of sequence divergence leading to non-reliable alignment.

We designed multiple taxon-specific primers by using the Primer3 program [42] and considering physical and structural properties of the oligonucleotides (annealing temperature ≥ 60°C, G + C percentage over 60%, and self-complementarity, primer dimers and hairpins). Forward and reverse primers for each trematode species were designed (see Table 1). In the process, care was taken that amplicons of co-infecting species were differentiable by size, and that they did not align with other species parasitizing the snail host. To select the appropriate annealing temperature for the 4 primers of each duplex PCR, a temperature gradient was used, with an artificial mixed infection (DNA of two species was combined). For all primers listed in Table 1, duplex PCR conditions were as follows: denaturation (94°C for 3 min); 35 cycles of amplification (94°C for 50 s, 66.6°C (for the Ebro samples) or 64°C (for the Otago samples) for 1 min, and 72°C for 1 min); and 4 min final extension at 72°C. PCR products were visualized on a 1.5% agarose gel, stained with ethidium bromide. The band sizes were checked against a GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific). To confirm the identity of the bands, the amplicons of two duplex PCR products with previously known samples, were gel-excised, purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) in accordance with the manufacturer's instructions and sequenced. Positive controls consisted of target DNA from an artificial mixed infection, while in negative controls nanopure water was used instead of DNA sample. Parasite prevalence was determined for each separate sample (i.e. each sampling year for the Ebro, each bay sampled for Otago). When amplification was negative for both species, PCR was repeated with a 1:50 dilution of the template, thus eliminating false negatives due to inhibition.

Statistical analyses

The results from the classical detection method and the duplex PCR detection method were transformed in two dimensional contingency tables (2 × 2). Statistical differences between prevalences obtained by the two methods were evaluated by McNemar's Chi-squared test for paired proportion ($\chi 2$, critical p-value < 0.05) (R, package stats, version 2.15.0) [43]. Additionally, the Cohen's Kappa Statistic (K, critical p-value < 0.05) for agreement between both techniques was calculated (R, package fmsb, version 0.4.1) [44], using the estimated Kappa to assess the extent of agreement (following [45]). If Kappa is less than 0, the prevalences obtained from the two methods show "No agreement", if 0–0.2, "Slight agreement", if 0.21-0.4, "Fair agreement", if 0.41-0.6, "Moderate agreement", if 0.61-0.8, "Substantial agreement", and if 0.81-1.0, "Almost perfect agreement". The Cohen's Kappa tests the null hypothesis that the agreement between the two methods is the same as random, with Kappa =0. A higher Kappa shows a higher extent of agreement. Statistical analyses were conducted in the software R (version 3.0.1 [46]).

Table 1 Species-specific ITS rDNA primers

Species [GenBank Acc. Number]	Annealing temperature	Primer name	Primer sequence 5'-3'	PCR product size (nt)	Amplified region
C. labracis [JQ694148]	66.6°C	Caino_F	ACGTGCAGCTCATGACACGG	301	ITS1
		Caino_R	TCAGTCAAGCCAGGGGAAGG		
M. obovata [JQ694145]		Macv_F	CCCGAGGCACTCAAAGACTG	537	ITS1
		Macv_R	TCAGTCGAGCCCAGGATAGG		
M. novaezealandensis [KJ540203]	64°C	Maritr_F	TTGACATTCGGCCGGGGTGC	214	ITS2
		Maritr_R	ACCGGCCTAAAGCGCACAGA		
Philophthalmus sp. [KJ540204]		Philsp_F	CGTGAGAGATCACGCGAGG	352	ITS2
		Philsp_R	TGTGCGCCTCACCAAGTGAG		

Species-specific ITS rDNA primers designed in this study for amplification of i) *C. labracis* and *M. obovata*, infecting *G. adansonii* snails from the Ebro Delta (Spain) and ii) *M. novaezealandensis* and *Philophthalmus* sp., infecting *Z. subcarinatus* snails from Otago Habour (New Zealand).

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Results

Prevalences comparison: Classical detection method and duplex PCR

All PCR amplicons, which were sequenced for control confirmed the expected identity of each single and double infection. As shown in Figure 1, infections of the snail tissue samples could be easily differentiated after simple gel visualization.

In both snail-parasite systems, overall infection prevalences determined by duplex PCR were considerably higher than those determined by the classical cercarial shedding method. Depending on the year of sampling (2011 *versus* 2013), among the snails from the Ebro delta 17.9% and 60.1% more snails were found infected using the molecular method. In the Otago samples, the difference between prevalences based on the classical and duplex PCR method was not quite as pronounced but still important with 9.9% and 20.6% more infections detected by PCR, depending on the sampling site (Lower Portobello Bay *versus* Oyster Bay).

Estimates of prevalence of both single and double infections were higher with the duplex PCR method, the difference ranging from 0.6% to 54.2% for single infections and 2.4% to 9.5% for double infections. In the Ebro data (Table 2), the detection rate of single infections with the duplex PCR method was between 1.1% and 3.5% higher for *M. obovata*, and between 7.9% and 54.2% higher for *C. labracis*, the latter species being overlooked in 18.4% and 66.9% of the infections by the classical method. In the Otago data, the detection rate of single infections with the duplex PCR method was between 6.2% and 15.9% higher for *M. novaezealandensis*, and between 0.6% and 2.4% higher for *Philophthalmus* sp., the latter species being overlooked in up to 50% of the infections by the classical method. The increased detection of double infections with the duplex PCR method ranged between 9% and 9.5% in the Ebro data and between 2.4% and 3.2% in the NZ data. Given their low frequency, the most important difference lay in the detection of double infections. Indeed, depending on the sample, from 41.6% to 80% of the double infections detected by duplex PCR were not detected by the classical method (Table 2). Only in single infections of *M. obovata* in 2013 (Ebro samples), was the prevalence detected by duplex PCR lower (3.6%) than that of the classical method (7.1%); however, this was due to the detection of double infections in the same snails thus causing a strong increase in the number of double infections in that sample, from 2.4% (classical method) to 11.9% (duplex PCR method).

The two methods were compared using 2 × 2 contingency tables, for each sample and type of infection (Table 3). The results of McNemar's test revealed statistically significant differences between the prevalences obtained by the two detection methods. The majority of comparisons (7 out of 12) between the two detection methods show that the detection by duplex PCR is significantly higher. The lack of significance of some comparisons (Table 3) is probably due to the modest sample sizes. Kappa values generally indicated a fair to substantial agreement between the classical method and PCR results (22 to 78% of agreement). Importantly, the Kappa values obtained from the Ebro samples show a lower agreement between both detection methods than those of the Otago samples, with double infections showing generally a much lower agreement between the methods. In samples with low agreement between the methods, the strength of that agreement was non-significant, maybe due to the low prevalences of infections (i.e. double infections).

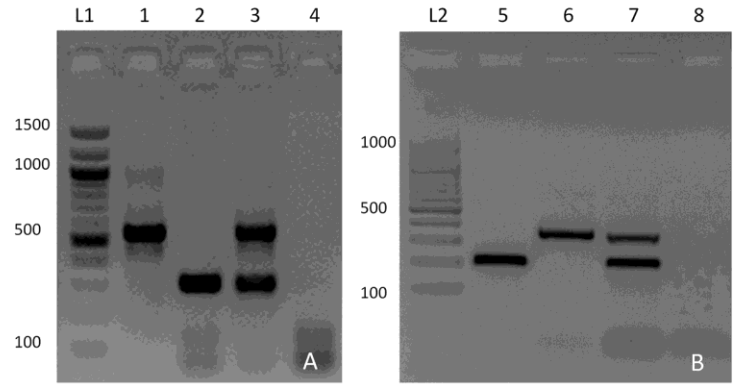

Figure 1 Single-round duplex PCR detection method for single and double trematode infections in snails. Agarose gels showing amplicons produced by single-round duplex PCR reactions for single and double trematode infections in snails, based on ITS rDNA sequences: **(A)** PCR products of infected *G. adansonii* tissues (Ebro samples). From left to right, L1 shows a 1500 bp DNA ladder, lane 1 *M. obovata* (537 bp), lane 2 *C. labracis* (301 bp), lane 3 artificially mixed infection, lane 4 negative control. **(B)** PCR products of infected *Z. subcarinatus* tissues (Otago samples). From left to right, L2 shows a 1000 bp DNA ladder, lane 5 *M. novaezealandensis* (214 bp), lane 6 *Philophthalmus* sp. (352 bp), lane 7 artificially mixed infection, lane 8 negative control.

Table 2 Prevalence of infections detected by the classical detection method and the duplex PCR detection method

		Total no. snails	Infection	No. infected Emission	Prevalence (%) Emission	No. infected duplex PCR	Prevalence (%) duplex PCR	% infections that failed detection by emission
Ebro samples	**2011**	89	*C. labracis*	31	34.8	38	42.7	18.4
			M. obovata	21	23.6	22	24.7	4.5
			Double infection	6	6.7	14	15.7	57.1
			Total 2011 infected	58	65.2	74	83.1	21.6
	2013	168	*C. labracis*	45	26.8	136	81	66.9
			M. obovata	12	7.1	6	3.6	−1
			Double infection	4	2.4	20	11.9	80
			Total 2013 infected	61	36.3	162	96.4	62.3
Otago samples	**LP**	161	*M. novaezealandensis*	81	50.3	91	56.5	11
			Philophthalmus sp.	10	6.2	11	6.8	9.1
			Double infection	7	4.3	12	7.5	41.6
			Total LP infected	98	60.9	114	70.8	14.0
	OB	126	*M. novaezealandensis*	62	49.2	82	65.1	24.4
			Philophthalmus sp.	3	2.4	6	4.8	50
			Double infection	1	0.8	4	3.2	75
			Total OB infected	66	52.4	92	73.0	28.3

Prevalence and numbers of infections detected based on the classical method (emission of parasites) and the duplex PCR method. i) Ebro samples: *C. labracis*, *M. obovata* and double infections in *G. adansonii*, from the Ebro Delta (Spain), years 2011 and 2013; and ii) Otago samples: *M. novaezealandensis*, *Philophthalmus* sp. and double infections in *Z. subcarinatus*, from Otago Habour (New Zealand), sampling sites (LP: Lower Portobello Bay, OB: Oyster Bay). Percentage infections not detected by emission = (no. infected duplex PCR minus no. infected classical method) / no. infected duplex PCR.

Unusual events

Fourteen infections detected by the classical method had an unusual duplex PCR outcome in the Ebro samples: 1. Cercarial emission was identified as *C. labracis* but duplex PCR detected only *M. obovata* (four samples, 1.6%), 2. The opposite case (seven samples, 2.7%), and 3. Double infections detected by emission were identified as single *M. obovata* infections by duplex PCR (three samples, 1.2%). In the Otago samples, only one unusual event occurred: Two snails identified with double infections by cercarial emission were determined to be single infections by duplex PCR (one each for *M. novaezealandensis* and *Philophthalmus* sp. respectively, 0.7%).

Discussion

Traditionally, studies evaluating the influence of parasitism on snails or changes induced in parasitized snails determine the infection status of snails used in experiments based on parasite emission following incubation (i.e. the classical method). Additionally snail dissection may be used, and provides more reliable results than those based on cercarial emission [15-17,20]. However, as highlighted here, these methods may not be accurate enough, especially for the detection of immature and double infections. More recently, some studies have used species-specific primers for the identification of single larval individuals [47], and many studies use molecular methods for parasite detection and identification (as example [48-50]), quantification of infection levels within a host [51], or co-infection prevalence of trematode eggs in stool samples [52,53]. Martínez-Ibeas *et al.* [18] used specific primers for the detection of *Dicrocoelium dendriticum* single infections in snail tissues, and documented a higher accuracy of PCR over cercarial release. Later, Martínez-Ibeas *et al.* [54] designed a mtDNA multiplex PCR for identification and discrimination of *Caliophoron daubneyi* and *Fasciola hepatica* in the snail *Galba truncatula*, but they did not find natural double infections, neither by microscopy nor by PCR. As far as we know, our is the first study where species-specific primers have been designed in a duplex PCR for the accurate assessment of single and double infections with a blind sample of snail tissue. Results of our duplex PCR method have been statistically compared with those of the classical detection method, and shown to consistently outperform the latter. With this methodology, low parasite burden, prepatent, immature or covert infections, and death of the molluscs after collection do not prevent estimation of 'true' prevalence.

Curtis [11] noted that the magnitude of the influence of parasitism on snails used in experiments has often been ignored or underestimated. The consequences of underestimating the effects of parasitism are compounded if the

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Table 3 Comparison of trematode infections by statistical methods

		Parasite species	Classical method detection	Duplex PCR detection		McNemar		Cohen's Kappa	
				+	-	X^2-test	P-value	K	P-value
Ebro samples	**2011**	*C. labracis*	+	30	7	6.76	0.01	0.37	<0.001
			-	22	30				
		M. obovata	+	24	4	3.06	0.08	0.61	<0.001
			-	12	49				
		Double infection	+	3	5	18.27	<0.001	−0.01	0.53
			-	32	49				
	2013	*C. labracis*	+	49	0	63.02	<0.001	0.33	<0.001
			-	65	54				
		M. obovata	+	11	5	2.72	0.1	0.49	<0.001
			-	13	139				
		Double infection	+	3	1	12.5	<0.001	0.22	0.13
			-	17	147				
Otago samples	LP	*M. novaezealandensis*	+	87	1	11.53	<0.001	0.78	<0.001
			-	16	57				
		Philophthalmus sp.	+	16	1	3.13	0.08	0.77	<0.001
			-	7	137				
		Double infection	+	6	1	2.29	0.13	0.61	0.003
			-	6	148				
	OB	*M. novaezealandensis*	+	62	1	19.36	<0.001	0.6	<0.001
			-	24	39				
		Philophthalmus sp.	+	4	0	4.17	0.04	0.55	0.02
			-	6	116				
		Double infection	+	0	1	0.8	0.37	−0.01	0.51
			-	4	121				

Comparison of trematode infections in snails detected by the classical method (emission of parasites) and the duplex PCR method. i) Ebro samples: *C. labracis*, *M. obovata* and double infections in *G. adansonii*, from the Ebro Delta (Spain), years 2011 and 2013; and ii) Otago samples: *M. novaezealandensis*, *Philophthalmus* sp. and double infections in *Z. subcarinatus*, from Otago Habour (New Zealand), sampling sites (LP: Lower Portobello Bay, OB: Oyster Bay). Data transformed in two-dimensional contingency tables (2×2). Results from McNemar's Chi-squared test for paired proportions ($\chi 2$) and Cohen's Kappa Statistic (K) for agreement between both methods. Kappa values can range from <0 (no agreement) to 1 (perfect agreement).

prevalence of infection or the types of species combinations in multiple infections are underestimated [17], for example in the detection of potential seasonal changes [9]. Thus, determining true infection prevalences is extremely important. In the present study we demonstrate that up to 66.9% of the single and up to 80% of the double infections are overlooked when the commonly used classical emission method is employed to determine parasite prevalences. We demonstrate that molecular detection of single and double infections by single round duplex PCR strongly outcompetes the classical method; it also avoids misidentifications in case of morphologically similar species infecting the same host or when immature larval stages are present. Caron *et al.* [14] pointed out that prevalence appears higher with PCR-based methods than microscopy-based techniques, because the former is more sensitive. Cucher *et al.* [19] proved that the detection rate of PCR methods is statistically higher than that of shedding and dissection, but until now this difference had not been tested in a mixed infection parasite-snail system.

Two different ITS rDNA regions that can be used to identify single species unequivocally [55,56] were chosen, ITS1 for the Ebro species, and ITS2 for the Otago species, selecting highly variable and thus species-specific sequence regions (see Table 1). Some authors have proven that ITS2 is too conserved for distinguishing closely related taxa [39,57], with ITS1 showing greater divergences between species [58]. The greater sequence variation found in the ITS1 [39,59] also permits the detection of intra-specific patterns of variation [47] and the study of closely related species, while ITS2 is more appropriate for the analysis of more distant relationships [59] and can be used as marker at species or genus level [57,60,61]. Since both Ebro species belong

to the same family, are morphologically very similar, and have highly similar ITS2 regions, primers were designed in the ITS1 regions. The Otago species can be easily differentiated morphologically as they belong to different families, thus primers were designed to amplify ITS2 regions.

The percentage of PCR positive samples not detected by emission, especially high for double infections, demonstrates the greater accuracy of the duplex PCR over the classical method. Cercarial emission misses many infections and underestimates true prevalence. The difference between the detection methods was higher for the Ebro samples, also showing much variability between years (17.9% in 2011 *versus* 60.1% in 2013) that could be due to immature infections of *C. labracis* during 2013. The percentage difference between methods for the Otago samples (9.9% in LP *versus* 20.6% in OB) highlights the differences of infection prevalences between sampling sites, as reported previously [29].

A noteworthy finding is the higher detection of double infections with the PCR method, implying that the prevalence of single infections is in fact lower. This is of special importance for research on parasite community structure. As an example, in the present study, simple infections that were found to be double infections represented 6.2% of the Ebro samples and 2.8% of the Otago samples. Detection failure is most likely due to immature infections, and the higher incidence of misidentified double infections in the Ebro samples must arise from problems with morphological identification of closely-related trematode species.

Thus, the few unusual findings can be explained by misidentification of the species, especially in the two opecoelid species infecting *G. adansonii* in the Ebro, since two morphologically similar larval stages infecting the same host may mask multiple infections [24]. After the removal of the snail tissue, a few "non-infected snails" in the Otago samples were found to have *Philophthalmus* sp. rediae; however, detection success by dissection is still much lower than that of the duplex PCR, especially regarding mixed infections not found by dissection. In any case, these results are included in the contingency table and the high power of detection of the PCR method has been statistically proven.

The difference between the methods in the case of *Philophthalmus* sp. (Otago samples) and for double infections in all samples is small at first sight, but their actual frequencies are also low, so that the higher detection levels when using the duplex PCR method is in fact unambiguous. Samples showing high agreement between both detection methods are probably the ones involving mature infections, which are easier to detect by the classical method. Ebro samples show generally a lower agreement between methods, due to the similarity in cercarial morphology between species making them harder to distinguish with the classical method. In such cases, the duplex PCR method is the best method to identify and detect infections. On the contrary, the Otago samples show generally a high agreement between methods, which confirms that the parasites are easier to differentiate. However, the agreement is far from perfect, which demonstrates that, even with morphologically dissimilar species, duplex PCR clearly outcompetes the classical method, allowing detection of immature infections.

Research on interactions between trematode species within snails generally focuses on immunity, altered attractiveness or interspecific antagonism. Kuris and Lafferty [2], in an exhaustive meta-analysis on interspecific interactions affecting the community structure of larval trematodes in snail hosts, concluded that as a general rule, fewer multispecies infections are observed than expected by chance ($f_e > f_o$). We have applied the formula proposed by Cort *et al.* [6] and used in Kuris and Lafferty [2] for calculating the expected frequency of multiple infections (f_e), and compared these with the observed prevalences (f_o) obtained separately with the different detection methods. Generally, the data obtained by the classical detection method followed the rule $f_e > f_o$, i.e. frequencies of double infections are lower than expected, suggesting competitive exclusion between trematode species in both the Ebro and Otago systems [2,4]. But when we calculated expected frequencies based on the duplex PCR method, a more complicated pattern emerged. With the exception of double infections in Oyster Bay, Otago, double infections were more frequent than expected by mere chance ($f_e < f_o$). This involved parasite species having only sporocyst larval stages (Ebro), and a species with rediae interacting with another developing only sporocysts stage (Otago). Sousa [1,7] and Kuris [26] documented cases of positive or neutral associations between certain species, involving two sporocysts-only species or species with different larval stages (rediae *versus* sporocysts). Within-host interactions between trematodes in *G. adansonii* in the Ebro system have never been studied, while interspecific competition between *Philophthalmus* sp. and *M. novaezealandensis* is known to occur in the Otago system [25,27]. Some studies have shown that a rediae-sporocysts confrontation results in a patent decrease in the cercarial production of the subordinate species [23] causing double infections to be less frequent than expected. Perhaps competition between the two species in the Otago system remains relatively weak and does not really affect the probability of mixed infection. This hypothesis is in accord with our findings based on the duplex PCR method: if double infections are more frequent than expected by chance and competition is weak, this interspecific interaction may have little effect at the infracommunity level. The higher accuracy of the duplex PCR method can

therefore change our interpretation of community structure of larval trematodes in snail hosts, by providing a clearer assessment of the importance of interspecific competition within the host and suggesting that other mechanisms may facilitate double infections.

Two notes of caution apply to these comments about double infections, however. First, the rough calculation of expected frequencies (f_e) performed here provides only an approximation of species co-occurrences in these systems. A more exhaustive study with higher numbers of snails would be necessary to test the significance of differences between f_e and f_o in relation to interactions between species in mixed infections. Second, the duplex PCR method may also somewhat overestimate the frequency of double infections, by detecting the earliest stages of infection by one species, soon to be eliminated by the previously established competing species. In this sense, the two species will sometimes coexist very briefly in the same host, because infection by the subordinate species will not always become fully realised. Findings of double infections made possible by the greater sensitivity of the duplex PCR must therefore be interpreted with caution.

The inaccuracy of cercarial emergence as a sure sign of infection derives from infections that do not release mature cercariae at the time of study [17]. Because of temporal variability in cercarial production and species-specific emergence conditions [32,33,37,38], prevalences can only be obtained after long-term monitoring of cercarial shedding. Despite being time-consuming, the advantage of the classical shedding method is that it requires only basic laboratory equipment, like a microscope. The duplex PCR, on the other hand, is more costly as it requires DNA extraction and PCR reagents. However, several samples can be analyzed simultaneously in 96-well plates. Caron *et al.* [14] showed that an optimized PCR protocol allows the simultaneous analysis of up to 200 samples in less than 10 h, with a cost of about 0.3 Euro per snail. The obvious advantage is that the incubation period is skipped and samples can be analyzed immediately with low human error. In addition, snail tissue can be stored in 100% ethanol or as extracted DNA in the freezer until further analysis or for the possibility of future studies. Given its precision, the number of snail samples needed for accurate estimates of prevalence is also lower with the duplex PCR. The principal advantage of the duplex PCR remains its high sensitivity and specificity because the sequence amplified is always accurately targeted with primers [14], so that immature and covert infections can be also identified.

Conclusions

Our species-specific ITS-based PCR assay for the calculation of 'true' prevalences may be extrapolated to other systems, even those including more than two parasite species. We feel this method should be considered as an additional tool for determining prevalences of larval stages with high accuracy. The findings presented here also challenge previous conclusions based on cercarial emission studies, concerning interspecific competition in mixed infections, parasite population parameters (prevalence and intensity of infections in snails) and seasonal fluctuations in parasite recruitment into snail populations.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AB-T and ASH designed the study. AB-T carried out the field activities, analyzed the data and prepared the manuscript. ASH supervised the molecular study and RP the statistics. ASH, RP and JAR critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

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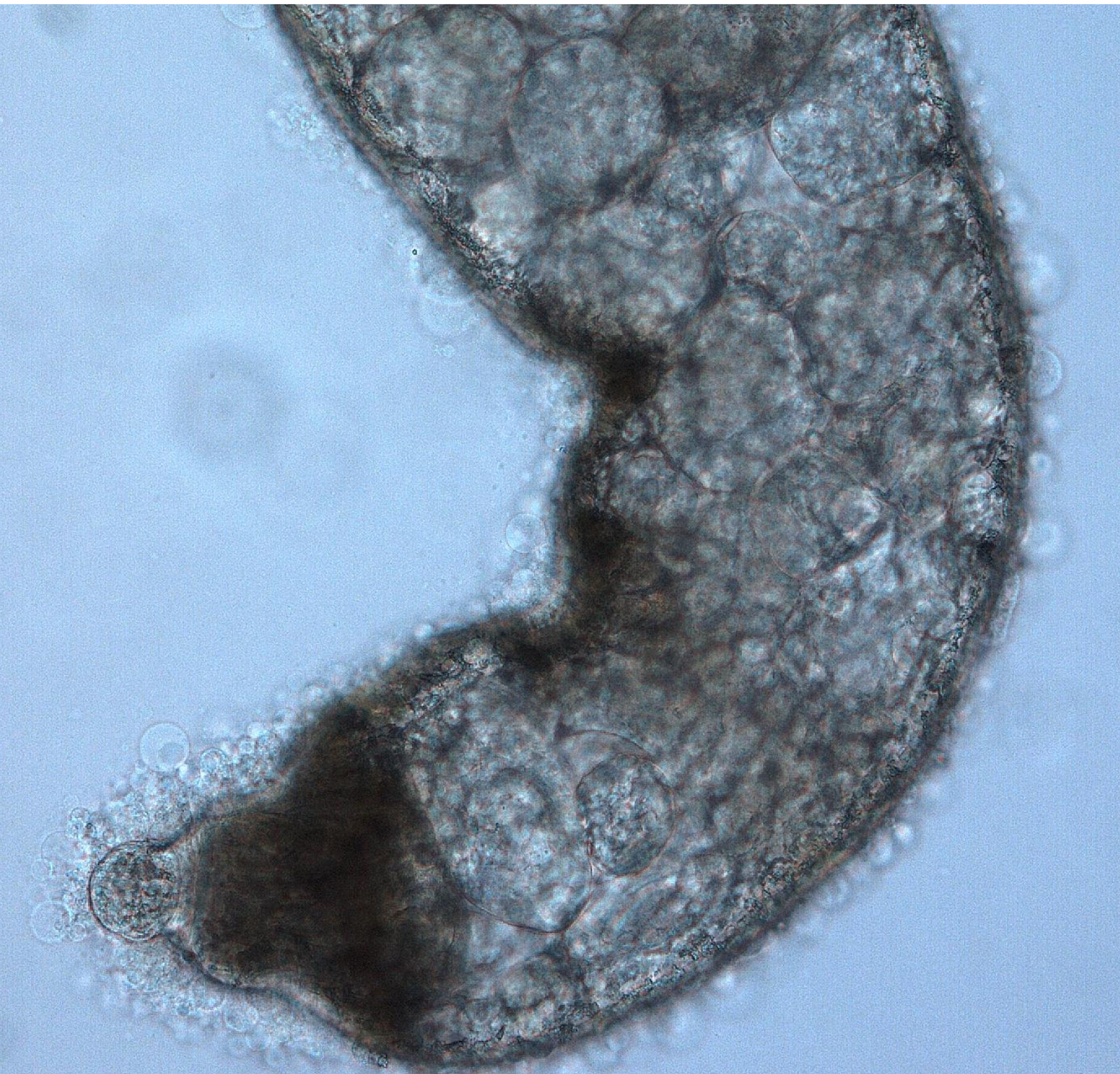
~Chapter 7~

Trematode maturation patterns in a migratory snail host: What happens during upshore residency in a Mediterranean lagoon?



~Chapter 7~

Trematode maturation patterns in a migratory snail host: What happens during upshore residency in a Mediterranean lagoon?

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ABSTRACT: Gastropods act as first intermediate hosts to many trematode species in intertidal habitats. A variety of factors affect cercarial development and transmission, as well as their hosts' distribution, and cercarial release is expected to be temporally and spatially adapted to maximise the encounter of the adequate downstream host. In the studied system, *Cainocreadium labracis* (Dujardin, 1845) and *Macvicaria obovata* (Molin, 1859) (Opecoelidae Ozaki, 1925) parasitize the same snail host, *Gibbula adansonii* (Payraudeau, 1826) (Trochidae), in the shallow waters of a Western Mediterranean lagoon. Both cercariae attach to a surface of seagrass after their emergence, waiting to encounter or being preyed upon by their downstream hosts (in *C. labracis* small benthic fish and in *M. obovata* a snail). In order to better understand the patterns of parasite maturation in the snail host, the larval development inside sporocysts was monitored during upshore residency of the snail, i.e. from March to May. Data on the relative quantity of different maturation stages as well as on snail and sporocyst size were explored using linear models and linear mixed models. We demonstrate that the effect of the trematodes on snail growth is species-specific, and that snail and sporocyst size are proxies only of the reproductive capacity of *M. obovata* but not that of *C. labracis*. Surprisingly, monthly variation of the number of cercarial embryos and germinal balls was not detected in either parasite species, but a higher number of mature stages and the highest maturity index (average maturity of each sporocyst) was found in April. *Gibbula adansonii*'s presence in the upshore waters of the lagoon is limited to its spawning-period in spring, during which we observed a continuous production and maximum output of infectious cercariae. This indicates a link between larval maturation and snail migration, since the synchronisation of maximum parasite production with host maturation guarantees the co-ocurrence of snails, mature parasite transmission stages and downstream hosts in time and space, and thus guarantees successful completion of the life cycle.

KEY WORDS: Larval maturation · Snail migration · Trematodes · Opecoelidae · *Cainocreadium labracis* · *Macvicaria obovata* · Mediterranean lagoon

7.1. Introduction

Molluscs are an important component of European lagoons (Barnes 1980), with gastropods acting as first intermediate hosts to many trematode species in these marine environments (Torchin *et al.* 2002, Bartoli & Boudouresque 2007, Bartoli & Gibson 2007). In Europe, 56 taxa, i.e. approximately 9.6%, of the 'marine' digenean species reported by Bartoli & Gibson (2007) complete their life cycles in lagoons, where the first and second intermediate host naturally occur close to each other (Bartoli & Prévot 1986). However, parasites in lagoons are exposed to extreme changes in environmental conditions due to the high habitat heterogeneity and diurnal as well as seasonal variation of environmental factors in lagoons (e.g. Palacín *et al.*1991, Koutsoubas *et al.* 2000). The complex life cycles of digenean trematodes involve multiple life cycle stages and transmission events (Galaktionov & Dobrovolskij 2003, Poulin 2007). The definitive host, usually a bird or a fish, acquires the parasite via the trophic chain, and the adult parasite reproduces sexually in their digestive tract. Eggs are released via host faeces into the water, where a free-living miracidium hatches and infects the first intermediate host, usually a gastropod. Thereafter it develops into a sporocyst or a redia, in which asexual multiplication occurs resulting in the production of a high number of dispersive offspring, i.e. cercariae that emerge from the snail once they are mature. The cercariae infect a second intermediate host within a short period of time (hours to days), usually another mollusc or a fish, and encyst as metacercariae before being consumed by the definitive host.

Transmission pathways and factors affecting cercarial dispersion and release have been extensively studied (Curtis 1987, Bao-Zhen *et al.* 1997, McCurdy *et al.* 2000, Miura & Chiba 2007, Thieltges 2007, Fermer *et al.* 2010, Born-Torrijos *et al.* 2014a, see Pietrock & Marcogliese 2003 and Thieltges *et al.* 2008 for reviews), as well as seasonal and spatial heterogeneity of cercariae (Lang & Dennis 1976, Hughes & Answer 1982, Muñoz *et al.* 2013). However, while detailed descriptions of the parthenogenetic generations inside rediae or sporocysts are available (e.g. Touassem & Théron 1986, Dobrovolskij *et al.* 2000), the information on the variation of the proportion of developmental stages over time is scarce (Korniychuk 2008, Fermer *et al.* 2010, Prinz *et al.* 2010) but important in a context of parasite transmission.

During infection, hosts and parasites affect each other, with each host-parasite combination being governed by its own rules, which are associated with the ecological

and physiological peculiarities of the snail and trematode species (Gorbushin & Levakin 1999). Hosts that were castrated by the parasite often divert energy toward somatic growth instead of reproduction (Baudoin 1975, Poulin 2007), although the extent of this condition can vary depending on the parasite species (Fernandez & Esch 1991, Gorbushin & Levakin 1999). At the same time, the parasite may use the host's energy to their own benefit, since snails provide space and food, thus influencing the trematode's reproductive output (Cort *et al.* 1954, Baudoin 1975, Gérard *et al.* 1993, Poulin 2007). In principle, a larger host provides more resources and space for parasite growth (Lauckner 1980, Graham 2003).

In order to better understand the patterns and cycles of larval maturation in snail intermediate hosts in relation to host size as well as parasite transmission to downstream hosts, we investigated the development of trematode larval stages in the gastropod *Gibbula adansonii* (Payraudeau, 1826) (Prosobranchia, Trochidae) in a Western Mediterranean lagoon, throughout three months. *Gibbula adansonii* is abundant in the "Els Alfacs" lagoon of the Ebro Delta (Spain), only during the spring months. In the studied system, two species, *Cainocreadium labracis* (Dujardin, 1845) and *Macvicaria obovata* (Molin, 1859) (Opecoelidae Ozaki, 1925), infect the same snail host and both, single and double infections can occur (Born-Torrijos *et al.* 2014b). However, both species have different transmission strategies since their second intermediate hosts belong to different host groups: The cercariae of *C. labracis* infect small benthic fish such as gobiids (i.e. *Gobius niger* L.) as second intermediate hosts, and sea bass *Dicentrarchus labrax* (L.) as definitive host (Maillard 1971). *Macvicaria obovata* uses the gastropod *Cyclope neritea* (L.,1758) as second intermediate host and sparids, i.e. *Oblada melanura* (L.) and *Sparus aurata* L. as well as *Blennius* spp., as definitive hosts (Born-Torrijos *et al.* 2012, and references therein). Both cercariae have a cotylocercous-type tail that permits them to attach to the surface and adopt a 'sit-and-wait' strategy, i.e. cercariae wait until the second intermediate host comes close or preys on them to infect it (Born-Torrijos *et al.* 2014a). In this context, due to the restricted mobility of both cercariae, the synchronization of upstream and downstream hosts in time and space is vital for parasite transmission and the completion of their life cycles (Kube *et al.* 2002, Fredensborg *et al.* 2006, Thieltges 2007). Thus, snail distribution, and in some cases their displacement, is just as essential for optimizing trematode transmission success. Spawning-related migrations are common and extensive in trochid snails (Williams 1965, Underwood 1973, Kendall *et al.* 1987), as well as the usurpation of host behaviour to make the snail change

their location to more appropriate habitats for cercarial release and transmission (Curtis 1987, 1993, Lowenberger & Rau, 1994). This manipulation of the gastropod enhances the probability of successful transmission to the next host (Curtis 1987, McCarthy *et al.* 2000, McCurdy *et al.* 2000, Miura & Chiba 2007, Averbuj & Cremonte 2010).

We analysed the distribution of different intramolluscan larval stages and maturation patterns of *M. obovata* and *C. labracis* in *G. adansonii* during the period when this snail is found in the intertidal habitat, determining links between larval maturation and snail upshore residency during reproduction. Furthermore, the relation between host and sporocyst size and the total number of larval stages in sporocysts was studied since they sometimes play an important role in the reproductive success of trematodes. Results are discussed in the context of the co-occurrence of first and second intermediate hosts in space and time so that sessile cercariae can successfully transmit to their downstream hosts.

7.2. Materials and methods

7.2.1. Sampling and developmental stage determination

Gibbula adansonii was collected by hand in the intertidal habitat of Els Alfacs lagoon (40°37′35″N 0°44′31″E) (Ebro Delta, Spain). Small, sporadic water level changes occur in the shallow areas of the lagoon (Palacín *et al.* 1991), provoking temperature and salinity changes that range from 13 to 34 ºC and from 35 to 44 ‰ (measurements taken during samplings). The muddy sediment where these snails are found can reach temperatures up to 32ºC (Palacín *et al.* 1991). Monthly samplings were done during 2011 and 2013, with successful collection of up to 600 adult *G. adansonii* specimens (8.02 ± 1.26 mm (5.73–11.83)) during March, April and May, when the snails were abundant, with average infection prevalences of 23.1 % for *C. labracis* and 3.97 % for *M. obovata* (Born-Torrijos *et al.* 2012). Between October and December less than 50 specimens were collected, as snail density was considerably lower. In contrast to the adults collected in spring, these snails were juveniles (2-3mm) and were not found to emit cercariae, suggesting they belonged to the new settlement that was still uninfected or had immature infections. During the rest of the year, snails were found only occasionally in the sampled area.

Snails were haphazardly selected and the digestive gland and gonad were removed and examined for infections under a stereomicroscope. Prior to dissection, as a

measurement representative for snail size, the maximum shell height was obtained to the nearest 0.1mm, using callipers. Depending on the parasite load, 5 to 25 sporocysts per snail were randomly selected and transferred to a glass slide to determine the developmental stages of their contents under the microscope. The number of developmental stages contained in daughter sporocysts was determined, ascribing them to the categories described in Prinz *et al.* (2010) and Fermer *et al.* (2010), i.e.: (1) germinal balls, (2) cercarial embryos including developmental stages ranging from those with a "basic cercarial shape" to "immature cercariae with primordial characters but without mobility" and (3) fully developed cercariae, actively moving inside the sporocyst and exhibiting prominent penetration glands and excretory system. In addition, photographs of a subset of sporocsysts of each snail were taken and the average length was determined using ImageJ (v 1.44, National Institutes of Health, USA) (Table 7.1). After morphological identification of the trematode species under the stereomicroscope (Born-Torrijos *et al.* 2012) and record of the developmental stages, part of the digestive gland and the gonad of the snail were fixed in 100% ethanol, in order to confirm the identity of the parasite by PCR (Born-Torrijos *et al.* 2014b). Only single infections were included in the analyses, since the number of mixed infections is low (Born-Torrijos *et al.* 2014b).

7.2.2. Statistical analyses

In order to control for the relative abundance of the three larval stages in the sporocyst, and because maturity is determined by the farthest advanced embryo (Rakotondravao *et al.* 1992), we designed and calculated the following maturity index: Maturity index (MI) = ((No. mature cercariae x 3) + (No. cercarial embryos x 2) + (No. germinal balls x 1))/ No. total larval stages in sporocyst. Hence, MI fluctuates between 1 (only germinal balls) and 3 (only mature cercariae), therefore providing an intuitive univariate summary of the infection maturity per sporocyst while weighing for larval abundance at different developmental stages. In statistical models, both MI and total abundance of each stage in each examined sporocyst was used. Linear models were constructed, for average sporocyst length per snail with the correspondent average MI for each snail, and the average number of each of the three larval developmental stages. The original dataset with the raw numbers of the three types of larval developmental stages per snail and the MI for each sporocyst was used in linear mixed models.

Differences in snail shell size between *C. labracis* and *M. obovata* single infected snails and uninfected snails were evaluated with a linear model (i). Thereafter, several other linear models were constructed (summarized in Fig. 7.1.). First, both species were included to determine if there were interspecific differences in the response variable, the mean total number of larval stages (represented as the average sum of the three types of embryonic developmental stages contained in a sporocyst). In two linear models, the effect of ii) the snail size and iii) the sporocyst length as proxies of reproductive capacity, was evaluated. Secondly, separate linear models were constructed for each species to test the effect of sporocyst length (predictor variable) on iv) the maturity index (response variable), and the effect of the sampling month (predictor variable) on v) snail size and vi) sporocyst length, as response variable. Linear mixed models were used to control for correlated structures in the data arising from repeated measures from single snails, i.e. with snail identity as random-effect variable. The effect of the month as fixed-effect predictor variable was tested on vii) the maturity index, viii) the number of mature cercariae, ix) the number of cercarial embryos and x) the number of germinal balls, as response variables. These analyses were performed in R (package lme4, version 1.1-7, Bates *et al.* 2014). For the models that included comparisons between months (v to x), multiple comparisons were obtained using Tukey's all-pair comparisons (R, package multcomp, version 1.3-3, Hothorn *et al.* 2008). Bonferroni correction was applied to adjust the p-values of multiple comparisons. Independent variables used as predictors were centred in the case of continuous variables, ascribing a value equal to 0 to the sample mean. All statistical analyses were conducted in R (R Development Core Team, version 3.0.1).

7.3. Results

After molecular confirmation of the infection status, 36 *C. labracis* infected snails, with 407 examined sporocysts, and 15 *M. obovata* infected snails, with 202 examined sporocysts, were included in the present dataset (Table 7.1). The monthly average snail size, sporocyst length and MI as well as the number of mature cercariae, immature stages, germinal balls and the mean total number of total larval stages are summarised in Table 7.1, represented graphically in Figure 7.2, whereas the statistical results obtained are summarized in Figure 7.1. The majority of the infected snails (90.2%, 46/51) contained all three developmental larval stages, only two *C. labracis* infected snails lacked mature stages in March and three *M. obovata* infected snails lacked germinal balls

in April. The mean size of *M. obovata* infected snails was significantly bigger than that of uninfected snails and that of *C. labracis* infected ones, with the latter snails being on average 14.6% shorter than those infected with *M. obovata* (8.82 ± 1.47 *vs* 7.69 ± 1.14) ($b_{[C.\ labracis\ \text{snail size}]}$= -1.1233, SE= 0.4021, *T-value* = -2.794, *P-value* = 0.007), and *M. obovata* infected snails on average 15% bigger than uninfected snails (8.82 ± 1.47 *vs* 7.67 ± 1.67) ($b_{[\text{Uninfected snail size}]}$= -1.1479, SE= 0.5728, *T-value* = -2.004, *P-value* = 0.049) (Table S7.1, Fig. S7.1). Multiple comparisons showed significant differences in snail size only between infected snails (Table S7.1, Fig. S7.1).

There was no significant difference between the two species regarding the mean total number of larval stages (herein referred to as "number of larval stages") in snails of the same size (*T-value* = 1.491, *P-value* = 0.143, Table 7.2), but the snail size showed a positive relationship with the number of larval stages of *M. obovata* ($b_{[M.\ obovata\ \text{snail size}]}$=4.783, SE=1.810, *T-value*=2.642, *P-value* = 0.011, Table 7.2B), acting thus as a predictor of reproductive capacity for this species (Fig. 7.3).

Cainocreadium labracis had significantly less larval stages than *M. obovata* in sporocysts of the same size, with 28% difference ($b_{[C.\ labracis\ \text{total mean larval stages}]}$= -12.7176, SE=5.5234, *T-value* = -2.303, *P-value*= 0.026, Table 7.3). There was a positive correlation between the number of larval stages of *M. obovata* with sporocyst length (slope=0.0397, *C. labracis* as intercept *P-value* =0.033 in Table 7.3A, *M. obovata* as intercept *P-value* =0.009 in Table 7.3B), whereas there was no correlation between these variables for *C. labracis* (slope=0.0063, *C. labracis* as intercept *P-value* =0.1508 in Table 7.3A, and *M. obovata* as intercept *P-value* =0.033 in Table 7.3B). Thus, the sporocyst length acts as a proxy of reproductive capacity only in the case of *M. obovata* (Table 7.3B, Fig. 7.4). The linear model using sporocyst length as predictor showed no significant effect on the MI of either species (*C. labracis*, n=36, $b_{[C.\ labracis\ \text{mean sporocyst length}]}$= 5.581 e^{-05}, SE=9.191 e^{-05}, *T-value* = 0.607, *P-value*= 0.548; *M. obovata*, n=15, $b_{[M.\ obovata\ \text{mean sporocyst length}]}$= -0.0002, SE=0.0002, *T-value* = -1.266, *P-value*= 0.228).

Most importantly, neither the mean snail size nor the mean sporocyst length of *C. labracis* infected snails or of *M. obovata* infected snails differed significantly between months (*P-value* >0.05). Although pairwise comparisons did not reveal significant differe nces between months (Table 7.S2), linear mixed models showed that the maturity index of *C. labracis* significantly increased in April, with 9.2% difference to March ($b_{[C.\ labracis\ \text{matu rity index}]}$= 0.17678, SE=0.07737, *T-value* = 2.29, *P-value*= 0.022, Table S7.2A, Fig. S7.2A). The

LMM showed that the proportion of variance explained by random effect 'snail' in *C. labr acis* infections is 40%. The maturity index of *M. obovata* also increased significantly in A pril ($b_{[M.\ obovata\ \text{maturity index}]}$= 0.1526, SE=0.0651, *T-value* = 2.35, *P-value*=0.019, Table S7.2B, Fi g. S7.2B), with the proportion of variance explained by random effect 'snail' being 13% (L MM), and with the increase in April supported by the Tukey treatment in the pairwise co mparisons (Table S7.2B2, *T-value*= 2.346, *P-value*=0.047).

The number of mature stages of *C. labracis* increased significantly in April, with 89% diff erence to March ($b_{[C.\ labracis\ \text{mean mature stages}]}$= 3.0126, SE=1.0998, *T-value* = 2.739, *P-value*=0.00 6, Table 7.4A1, Fig S7.3A), with a proportion of variance explained by random effect 'snail ' 49% (LMM). This was supported by the multiple comparisons test that showed significa ntly higher values for mature stages in April (*T-value* = 2.739, *P-value*= 0.0185) and May (*T-value* = -2.424, *P-value*= 0.0460) (Table 7.4A2). On the contrary, mature stages of *M. ob ovata* did not show a significant variation between months (Table 7.4B, Fig S7.3B). The L MM showed that the proportion of variance explained by random effect 'snail' in *M. obov ata* infections was 23%. The cercarial embryos, i.e. developmental stages, of *C. labracis* di d not show significant differences between months (*P-value* >0.05, Table S7.4A, Fig S7.4A), explaining the random effect 'snail' a proportion of variance of 67% (LMM). The numb er of *M. obovata* cercarial embryos increased in May ($b_{[M.\ obovata\ \text{mean cercarial embryos}]}$= 19.883, S E=9.321, *T-value* = 2.133, *P-value*=0.033, Table S7.4B, Fig S7.4B), with the proportion of var iance explained by random effect 'snail' being 64% (LMM), although pairwise compariso n did not support this result due to the small sample size in May. The results of the linea r mixed models did not show any significant differences between months for the number of germinal balls of either of the studied species (*P-value*>0.05, LMM, proportion of varia nce explained by random effect 'snail'= 36% in *C. labracis* and 65% in *M. obovata*).

7.4. Discussion

The present study investigated larval maturation of two trematode species in the snail *G. adansonii* in relation to snail and sporocyst size, aiming at determining their maturation patterns during the months in which the snail is found in the upshore habitat of the lagoon.

7.4.1. Snail and sporocyst size in relation to parasite

Environmental conditions in the lagoon but also parasitism affect the snail's growth, with each host-parasite system responding differently (Fernandez & Esch 1991,

Gorbushin & Levakin 1999). Trematodes can replace the snail's gonadal tissue (Probst & Kube 1999), leading to its partial to total castration. The energy previously used for snail reproductions is deviated towards the parasites' development as well as towards tissue repair or somatic growth of the host (Fernandez & Esch 1991). Thus, although parasitized snails are larger (Lang & Dennis 1976, Hughes & Answer 1982, McCurdy *et al.* 2000, McCarthy *et al.* 2004, Karvonen *et al.* 2006, Averbuj & Cremonte 2010), in some cases parasitism does not affect the host's growth as all additional energy goes into tissue repair instead of growth or to the parasite (Sousa 1983, Fernandez & Esch 1991, Gorbushin & Levakin 1999). In the system investigated, the bigger shell size of *M. obovata* infected snails compared to uninfected and *C. labracis* infected snails, suggests that only in *M. obovata* infections, energy is deviated to somatic growth and parasite proliferation (Baudoin 1975, Sousa 1983, Poulin 2007). In contrast, it seems that snails infected with *C. labracis*, which have similar size as uninfected snails, destine the energy to other functions, demonstrating that energy allocation after parasitic infection/castration is strongly species-dependent (Sousa 1983, Fernandez & Esch 1991, Gorbushin & Levakin 1999). While both opecoelids infect gonads and digestive gland (Born-Torrijos *et al.* 2012) further histological studies could help to explain these differences, maybe related to different degrees of damage and/or parasitic castration (Miura & Chiba 2007).

The way that a parasite uses the energy from its host is related to the parasite's success, so that space and food availability in the snail host limits the reproductive capacity of sporocysts producing cercariae (Cort *et al.* 1954, Gérard *et al.* 1993, Poulin 2007). Thus, in some species such as *M. obovata*, the size of the sporocyst and especially of the snail is a predictor of the trematode reproductive capacity (Poulin 2007). Thus, independent from *M. obovata* infected snails deviating the additional energy towards somatic growth, the parasite also benefits from the greater spatial and energetic resources available in larger host individuals (Lauckner 1980, Graham 2003). As bigger snails and bigger sporocysts produce a higher number of larval stages per sporocyst (Lie 1969, Rondelaud & Barthe 1987, Gérard *et al.* 1993), *M. obovata* seems to develop with little to no constraints in *G. adansonii*. Some castrator digeneans specifically channel energy away from host reproduction toward their own growth (Baudoin 1975, Korniychuk 2008). On the contrary, the *C. labracis* reproductive output is not related to snail or sporocysts size, since it generally infects smaller snails. Its sporocysts and cercariae are bigger than those of *M. obovata* (Born-Torrijos *et al.* 2012; and Table 7.1), so

that its production is more costly, given that resources for production are limited (Loker 1983). Hence, *C. labracis* suffers a spatial constriction that limits its reproductive capacity (Rakotondravao *et al.* 1992, McCarthy *et al.* 2002).

7.4.2. Parasite maturity throughout snail upshore residency

Bigger and older sporocysts usually contain mature cercariae (Byrd 1954, Fermer *et al.* 2010), so that a relationship between MI and sporocyst length can be expected, however, we failed to detect this effect probably due to the shrinking of the sporocysts after cercarial release. This could be related also to the simplicity of the MI formula, which does not take into consideration that the distribution of stages does not follow a Gauss curve; therefore the present formula calculates the average maturity of the sporocyst but does not necessarily indicate the most common stage. Moreover, the large variability of the MI within a snail, i.e. intra-host variation, could provoke that some patterns between individuals remain unnoticed (see below).While the use of an index is easier for biological interpretation, this simple representation may not detect a more complex relationship between a specific stage and sporocyst length. In the future, this formula could be redefined by using a model that takes the distribution of stages into consideration.

The size of infected snails did not show monthly variation, indicating that they consist of adult specimens or aged snails (9.5 cm height according to Beck 1995). These snails are thus of 1+ age and have been exposed to infective miracidia of the trematode species studied here for at least 12 months. Adult stage as well as the gonadal maturity during upshore residency was confirmed in histological sections (unpublished data) of individuals belonging to the same population as those of the present study.

In the present study, sporocysts length did not show temporal variation and the majority of the infected snails (90.2%, 46/51) contained all three developmental stages, i.e. embryos of different ages are found simultaneously with mature cercariae in individual sporocysts. The co-occurrence of the three stages and the absence of sporocysts containing germinal balls exclusively indicates that *M. obovata* and *C. labracis* have a short prepatent infection , as demonstrated in e.g. *Echinostephilla patellae* (Prinz *et al.* 2010) or *Parvatrema minutum* (as *Meiogymnophallus minutes* in Fermer *et al.* 2009, 2010).This allows a continuous rapid production of mature cercariae and their emergence from the snail.

More detailed analyses showed that the number of mature stages of *C. labracis* and *M. obovata* as well as their MI was highest in April. The overall variance of the MI is mostly explained by the snail effect, i.e. variation is based on within-host variation rather than between-host variation. This may explain the lack of clear statistically significant p atterns during the three months. Embryonic development of premature stages in the spo rocysts can be stimulated by a constant release of mature cercariae into the environment (Bao-Zhen *et al.* 1997), so that an equilibrium between mature cercariae and the formati on and development of new embryos is established during the three months, allowing fo r continuous production of mature transmission stages during the residency of *G. adanso nii* in the intertidal habitat of the lagoon, during spring.-

7.4.3. Encounter with downstream hosts

The effectiveness of this mechanism has to be analysed in relation to the biology of the first and second intermediate host, and the transmission strategies of both trematode species. It is important to keep in mind that both studied cercariae, adopt a 'sit-and-wait' strategy of downstream host finding, so that their dispersal is not dependent on tidal changes. Thus, for the completion of life cycles, the temporal and spatial encounter between upstream and downstream host must be guaranteed. First, the abundance of the molluscan host is important for transmission (Torchin *et al.* 2002). In this system, *G. adansonii* snails migrate upshore and are numerous in the shallow waters of the lagoon from March to May. This snail species has been previously reported from a range of 0 to 30 m depth, occurring in deeper waters during June and July (Milazzo *et al.* 2000, Antoniadou *et al.* 2005, Soppelsa *et al.* 2007, Crocetta *et al.* 2009). In general, most trochid snails show displacement caused predominantly by reproductive triggers (Fretter & Graham1962, Williams 1965, Desai 1966, Underwood 1973, Kendall *et al.* 1987), so that migrations seem to be an essential part of their biology. Second, for sessile cercariae to come in contact with their downstream hosts, it is important that these hosts are present close to the sediment surface which cercariae attach to. Shallow waters force these vicinity conditions. In this case, the downstream hosts inhabit the shallow waters of the lagoons during the spring months (Arruda *et al.* 1993, Bouchereau & Guelorget 1998, Elliott & Hemingway 2002), and they were previously found in close vicinity (Koutsoubas *et al.* 2000, Soppelsa *et al.* 2007, Born-Torrijos *et al.* 2012, Ribeiro *et al.* 2012). Small benthic fish that act as second intermediate host of *C. labracis* (Maillard 1971, Bartoli & Gibson 2007) spawn in shallow coastal waters from March/April to August/September

(Bouchereau & Guelorget 1998, and references therein), usually with one spawning peak in April. Hence, it is during this period, when hosts and parasite transmission stages are most abundant (Arruda *et al.* 1993). The second intermediate host of *M. obovata*, the snail *C. neritea*, is found further upshore in the intertidal habitat (–0.8 and +3.1 m, Bachelet *et al.* 2004) moreover showing extremely high metacercarial infection prevalence (>98%, Born-Torrijos *et al.* 2012). Thus, although the prevalence in the first intermediate host of *M. obovata* is comparably low (7.7% versus 30.8% for *C. labracis*, Born-Torrijos *et al.* 2012) the accumulation of metacercariae in *C. neritea* suggests a successful or even increased transmission of this parasite in the intertidal habitat.

Parasite-induced behavioural changes of the host are difficult to demonstrate, especially if the mechanisms causing the changes were not identified and the enhancement of the transmission is not by predation of the final host on the intermediate host (Poulin 2007). However, usurpation of host behaviour by cercariae to enhance the transmission to a downstream host has been reported (Curtis 1987, 1990, 1993, Lowenberger & Rau 1994, Miller & Poulin 2001), with parasitized specimens changing their vertical distribution and thus facilitating cercarial transmission. Spawning-related upward migration is quite pronounced in trochids, and snail migration towards ideal sites for the release and transmission of cercariae in areas were suitable hosts are found could be enforced by the parasite and become a repeated and purposeful (adaptive) process to complete life cycles. Long-term associations between snails and digeneans (Blair *et al.* 2001) permit parasites to adapt their maturity patterns to that of the host, either benefiting from the snail's spawning-related migration or further manipulating snail migration behaviour.

7.5. Conclusions

We demonstrate that the reproduction-related migration of *G. adansonii* coincides with the cercarial maturation of two trematode species, with a maximum output of infective cercariae throughout the whole period of residency of the snail in shallow upshore habitats, guaranteeing the production of a maximum number of suitable parasites for transmission to downstream lagoon hosts. This aim can only be met if parasite and host maturation are synchronised in time and space (Kube *et al.* 2002). Close vicinity of first and second intermediate hosts is an absolute precondition in the case of sessile cercariae. We show that the cercariae make different use of the snail resources, however,

both benefit from, or even enforce the snail's reproduction-related migration. The present snail-parasite system opens the door to further studies on parasite-induced behaviour changes as a true adaptation for transmission. Thereby, a larger sample size would reduce statistical uncertainty and some tendencies could become clearer patterns, while a follow-on study should cover a wider range of depths in the lagoon allowing us to analyse snails of different ages for the parasite's reproductive patterns and seasonality throughout the whole year, thus providing a better understanding of biology of both host and parasites.

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7.6. Figures and Tables

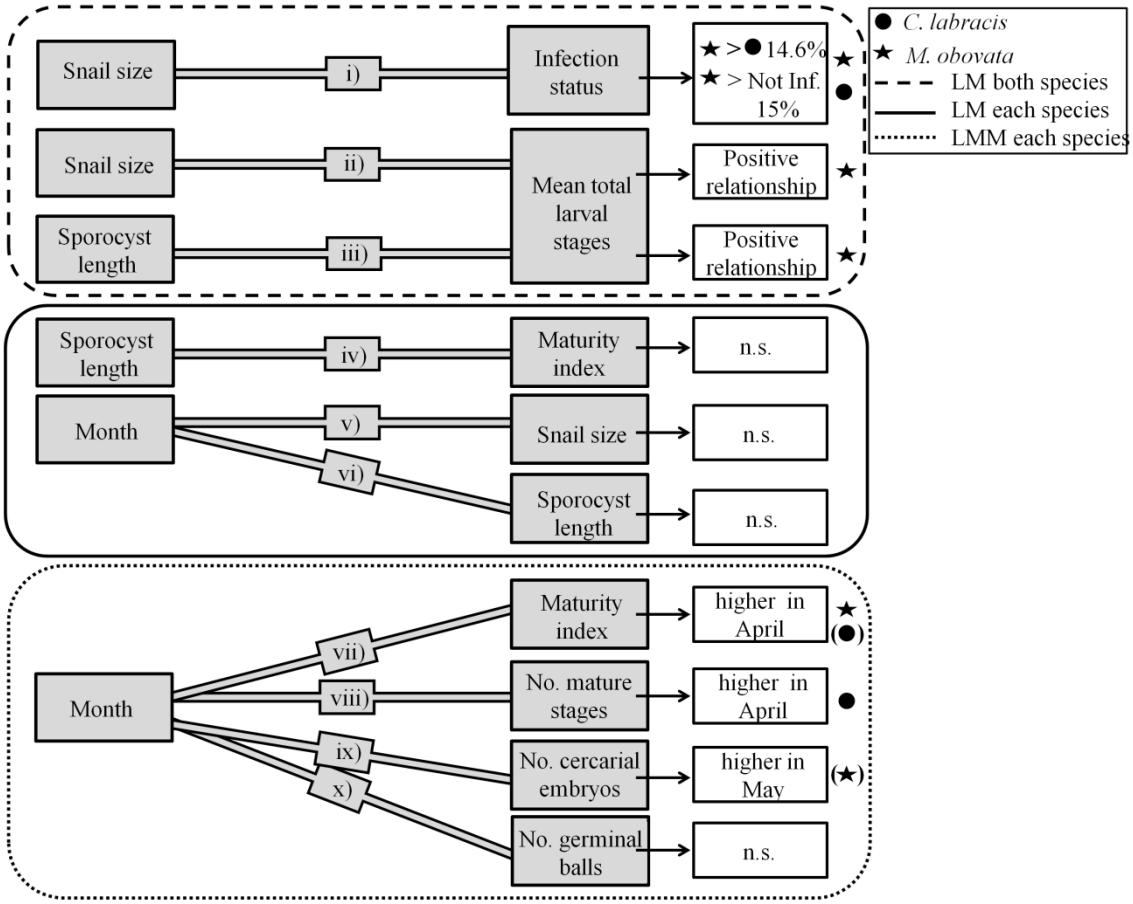

Figure7.1. Summary of the statistical models designed (i to x) and the results obtained in the present study. Solid circles represent significant results for *C. labracis* whereas stars r epresent significant results for *M. obovata*. Symbols (circles or stars) in brackets indicate significant results obtained by linear mixed models, but not by the Tukey treatment in p airwise comparisons.

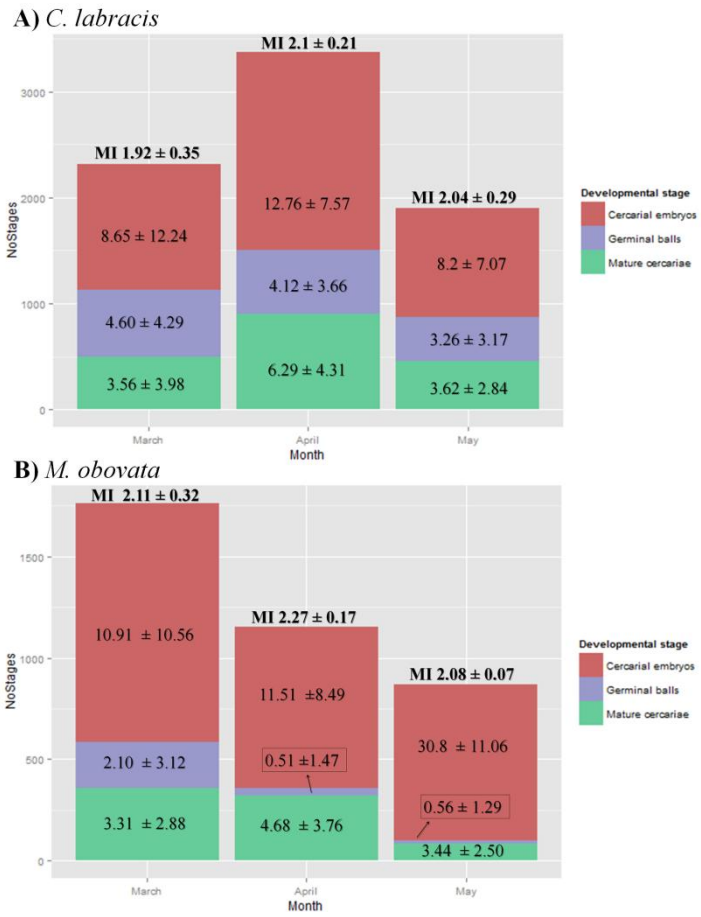

Figure 7.2. Representation of the total number of developmental stages and differentiated per developmental stage of *C. labracis* and *M. obovata*, giving the mean number of mature cercariae, cercarial embryos and germinal balls per sporocysts in each month (mean ± SD), with the mean maturity index (MI) on the top of the bar.

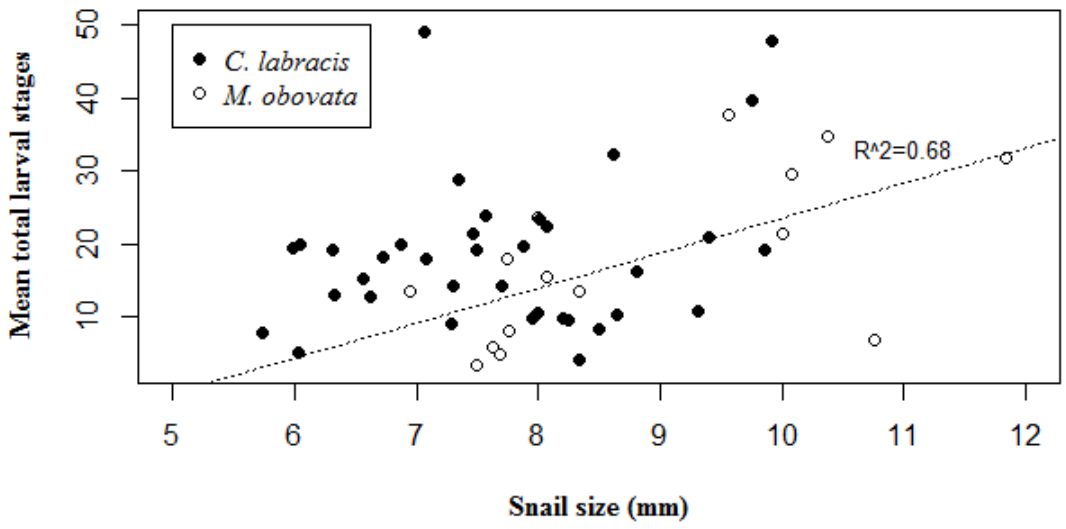

Figure 7.3. Representation of the results of a linear model evaluating the relationship bet ween the sum of all larval stages (mean total number of larval stages) as a proxy of repro ductive capacity and snail size for both *C. labracis* (N=36) and *M. obovata* (N=15) single i nfections. Only regression lines of significant relationships are shown (*M. obovata*, y= -2 4.22 + 4.78x, *P-value*=0.01, R^2=0.68). Solid circles represent *C. labracis* samples, whereas o pen circles with the dashed regression line represent *M. obovata* samples.

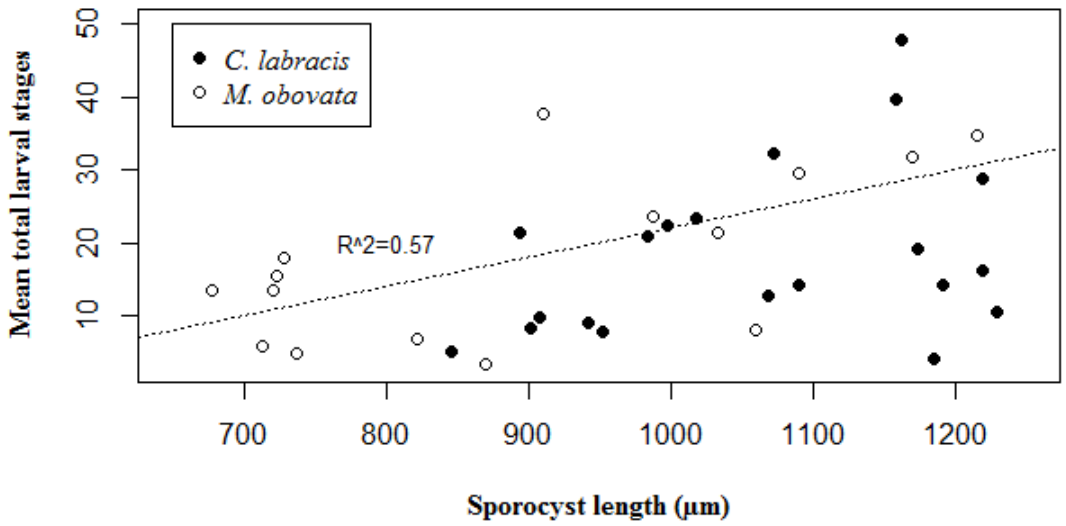

Figure 7.4. Representation of the results of a linear model evaluating the relationship bet ween the sum of all larval stages (mean total number of larval stages) as a proxy of the re productive capacity and sporocyst length of both, *C. labracis* (N=36) and *M. obovata* (N=1 5) single infections. Only regression lines of significant relationships are shown (*M. obov ata*, y= -17.67+ 0.04x, *P-value*=0.01, R^2=0.57). Solid circles represent *C. labracis* samples, w hereas open circles with the dashed regression line represent *M. obovata* samples.

Table 7.1. Summary of dataset used in the present study with details of host and parasites (C, *Cainocreadium labracis*; M, *Macvicaria obovata*). Snail size (shell length), sporocyst length and maturity index (MI=No. mature cercariae x 3) + (No. cercarial embryos x 2) + (No. germinal balls x 1)/ No. total larval stages in sporocyst) are presented as mean ± SD; number of mature cercariae, cercarial embryos, germinal balls and total larval stages are presented as mean ± SD (range).

Month	Species	No. snails	No. sporocysts	Snail size (mm)	Sporocyst length (µm)	Maturity index	No. mature cercariae/per sporocyst	No. cercarial embryos/per sporocyst	No. germinal balls/per sporocyst	Total larval stages/per sporocyst
March	C	12	138	7.71 ± 1.39	1327.3 ± 307.46	1.92 ± 0.35	3.56 ± 3.98 (0-16)	8.65 ± 12.24 (0-80)	4.60 ± 4.29 (0-27)	16.8 ± 15.60 (1-106)
	M	9	108	8.69 ± 1.55	931.93 ± 263.56	2.11 ± 0.32	3.31 ± 2.88 (0-13)	10.91 ± 10.56 (0-38)	2.10 ± 3.12 (0-14)	16.33 ± 3.25 (2-56)
April	C	15	143	7.28 ± 1.03	1430.77 ± 280.11	2.1 ± 0.21	6.29 ± 4.31 (0-19)	12.76 ± 7.57 (2-38)	4.12 ±3.66 (0-26)	23.17 ± 1.16 (5-66)
	M	5	69	8.73 ± 1.46	770.78 ± 272.40	2.27 ± 0.17	4.68 ± 3.76 (0-18)	11.51 ±8.49 (2-39)	0.51 ±1.47 (0-10)	16.7 ± 11.74 (4-54)
May	C	9	126	8.37 ± 0.60	1204.79 ± 308.23	2.04 ± 0.29	3.62 ± 2.84 (0-15)	8.2 ± 7.07 (0-30)	3.26 ± 3.17 (0-16)	15.10 ± 8.49 (4-52)
	M	1	25	10.37	1215.98 ± 338.04	2.08 ± 0.07	3.44 ± 2.50 (0-8)	30.8 ± 11.06 (6-48)	0.56 ± 1.29 (0-5)	34.8 ± 13.32(6-56)

Table 7.2. Results of the linear model evaluating the relationship between the sum of all lar val stages (mean total number of larval stages) as a proxy of reproductive capacity and snai l size for both *C. labracis* (N=36) and *M. obovata* (N=15) single infections. The snail size is centered. The intercept value stands for mean total larval stages of (A) *C. labracis* or (B) *M. obovata* infections. Statistically significant results (at α=0.050) are indicated in bold.

	Estimate	SE	*T-value*	*P-value*
(A) *C. labracis* infection				
(Intercept)	19.258	1.735	11.103	**<0.001**
Mean snail size	2.436	1.475	1.652	0.105
M. obovata	-5.106	3.423	-1.491	0.143
M. obovata x Mean snail size	2.346	2.335	1.005	0.320
(B) *M. obovata* infection				
(Intercept)	14.152	2.951	4.795	**<0.001**
Mean snail size	4.783	1.810	2.642	**0.0112**
C. labracis	5.106	3.423	1.491	0.1425
C. labracis x Mean snail size	-2.346	2.335	-1.005	0.3201

Table 7.3. Results of a linear model evaluating the relationship between the number of larv al stages (i.e. mean total number of larval stages) as a proxy of reproductive capacity and s porocyst length for both, *C. labracis* (N=36) and *M. obovata* (N=15) single infections. The s porocyst length is centered. The intercept value stands for mean total larval stages of (A) *C . labracis* or (B) *M. obovata* infections. Statistically significant results (at α=0.050) are indic ated in bold.

	Estimate	SE	*T-value*	*P-value*
(A) *C. labracis* infection				
(Intercept)	17.6306	1.7595	10.020	**<0.001**
Mean sporocyst length	0.0063	0.0043	1.460	0.1508
M. obovata	12.7176	5.5234	2.303	**0.026**
M. obovata x mean sporocyst length	0.0334	0.0152	2.195	**0.0331**
(B) *M. obovata* infection				
(Intercept)	30.3482	5.2356	5.796	**<0.001**
Mean sporocyst length	0.0397	0.0146	2.723	**0.009**
C. labracis	-12.7176	5.5234	-2.303	**0.026**
C. labracis x Mean sporocyst length	-0.0334	0.0152	-2.195	**0.033**

Table 7.4. Results of linear mixed models (a.1, b.1) and pairwise comparisons (a.2, b. 2) evaluating the effect of month on the number of (A) *C. labracis* (N: March=138, April=143, May =126) and (B) *M. obovata* (N: March=108, April=69, May=25) mature stages. The intercept value in the LMM stands for the mean number of mature stages in the first month, i.e. March, to which the other months are compared. The estimate of a month is added or subtracted from the intercept value. Statistically significant results (at α=0.050) are indicated in bold, with the corresponding P-value obtained after Bonferroni correction given in parentheses. (A) *C. labracis* infections: Random effect 'snail' variance =7.300, (B) *M .obovata* infections: Random effect 'snail' variance =2.424.

	Estimate	SE	*T-value*	*P-value*
(A) *C. labracis* infection				
(a.1)				
(Intercept)	3.3884	0.8157	4.154	**<0.001**
April	3.0126	1.0998	2.739	**0.0062**
May	0.1180	1.2439	0.095	0.9243
(a.2)				
April-March	3.013	1.100	2.739	**0.0168 (0.0185)**
May-March	0.118	1.244	0.095	0.9950 (1.000)
May-April	-2.895	1.194	-2.424	**0.0404 (0.0460)**
(B) *M. obovata* infection				
(b.1)				
(Intercept)	3.3148	0.5874	5.643	**<0.001**
April	1.4566	0.9747	1.494	0.1352
May	0.1252	1.7596	0.071	0.9434
(b.2)				
April-March	1.4566	0.9747	1.494	0.283 (0.405)
May-March	0.1252	1.7596	0.071	0.997 (1.000)
May-April	-1.3314	1.8320	-0.727	0.740 (1.000)

7.7. Supplementary Materials

7.7.1. Figures and Tables

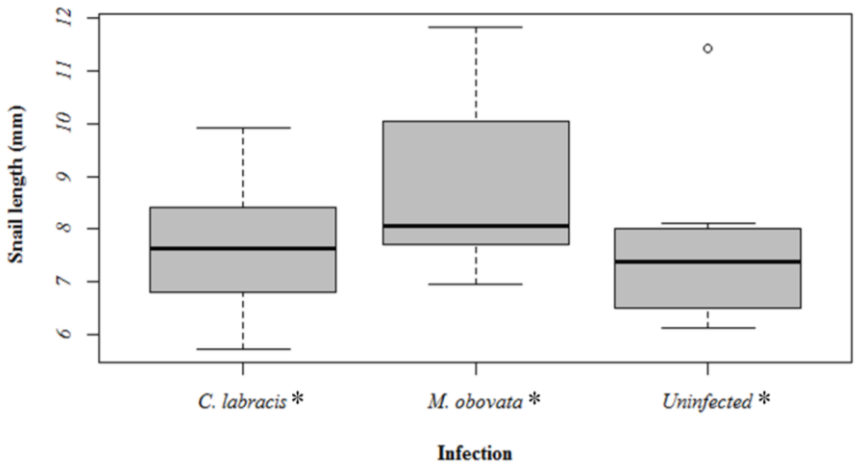

Figure S7.1. Differences in snail size between *C. labracis*, *M. obovata* infected snails and uni nfected snails. Asterisks indicate statistically significant differences (*P-value C. labracis* and *M. obovata* infected snails <0.01, *P-value* uninfected snails=0.049).

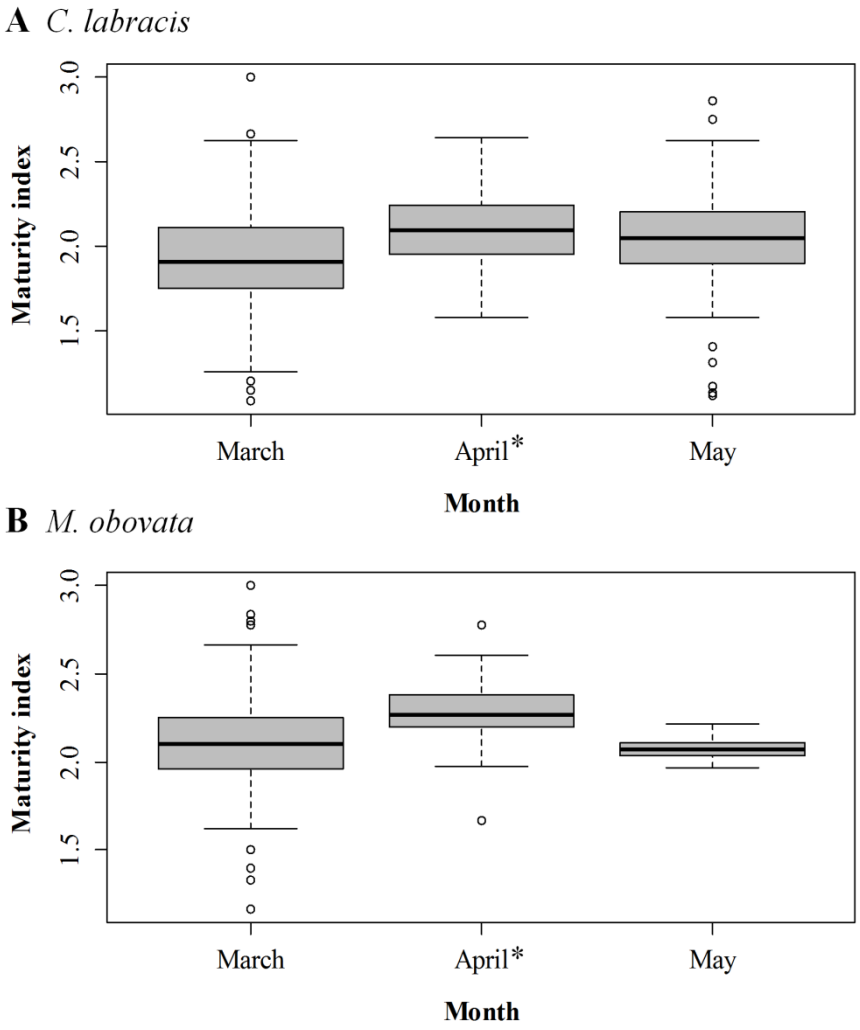

Figure S7.2. Monthly differences in maturity index in (A) *C. labracis* and (B) *M. obovata* infections. Asterisks indicate statistically significant differences (*P-value*=0.02 in *C. labracis* and *M. obovata* infections).

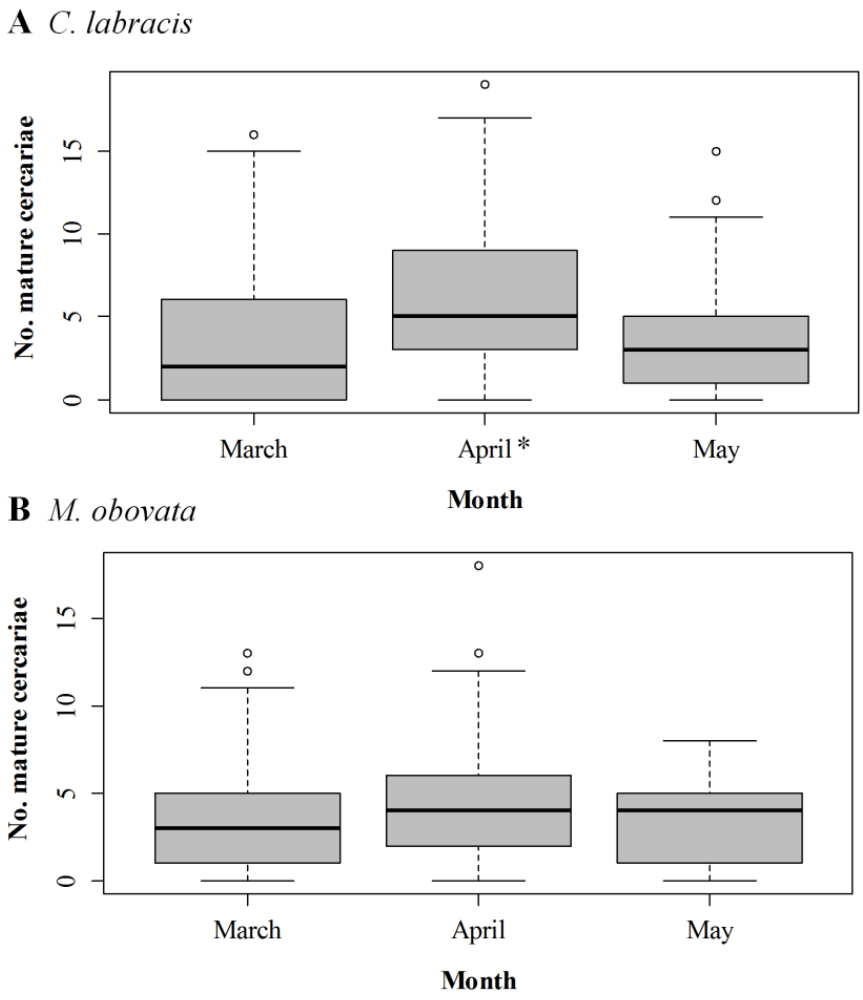

Figure S7.3. Monthly differences in number of mature stages in (A) *C. labracis* and (B) *M. obovata* infections. Asterisks indicate statistically significant differences (*P-value*<0.001).

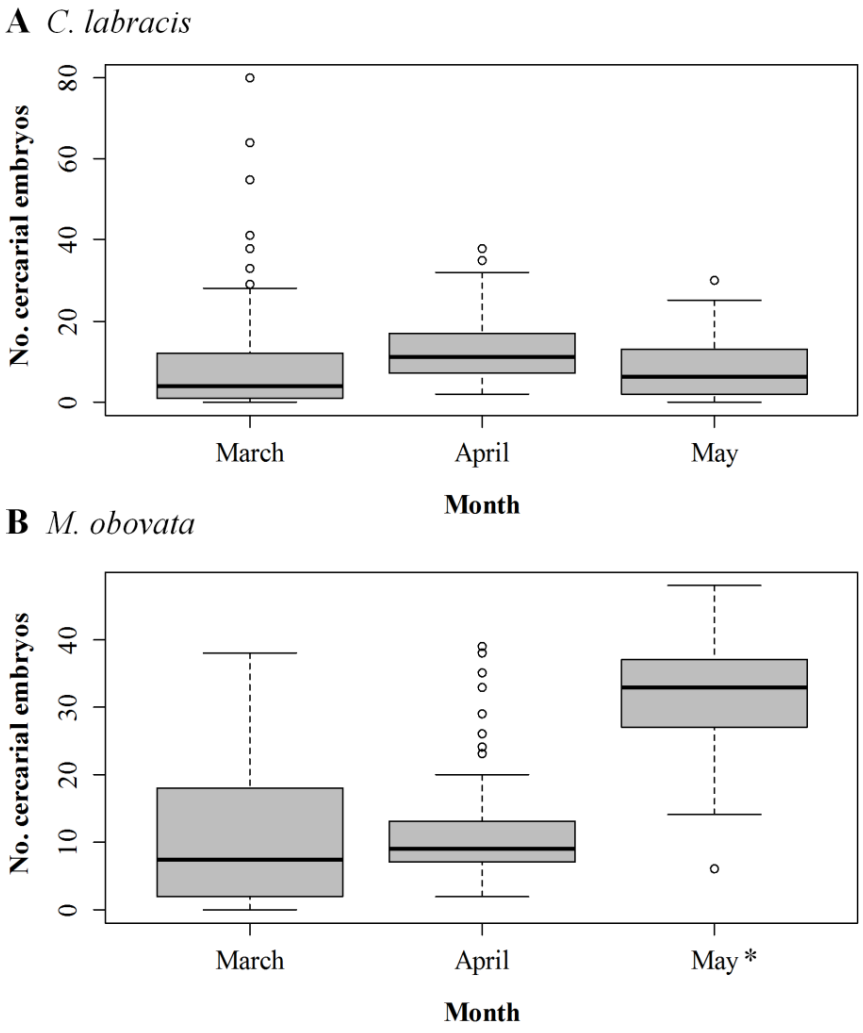

Figure S7.4. Monthly differences in number of cercarial embryos in (A) *C. labracis* and (B) *M. obovata* infections. Asterisks indicate statistically significant differences (*P-value*=0.03).

Table S7.1.Results of (a) linear model and (b) pairwise comparisons evaluating the differences in snail size in *C. labracis* (N=36) and *M. obovata* (N=15) single infected snails and uninfected snails (N=8). The intercept value stands for snail size in *M. obovata* infected snails, to which the size of *C. labracis* infected snails is compared, showing the difference between means. Statistically significant results (at α = 0.050) are indicated in bold type, with the corresponding P-value obtained with Bonferroni correction given in parentheses (b).

	Estimate	SE	*T-value*	*P-value*
(a)				
(Intercept)	8.8167	0.3378	26.101	**<0.001**
C. labracis	-1.1233	0.4021	-2.794	**0.007**
Uninfected	-1.1479	0.5728	-2.004	**0.049**
(b)				
M.obovata- C.labracis	1.1233	0.4021	2.794	**0.019 (0.021)**
Uninfected- *C.labracis*	-0.0246	0.5114	-0.048	0.999 (1.00)
Uninfected- *M.obovata*	-1.1479	0.5728	-2.004	0.118 (0.15)

Table S7.2. Results of linear mixed model (a.1, b.1) and pairwise comparisons (a.2, b.2) evaluating the effect of month on the maturity index of (a) *C. labracis* and (b) *M. obovata* sporocysts. The intercept value in the LMM stands for the mean maturity index in the first month, i.e. March, to which the other months are compared. The estimate of a month is added or subtracted from the intercept value. Statistically significant results (at α=0.050) are indicated in bold type, with the corresponding P-value obtained with Bonferroni correction given in parentheses. (A) *C. labracis* sporocysts: N=407 (March=138, April=143, May=126). Random effect 'snail', variance =0.035, (B) *M. obovata* sporocysts: N=202 (March=108, April=69, May=25). Random effect 'snail', variance =0.009.

	Estimate	SE	*T-value*	*P-value*
(A) *C. labracis* infection				
(a.1)				
(Intercept)	1.92244	0.05729	33.56	**<0.001**
April	0.17678	0.07737	2.29	**0.022**
May	0.10205	0.08729	1.17	0.242
(a.2)				
April-March	0.17678	0.07737	2.285	0.0577 (0.0669)
May-March	0.10205	0.08729	1.169	0.4710 (0.7271)
May-April	-0.07473	0.08391	-0.891	0.6456 (1.0000)
(B) *M. obovata* infection				
(b.1)				
(Intercept)	2.11305	0.03944	53.57	**<0.001**
April	0.15264	0.06507	2.35	**0.0188**
May	-0.03353	0.11372	-0.29	1.2282
(b.2)				
April-March	0.15264	0.06507	2.346	**0.0468** (0.057)
May-March	-0.03353	0.11372	-0.295	0.9516 (1.000)
May-April	-0.18617	0.11855	-1.570	0.2488 (0.349)

Table S3. Results of linear mixed model (a.1, b.1) and pairwise comparisons (a.2, b.2) evaluating the effect of month on the number of (A) *C. labracis* and (B) *M. obovata* developmental stages (cercarial embryos). The intercept value in the LMM stands for the mean of developmental stages in the first month, i.e. March, to which the other months are compared. The estimate of a month is added or subtracted from the intercept value. Statistically significant results (at α=0.050) are indicated in bold type, with the corresponding P-value obtained with Bonferroni correction given in parentheses. (A) *C. labracis* sporocysts: N=407 (March=138, April=143, May=126). Random effect 'snail', variance =57.64. (B) *M. obovata* sporocysts: N=202 (March=108, April=69, May=25). Random effect 'snail', variance =76.28.

	Estimate	SE	*T-value*	*P-value*
(A) *C. labracis* infection				
(a.1)				
(Intercept)	8.833	2.240	3.943	**<0.001**
April	3.627	3.013	1.204	0.2286
May	-2.653	3.419	-0.776	1.5622
(a.2)				
April-March	3.627	3.013	1.204	0.450 (0.686)
May-March	-2.653	3.419	-0.776	0.717 (1.000)
May-April	-6.280	3.276	-1.917	0.133 (0.166)
(B) *M. obovata* infection				
(b.1)				
(Intercept)	10.917	2.979	3.665	**<0.001**
April	1.162	4.976	0.234	0.815
May	19.883	9.321	2.133	**0.033**
(b.2)				
April-March	1.162	4.976	0.234	0.9692 (1.000)
May-March	19.883	9.321	2.133	0.0779 (0.0987)
May-April	18.721	9.690	1.932	0.1223 (0.1600)

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~Chapter 8~

From benthos to seagulls: host spectra and transmission strategies of Cardiocephaloides longicollis (Strigeidae, Trematoda) in the Mediterranean

~Chapter 8~

From benthos to seagulls: host spectra and transmission strategies of *Cardiocephaloides longicollis* (Strigeidae, Trematoda) in the Mediterranean

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ABSTRACT: The trematode *Cardiocephaloides longicollis* (Strigeidae, Trematoda) is distributed mainly in the Mediterranean; it has a complex three-host life cycle, including a snail, a fish and a definitive bird host. It is trophically transmitted from fish to bird and, since infection rates in these hosts are often high, it likely exerts a strong effect on the food web structure in Mediterranean habitats. Despite punctual records on infections, the full host spectrum of *C. longicollis* is unknown. In the present study, we aimed to extend the knowledge of the host-spectrum and host-specificity of *C. longicollis* on all host levels, and to analyse this information in the context of transmission dynamics, dispersion and accumulation of the parasite in different hosts or at different geographic localities. Screening of 3351 molluscs (24 species), 2108 fish (25 species), 154 birds (17 species) and 158 bird faecal samples for *C. longicollis* infection yielded new host records at the first intermediate host level (1 taxon) but especially the second intermediate host level (12 taxa), the latter including two new host families. Statistical analyses showed that the infection level in fish was highest in autumn and winter, possibly due to parasite-induced mortality in spring. It also revealed that demersal and benthic fish were more frequently targeted by cercariae as their parasite load was generally higher than that of pelagic fish. Furthermore, accumulation of cerebral metacercariae with fish size was observed, but parasite numbers stagnate in the largest fish, which strongly points to parasite-induced mortality at a specific fish size. Infection levels of *C. longicollis* in areas with extensive fishing activity were higher than previously recorded and we found fish species and fish sizes with the highest number of parasites to represent an important fraction of bycatch in the Mediterranean. Thus, the present results strongly suggest an enhanced transmission by anthropogenic impact from Mediterranean fisheries. For the first time, *C. longicollis* was also detected in aquaculture facilities, and the impact of these installations on the parasite life cycle is yet to be evaluated.

KEY WORDS: Transmission · Trematodes · Strigeidae · *Cardiocephaloides longicollis* · Anthropogenic influence · Host spectra · Mediterranean

8.1. Introduction

Trophically transmitted parasites are central elements in most aquatic food webs (Lafferty *et al.* 2006, 2008), playing an important role in host community structure and exerting strong effects on food web structure when infecting a high number of host species (Thompson *et al.* 2005). However, information on the full spectrum of host species used by individual parasites is scarce or unavailable for most species (Poulin 2001). In parasites with complex life cycles, the degree of specialization (i.e. the number of host species used) can vary widely from one life stage to the next. To complete their life cycle, polyxenous parasites use both active transmission modes, involving free-living stages, as well as passive transmission modes, exploiting existing food chains. Thereby, species-specific and stage-specific adaptive strategies ensure transmission (Sukhdeo & Sukhdeo 2004, Poulin 2007). The full set of stage-specific host spectra and transmission strategies characterizes the life cycle ecology of trophically transmitted parasites (Poulin 2007).

The digenean trematode *Cardiocephaloides longicollis* (Rudolphi, 1819) Dubois, 1982 (Strigeidae) is widespread along European coastlines, especially in the Mediterranean (Fig. 8.1), and its complex life cycle is embedded in local food webs. Adult *C. longicollis* colonize the digestive tract of their definitive hosts, seagulls, where they reproduce sexually. Eggs are released via faeces, which drop to the seafloor where free-swimming miracidia hatch. Following entry into the first intermediate host, the snails *Nassarius corniculum* (Olivi, 1792) and *N. reticulatus* (L.) (Nassariidae), the parasite develops cercariae-producing sporocysts by massive asexual reproduction. Cercariae emerge from the snails and infect a fish second intermediate host by burrowing into the skin. This event is followed by migration to and encystment (metacercaria formation) in the optic lobes of the fish brain (Prévot & Bartoli 1980). Infected fish species generally inhabit marine environments but also brackish waters with variable salinity and temperatures, such as lagoons or estuaries. Nineteen fish species from 7 fish families have been reported as second intermediate hosts, most frequently sparids, followed by bleniids, gobiids, and labrids (Prévot & Bartoli 1980, Bartoli & Prévot 1986, Machkevsky *et al.* 1990, Korniychuk 1997, Zander 2004, Osset *et al.* 2005). *Cardiocephaloides longicollis* is trophically transmitted to the definitive host when infected fish are consumed by seabirds (Bartoli & Prévot 1986, Fredensborg & Longoria 2012).

The main area of distribution of *C. longicollis* appears to be the Mediterranean; however, the parasite has been reported from a wide range of geographical locations with contrasting physical conditions such as the Baltic and Black Sea, and even Lake Baikal as well as the North Atlantic around the Welsh Islands, parasitizing migratory bird hosts, usually members of the Laridae and Sternidae families (Fig. 8.1).

Cardiocephaloides longicollis uses a variety of strategies to ensure transmission to the next host. Eggs, miracidia and cercariae are at the mercy of environmental conditions and water currents. Cercariae of *C. longicollis* overcome the distance between their benthic/sedentary snail hosts and their target fish hosts by following a strategy of alternation between swimming and resting periods, using positive phototaxis (Bartoli & Prévot 1986, Combes *et al.* 1994). A common adaptation of parasites is to manipulate their host's behaviour in order to facilitate transmission, for example by inducing behaviours in the intermediate host that make it more susceptible to predation by the definitive host (Poulin 2001, Moore 2002). *Cardiocephaloides longicollis* is such a manipulative parasite, whose metacercariae strategically locate in the optic lobes of the mesencephalon of fish (Prévot & Bartoli 1980, Osset *et al.* 2005, Bartoli & Boudouresque 2007), causing a fundamental change in host behaviour caused by hampered vision and swimming ability (Prévot 1974, Barber & Crompton 1997). This results in fish dwelling in the upper part of the water column as well as in body oscillations that make the bright flanks visible to flying birds (Osset *et al.* 2005, Bartoli & Boudouresque 2007). Infected fish could thus be more prone to predation by birds (Moore 2002, Seppälä *et al.* 2004, Osset *et al.* 2005, Fredensborg & Longoria 2012).

Increasing intensities of infection by metacercariae may have an enhanced effect on fish behaviour as demonstrated e.g. for *Euhaplorchis* sp. (see Fredensborg & Longoria 2012) and *Diplostomum phoxini* (see Barber & Crompton 1997). In habitats where *C. longicollis* is numerous and increased predation rates by birds occur, energy flow through the food chain may be altered. As a consequence, changes in predator-prey links, food web structure and dynamics are likely to occur (Thomas & Poulin 1998, Lefevre *et al.* 2009). Anthropogenic influences, with both aquaculture and fisheries being particularly relevant, may further enhance the transmission of *C. longicollis* to their final hosts. Osset *et al.* (2005) hypothesized about the potential impact of fish discards on the life cycle of *C. longicollis*, but evidence was not provided. An increased transmission of *C. longicollis* could occur in areas of intensive fisheries, if discards, which are frequently consumed by seabirds (Witt *et al.* 1981, Arcos *et al.* 2001, Christel *et al.* 2012), harbour

large numbers of metacercarial stages. Similarly, aquaculture installations attract birds, however, knowledge about the occurrence of *C. longicollis* in aquaculture stocks is lacking to date.

Despite existing records on the occurrence and abundance of *C. longicollis* in several localities and host species, this information is not comprehensive and has not previously been synthesised in a way that would allow a more holistic appraisal of the parasite's transmission dynamics. Here, we explore the successful life cycle strategies of *C. longicollis* by (i) extending our knowledge of the host spectrum used by *C. longicollis* in the Mediterranean Sea, reviewing its degree of specialization at all levels, i.e. in snail, fish and bird hosts; (ii) critically determining the marine microhabitat targeted by cercariae, by comparing infection levels in benthic, demersal and pelagic fish species, using both, new and previously-published data; (iii) analyzing the effect of fish size on the accumulation of *C. longicollis* metacercariae and the possible implications of different parasite loads; and (iv) exploring which factors (anthropogenic and physical) could enhance the transmission of the parasite, such as fisheries and aquaculture .

8.2. Materials and Methods

8.2.1. Sampling sites and habitat definition

An opportunistic sampling strategy was adopted for this study, focussing on the examination of a diverse range of hosts suspected to be part of the life cycle of *C. longicollis*. All sampling sites are indicated in Fig. 8.1 and described in detail in Table S8.1. Molluscs were hand-collected in shallow areas that are likely important for *C. longicollis* transmission but differ in their biological conditions and anthropogenic influence (Table S8.1). In contrast to molluscs, birds move over large distances, dispersing parasite eggs, so that their site of capture represents only a small dot on their large distribution map. Thus, samples of bird specimens were divided in two large zones, approx. 200 km from each other, one around the Ebro Delta and the other around Valencia. All birds were obtained from bird sanctuaries. Additionally, bird faeces were collected from an uninhabited island (off Benidorn, Spain) with an isolated, large colony of *Larus michahellis*. Fish species were sampled in 14 localities with different habitat characteristics (see Fig. 8.1 and Table S8.1), along the western Mediterranean coast (Spain) and in three lagoons in southern Sardinia (Italy), including one *S. aurata* aquaculture installation (netpens). The largest number of fish individuals and species

were collected in Carboneras and Santa Pola (Spain), areas of high fishing activity. Demersal, benthic and pelagic fish were obtained by trawling, seine net and trapping. In the lagoons of Sardinia, V-shaped nets (so called "lavorieri") were used to catch fish that migrate from the lagoon into the sea.

8.2.2. Host taxa and screening methodology

A total of 3351 molluscs belonging to 24 species and 18 families were sampled (Table S8.2). The specimens were dissected under a stereomicroscope and examined for the presence of sporocysts or mature cercariae of *C. longicollis*. A total of 2108 fish belonging to 25 species and 10 families was examined for the presence of metacercariae of *C. longicollis* in the brain (Table S8.3). After capture, all fish were immediately transported to the laboratory and examined within 48 hours. Fish were measured and weighed before dissection. The whole brain was carefully removed from the skull and examined under the stereomicroscope for the presence of metacercarial cysts, which were recovered with a pipette, cleaned with saline and excysted for their identification, using fine needles. Two samples of 30 and 24 fish (see Table S8.3) were instead examined as a pool, for which the total number of parasites was determined after chloropeptic tissue digestion. A total of 154 birds belonging to 17 species and 4 families were obtained from the bird sanctuaries (Table S8.4). Given the opportunistic nature of sampling, sample sizes of some species are low. Birds were frozen until necropsy. The alimentary tract (from oesophagus to cloaca) was removed and examined for adult specimens of *C. longicollis*. A total of 158 bird faeces were obtained during four consecutive seasons (Table S8.5). Individual *L. michahellis* faeces were sampled, using a pipette and filtered seawater (5µm filter). After their visual quantification into categories (small, medium, large and extra large) based on the apparent mass of faeces, each individual collection container was filled up to 30ml with seawater. After mixing, two subsamples of 2ml each were examined under the stereomicroscope and *C. longicollis* eggs were quantified.

8.2.3. Parasite identification

All stages of *C. longicollis*, i.e. adults as well as sporocysts, cercariae, metacercariae and eggs, were identified following the descriptions by Prévot & Bartoli (1980) and Dubois (1968), and preserved in 100% ethanol for molecular analyses Additional samples were preserved in 70% ethanol for morphological examination. Random samples were analysed molecularly by rDNA sequencing, to confirm microscopic parasite identification. DNA extraction, rDNA amplification and sequencing methods were the

same as in Born-Torrijos *et al.* (2012). For faecal samples, either a higher concentration of Proteinase K was used (200 μgmL^{-1} versus 100 μgmL^{-1}) in order to rupture the egg shells, or 3 freeze-thaw cycles with liquid nitrogen were performed. For the amplification and sequencing of ITS2 rDNA sequences, primers 3S (forward 5′- GGT ACC GGT GGA TCA CGT GGC TAG TG-3) (Morgan & Blair 1995) and ITS2.2 (reverse 5′- CCT GGT TAG TTT CTT TTC CTC CGC-3′) (Cribb *et al.* 1998) were used, with an annealing temperature of 54ºC for 50s.

8.2.4. Statistical analyses

In all hosts, infection prevalence (%), mean intensity and range, as well as mean abundance (± standard deviation, SD) were calculated according to Bush *et al.* (1997). The data acquired in the present study were combined with published data from existing records, obtained through a search of the Web of Science (ThomsonReuters) (search string: Cardiocephal*), expanded by a manual bibliographic search of lists of references in publications found by the primary search. Inclusion of reports was restricted to those of Europe and Russia. For statistical analyses, only true zero prevalences were included, i.e. local prevalence is zero but there are existing records that show this host species can actually be infected. Nevertheless, host species examined in this study or in the existing literature found to be uninfected were added to Table S8.3, to indicate host taxa where *C. longicollis* has never been found. In order to estimate whether fish habitat (benthic, demersal and pelagic) and season of capture can be related with the prevalence or abundance of *C. longicollis*, fish data were analysed using generalized linear mixed models (GLMM) and linear mixed models (LMM). To check for congruent patterns in prevalence or abundance depending on fish habitat, our data combined with previously published records were analyzed with different linear models (LM). The correlation between the prevalence and mean intensity of *C. longicollis* infection was calculated for all fish samples, using a Spearman rank correlation. We initially also aimed at analysing whether certain zones of the Mediterranean show higher parasite densities in fish but due to the effect of the non-homogeneous sampling of host taxa, resulting in unusually high prevalences in particular fish species sampled only at one or two sites, weak statistical significance and skewed effects were observed so that the analysis was discarded. To investigate the relationship between fish length and *C. longicollis* abundance, two subsets of 198 *D. vulgaris* and 196 *D. annularis* were used. Fish size data were stratified into size classes and the effect of size on the number of metacercariae per individual fish was analysed using a generalized linear model (negative binomial GLM).

Thereafter, the degree of aggregation was calculated as an index of discrepancy (Poulin 1993), using the software package Quantitative Parasitology (QP web, powered by R, Version 1.0.9) (Rozsa *et al.* 2000, Reiczigel *et al.* 2013). Similar to fish data, bird data were analysed for the correlation between parasite prevalence and abundance, and for the effect of season on the number of *C. longicollis* eggs in bird faeces (negative binomial GLM). Data from snails were scarce and thus not analysed statistically. All analyses were conducted with different packages in R (R Development Core Team, version 3.0.1) and detailed information on all models and conditions can be found in Supplementary Materials-Detailed statistical methods.

8.3. Results

8.3.1. *Cardiocephaloides longicollis* hosts

In molluscs, the prevalence of *C. longicollis* was relatively low and the occurrence of the parasite was restricted to nassariid snails, with only one new host record, *Cyclope neritea* (L.), thus indicating a high degree of host specificity at this level. Prevalence was relatively low in *C. neritea* (0.65% in the Ebro Delta, and 1 of 2 snails in Carboneras), whereas in the more abundant host *N. reticulatus*, the prevalence was higher with 8.3%, exceeding previous records of *C. longicollis* in mollusc hosts. Our data demonstrate that *C. longicollis* has a much wider host spectrum in the second intermediate host than previously reported, by adding 12 new host records: *Diplodus sargus* (L.), *Dentex dentex* (L.), *Spicara maena* (L.), *Spondyliosoma cantharus* (L.), *Pagellus acarne* (Risso, 1827), *Pagellus erythrinus* (L.), *Pagellus bogaraveo* (Brünnich, 1768), *Oblada melanura* (L.), *Zosterisessor ophiocephalus* (Pallas, 1814), *Coris julis* (L.), *Chromis chromis* (L.), *Serranus scriba* (L.). This includes the Sparidae, the Gobiidae and the Labriidae as previously recorded host families and the Pomacentridae and Serranidae as newly reported host families. For the first time, *C. longicollis* was detected in *S. aurata* from aquaculture netpen facilities, located in the southwestern Mediterranean, with 53.9% prevalence. Wild *S. aurata* from the coast of Italy showed similar infection prevalences (40–62.5%). With regard to final hosts, the infection levels found in three larid species were high with ≥50% prevalence and thus up to 22 times higher than in previous reports (Table S8.4). However, *C. longicollis* was absent from terns and smaller larid species in the present study that had been reported as hosts in previous studies (see Table S8.4).

We identified all specimens of *C. longicollis* from different hosts morphologically, and confirmed their identity molecularly for the following: Metacercariae from *D. dentex*, *C. chromis*, *D. puntazzo* and *L. mormyrus*, cercariae from *N. reticulatus* and *C. neritea*, and adults parasitizing *L. michahellis* and eggs from faecal samples of the same species. Sequence identity of 28S rDNA sequences was 100% when compared with the sequence of *C. longicollis* available on Genbank (AY222171) (bp compared: 1307bp of *D. dentex*, 1127bp of *C. chromis,* 437bp of *D. puntazzo,* 607bp of *L. mormyrus,*1216bp of *N. reticulatus* and 1097bp of *C. neritea*). ITS rDNA sequences of eggs and adults showed an identity between 99.7 and 100% and were deposited in Genbank (GenBank accession numbers XXXX, XXXX).

8.3.2. Site, season and habitat effects in fish hosts

Cardiocephaloides longicollis infection levels did not differ substantially between sites, when comparing data on the same fish species captured at different sites with similar high fishing activity, i.e. *D. vulgaris*, *S.aurata* and *L. mormyrus* (Table S8.3). Although the fish species *L. mormyrus* shows generally very high infection rates, it only reaches 100% in an area surrounded by aquaculture facilities. Moreover, *D. annularis* shows higher infection values in areas with high fishing activity (76.9% to 99.6%) than in areas with low fishing activity (3.3%, 3/5 infected fish; Table S8.4). Areas high fishing and aquaculture tradition such as Santa Pola and Carboneras show high mean intensity values of infection (19 to 26), as well as the Italian lagoons (19.8 to 49.9). The infection level of fish habiting sandy (14.3 to 100%) as well as rocky areas (5.3 to 100%) (Table S8.1) is similar, with a wide range of parasitic loads. High infection rates were also detected in snails (up to 8.3%), fish (40 to100%) and birds (27 to 69%) close to lagoons and bird sanctuaries, which conditions may favourable for *C. longicollis* transmission.

With regard to the seasonal abundance of *C. longicollis* in its fish hosts, the GLMM and pairwise comparisons showed that the overall prevalence of *C. longicollis* is significantly higher during winter and autumn compared to spring and summer (Fig. 8.2, Table S8.6, Fig. S8.1). This analysis showed no significant differences between habitats ($P>0.05$), possibly due to the heterogeneous infection levels in demersal and pelagic habitats and the single benthic sample (GLMM, proportion of variance explained by random effect 'fish species'= 72%). However, when looking for differences in mean abundance between habitats and accounting for fish species (Table 8.1A), we found that benthic fish had significantly higher levels than demersal and pelagic fish (LMM,

proportion of variance explained by random effect 'fish species'=91%). When focusing on the habitat effect (Table 8.1B, Fig. S8.2), with the overall prevalence not accounting for the fish species, fish from demersal and benthic habitats showed higher prevalence and mean abundance of *C. longicollis* compared to those from the pelagic habitat, with no significant differences between these two habitats. Including both new and previously published data, whether or not true zero data were included, the mean abundance was approx. 4 times greater in demersal, and approx. 23 times greater in benthic fish than in pelagic fish (Table 8.1B). The difference in prevalence, although not so drastic, is still considerable, being 38.3% and 131.6% greater in the demersal and benthic habitat, respectively, compared to the pelagic one. Generally, pairwise comparisons were concordant with linear model results.

8.3.3. Correlation between prevalence and abundance of *C. longicollis* in fish

We detected a strongly positive correlation between prevalence and mean intensity of *C. longicollis* infection across all samples in fish (rho=0.76, S= 2516.541, P<0.001) (Fig. 8.3), even when data from the literature were included (rho=0.69, S=6923.835, P<0.001). When analyzing this correlation with regard to habitat type, we found that, in the demersal habitat, where sample size is high, prevalences and abundance show high variability, whereas in pelagic and benthic habitats there is little variation, with a medium prevalence in pelagic fishes and a high prevalence in the benthic habitat. Thus, overall, demersal samples showed the highest and most diverse infection levels.

8.3.4. Accumulation of *C. longicollis* metacercariae with fish age

Data on prevalence, mean abundance and mean intensity of *C. longicollis* in the size-stra tified sample of *D. vulgaris* and *D. annularis* are given in Table S8.7 and S8.8, and are vis ualized in Fig. 8.4. In *D. vulgaris*, both parasite intensity and abundance showed a tende ncy to increase with host size, but reached their highest values in intermediate size class es 4 to 6 (120 to 148mm), where the highest number of metacercariae was recovered (aver age of 33–38 individuals per fish) (Fig. 8.4, Table S8.7). Prevalence showed a steady increa se with *D. vulgaris* size, however, in the two largest size classes, although prevalences we re the highest with 93–100% of fish infected, the abundance and the intensity of *C. longic ollis* decreased relative to medium-sized classes (Fig. 8.4, Table S8.7). The aggregation le vel, calculated by the discrepancy index, was similar in all size classes, expect for the high est class where it decreased markedly (Table S8.7). In *D. annularis,* the mean intensity an d abundance showed the same tendency of increase with host size, with the highest valu

es obtained in the two largest size classes (140 to 162 mm), where, once again, the aggreg ation of metacercariae was the lowest (Table S8.8, Fig. 8.4). Size class 8 contained the hig hest number of recovered metacercariae with an average of 73 parasites per fish brain. Inf ection prevalence showed a steep increase, from 46.2 to 79.0%, in class 1 to 2. In the four largest size classes it was greater than 95%, indicating that from approx.120cm total lengt h, almost every fish is infected. The highest mean abundance and mean intensity occurre d in both species at standard lengths around 140 to 150mm. Negative binomial GLM sho wed that the number of metacercariae in *D. vulgaris* was significantly higher in size class es 3 to 8 compared to the lowest size class, i.e. 1 ($P< 0.01$), and in *D. annularis*, higher in a ll size classes compared to size class 1 ($P< 0.01$).

8.3.5. Bird data

Among the bird species examined in this study, only larids were infected with *C. longicoll is*, with *L. michahellis*, *L. argentatus* and *L. andouinii* showing much higher prevalences t han previously reported, with 66.7%, 69.2% and 50% infected specimens respectively (Ta ble S8.4). *Chroicocephalus ridibundus* and species belonging to the family Sternidae were not infected with *C. longicollis* in our 19 samples, although 6 previous records exist for th ese hosts (see Table S8.4 for references). Our data together with that of the literature sho wed no significant relationship between prevalence and mean intensity across samples ($P>0.05$), possibly due to the low number of samples ($n= 12$).

The analysis of bird faeces showed that the number of eggs of *C. longicollis* was related to the amount of faeces in each sample (small, 3.5 ± 8.3, max 30 eggs; medium 20.6 ± 53.2, max 240 eggs, large 73.7 ± 269.6, max 1785; extra large 264.6 ± 587.3, max 2665 eggs). The prevalence of infected faeces was highest in spring (42.9%), followed by summer (35%), autumn (27.5%) and winter (22.2%) (Table S8.9, Fig. S8.3). However, the highest intensity, i.e. number of eggs in a single faecal sample, occurred in winter (150.2 ± 534.0), followed by spring (71.9 ± 170.7). Nevertheless, no significant seasonal differences in the mean number of eggs per sample were found by negative binomial GLM ($P>0.05$, Table S8.9).

8.4. Discussion

Parasites can have strong impacts on ecosystems and food webs, especially if their biomass is substantial such as that of trematodes in intertidal areas and estuaries (Kuris *et al.* 2008). In order to estimate the influence of a parasite on an ecosystem and its food

webs, it is essential to know its full host spectrum and its abundance in different hosts. It has been shown that generalist parasite species have the strongest effect on food web attributes (Thompson *et al.* 2005). In the present study, we investigated the host spectrum, distribution and transmission of *C. longicollis*, a trematode common along the Mediterranean coastline. We demonstrate that *C. longicollis* has an extremely wide host spectrum, especially at the level of the second intermediate host, where it infects phylogenetically distantly related hosts. Furthermore, we determined that infection rates in definitive bird hosts are higher than previously reported. So what factors could be responsible for the detected increase in parasite prevalence and hence transmission?

8.4.1. Seasonality of parasite transmission

We did not expect to find any seasonal patterns in the life cycle of *C. longicollis* as metacercariae can survive for a long time (at least 12 months, in some cases even years; Chubb 1979, and references therein) in their fish hosts, they are not eliminated by a host tissue response when in nervous tissues such as the brain, and fish are consumed by seabirds throughout the year. Seagulls are homoeothermic, and thus development in the definitive host probably takes place throughout the year as indicated by our results which show that eggs are released with the faeces all year long, without statistical differences between seasons.

However, we detected a seasonal pattern of accumulation in fish hosts with autumn and winter showing higher infection rates than spring. This result could be explained by the seasonal occurrence of cercariae. Molluscs are poikilotherm and cercarial production likely peaks at higher temperatures in summer, as in many other Mediterranean species (see Poulin 2006). This would lead to increased infection rates during the summer and accumulation of metacercariae during autumn and winter. This effect is likely skewed to a certain degree with increasing age of fish, as metacercariae, once located and encysted in the brain, are immobile; however if the same pattern occurs during following years, the effect would still be visible. Alternatively, it is possible that the decline in the infection levels in fish during spring is related to an increase in fish consumption by birds (Annett & Pierotti 1989), eliminating the fish with heaviest infections first (see parasite-induced mortality below). Overall we conclude that *C. longicollis* does not show a clear seasonality in its life cycle and, thus, infection levels are comparable across different studies/seasons.

8.4.2. Target hosts

Similar to other trematodes, *C. longicollis* strategically uses phototaxis to reach its preferred microhabitat (Bartoli & Prévot 1986, Combes *et al.* 1994, Smith & Cohen 2012). The behaviour of the cercariae thus determines the ecological niche where fish are going to be infected, and cercariae can be more specific to the host's environment than to the host itself (Combes *et al.* 1994), as demonstrated in the present case. To the best of our knowledge, this is the first time that infection levels of *C. longicollis* have been compared between fish species by focusing on their specific habitats. We demonstrate that the mean abundance of *C. longicollis* in demersal and benthic fish is 4 and 23 times higher, respectively, than in pelagic fish. The strong preference for the benthic habitat detected in our data is based on the high abundance of *C. longicollis* in the only two benthic fish species found infected in our analysis, i.e. *Z. ophiocephalus* and *S. scriba*. However, previous reports show that infection levels in bottom-dwelling fish are overall extremely low with prevalences <2.9% (Table S8.3). Only a few exceptions with high *C. longicollis* infection prevalences exist alongside *Z. ophiocephalus* (75.6%) and *S. scriba* (75%) in the benthic habitat: *Neogobius melanostomus* (58%) and *Zoarces viviparus* (11%). We believe that these four species (and probably some others) are potential biological sinks as they are unlikely prey items for birds. Their infection is possibly a result of the wide host range of *C. longicollis* which puts this parasite at risk of infecting fishes that will not enable transmission to the appropriate definitive host. Depending on their density in a specific marine habitat, these bottom-dwelling fish with high infection levels remove substantial numbers of *C. longicollis* from the system. Overall, demersal fish clearly showed the highest infection levels, reaching 100% prevalence in *D. vulgaris* and *L. mormyrus*, and a maximum of 220 parasites in a single brain (*D. annularis*). Thus, the targeted swimming behaviour of the cercariae increases their exposure to demersal fish and pays off as the bulk of infected fish species comes to visit surface waters and enables trophic transmission to bird hosts.

8.4.3. Accumulation of metacercariae in fish

The cumulative effect of parasites with host size has been studied in several species (Thomas *et al.* 1995, Rousset *et al.* 1996, Osset *et al.* 2005). A decrease in parasite aggregation at a certain host size may indicate parasite-induced mortality, i.e. heavily infected individuals are removed from the population, either by death or by being preyed on more intensively (Anderson & Gordon 1982, Rousset *et al.* 1996, Poulin 2001). In an

attempt to better understand the accumulation of *C. longicollis* and detect potential parasite-induced host mortality, we investigated the relationship between fish size and parasite number in two heavily infected hosts, *D. annularis* and *D. vulgaris*. In both species, an accumulative effect was observed, i.e. larger, hence older, individual hosts harbour higher numbers of metacercariae, the highest abundance and intensity occurring in hosts around 140 to 150 mm standard length, a smaller size than previously analysed (Osset *et al.* 2005). The aggregation of metacercariae (or D index) in *D. vulgaris* was overall higher than in *D. annularis* at similar fish sizes, potentially due to the different spatial distribution of these hosts and thus different exposure to cercariae (Poulin 2001). However, the decrease of parasite aggregation in the largest size classes of both species indicates that heavily infected individuals are removed from the population, especially in *D. vulgaris* since the mean abundance decreases significantly also in even larger sizes (Anderson & Gordon 1982). The modification of the behaviour of intermediate fish hosts by large parasites loads, i.e. interference with vision and increased surface "flashing" behaviour (Osset *et al.* 2005, Bartoli & Boudouresque 2007), due to the accumulation of metacercariae, may be responsible for the removal of the fish with the highest infection levels by predatory seabirds, and hence lower infection levels are found in the "remaining" fish that are able to survive and grow into a larger size class.

8.4.4. Birds hosts and enhanced transmission to birds

Despite a few parasitological studies on gull species (references in Table S8.3) and the description of an associated pathology in the bird's intestine (Abdel-Aal *et al.* 2004), the information on *C. longicollis* distribution in final bird hosts is scarce. Since many seagull parasites are transmitted trophically, variations in parasite abundance may be a consequence of the feeding habits of each species (Roca *et al.* 1999).Thus, following encounter and host compatibility/suitability filters (Combes 2001, Lagrue *et al.* 2011), differences in bird diets may explain differences in infection levels (Oro & Ruiz 1997, Arcos *et al.* 2001). For example, both species feed equally on fish approx. 100 to 140 mm long (Arcos *et al.* 2001), but *L. michahellis* prefers, i.e. preys more on larger and heavier fish than *L. audouinii* (Witt *et al.* 1981, Oro & Ruiz 1997, Arcos *et al.* 2001). Furthermore, *L. michahellis* preys also, with small competition with other species, on the largest fish (150 to more than 250 mm) (Arcos *et al.* 2001), choosing approx. 50% of the time fish larger than 200mm and 20% of the time 110–190 mm long fish (Oro & Ruiz 1997). First, and most importantly, these size ranges include the highest abundance and intensity rates of infection in *D. vulgaris* and *D. annularis*, the latter showing 100% infection

prevalence. Fish preyed by seagulls are the ones with highest metacercariae counts due to the accumulation over time are removed from the population, therefore maximising the number of parasites in the final host. In contrast, smaller bird hosts such as *C. ridibundus* or terns, include in their diet more than 50% small fish (2–10cm, Oro & Ruiz 1997), which transmit lower parasite numbers, thus resulting in lower infection levels or no infections, as in the present study.

Larus audouinii and *L. michahellis* can exhibit behavioural plasticity depending on food availability. Both species show a clear tendency to use food resources of anthropogenic nature, if available (Witt *et al.* 1981, Bartoli 1989, Ramos *et al.* 2009). Both have recently been observed to feed on demersal and benthic fish from fisheries discards (Arcos *et al.* 2001, Christel *et al.* 2012, and references therein). As adult digeneans are indicators for the type of habitat where the gull has taken its prey (Bartoli 1989), the high prevalences in seagulls reported here clearly supports the uptake of large numbers of the most infected second intermediate hosts, i.e. predominantly demersal fish species (see above). Thereby, in the present study, infection levels were up to 22 times higher than reported in the past (>50%). A possible explanation therefore can be found in enhanced parasite transmission caused by fishery activities, which are along the Spanish Mediterranean coast, where the birds were sampled. For example, in *L michahellis* infection with *C. longicollis* was detected with prevalence between 61.5 and 69.2%, much higher when compared to the low infection found in areas with low fishing activity (10.4%). Bycatch constitutes an important fraction (13–67% in bottom trawl fisheries in the Western Mediterranean; General Fisheries Commission for the Mediterranean, No. 74, 2004) of the total catch in the extensive Mediterranean fisheries (Sánchez *et al.* 2004, Tzanatos *et al.* 2007). In the Ebro Delta, with an important commercial fishery (Arcos *et al.* 2001), discards are estimated around 41% of the landed fish (Oro & Ruiz 1997, Coll *et al.* 2008). Discards therefore support large seabird populations of *L. audouinii L. cachinnans, L. michahellis* and *C. ridibundus* (Oro & Ruiz 1997, Arcos *et al.* 2001) and represent an easily available food source for opportunistic scavenging species (Oro & Ruiz 1997, Sánchez *et al.* 2004). Discarded fish size varies somewhat depending on the area (Oro & Ruiz 1997, Machias *et al.* 2004), but clearly overlaps with the fish sizes showing the highest infection rates, e.g. in *Diplodus* sp. (169 ± 79mm), *Pagellus* sp. (93 to 147 mean size depending on species; Oro & Ruiz 1997, Machias *et al.* 2004), *Spicara* sp. (145 ± 30mm), *Serranus* sp. (120 ± 71mm). This fish size is swallowed by seagulls with a success rate of 63 and 100% (Oro & Ruiz 1997). Furthermore, the fish host species of *C.*

longicollis (Table S8.3), constitute the majority of bycatch in some Mediterranean areas, i.e. *P. acarne*, *B. boops*, *P. erythrinus* and *D. annularis,* together with *Spicara* spp. and *L. mormyrus* (Sánchez *et al.* 2004, Tzanatos *et al.* 2007). Most of these species are highly infected with *C. longicollis*, so that increased transmission by fishery activities becomes obvious. Moreover, the high cercarial prevalence found in *N. reticulatus* (8.3%) from the lagoon in the Ebro Delta exceeds previous records (Table S8.2), pointing too to a successful transmission to first intermediate hosts via infected faeces dropped in the shallow waters of the lagoon, thus combining two factors that seem to increase *C. longicollis* transmission. In addition to the high infection rates of this mollusc in a lagoonal habitat, the high infection detected in fish (40 to 100%) and birds (27 to 69%) close to these areas indicates these confined habitats as most favourable for *C. longicollis* as they concentrate its potential hosts, thus facilitating the parasite's transmission.

In addition to fisheries, aquaculture installations could act as enhancers of *C. longicollis* transmission due to the close vicinity of all hosts. Nassariid snails are scavengers that inhabit coarse to muddy sediment in shallow areas up to 25m depth (Bachelet *et al.* 2004, Rueda & Salas 2008, Urra *et al.* 2013). Molluscs occur at higher densities near sea cages (Edgar *et al.* 2005). With piscivorous birds being attracted to aquaculture installations, trophic transmission may also be enhanced around fish cages. In this study, we report, for the first time, the presence of metacercariae of *C. longicollis* in *S. aurata* cultured in netpens in Carboneras, with 53.9% prevalence. Wild *S. aurata* from other areas of the Mediterranean showed similar infection prevalences; thus the impact of aquaculture activities on *C. longicollis* transmission is still difficult to estimate at present, although increased parasite transmission can be expected.

In spite of their important role, parasites are under-represented in food web studies (Lafferty *et al.* 2008) and their inclusion is recommended (e.g. Thompson *et al.* 2005, Lafferty *et al.* 2006). We demonstrated that, as a trophically-transmitted generalist species with a complex life cycle, *C. longicollis* affects a high number of host species. Thus, a strong effect of this parasite on the energy flow through the food web is expected, even under natural conditions. However, the enhanced transmission to seagulls caused by anthropogenic influences, i.e. fishing activities and discards, which we demonstrated in the present study, makes *C. longicollis* an ideal candidate to compare future food web structures in natural communities versus those impacted by fisheries.

Acknowledgments. This research was supported by projects Prometeo/2015/018 and Revidpaqua ISIC/2012/003 of Valencian Regional Government (JAR), and by the European Centre of Ichtyoparasitology (Czech Science Foundation, grant 505/12/G112, Center of Excellence) (ASH). AB-T is the recipient of a doctoral fellowship (Ministry of Education and Science, Spain; grant AP2009–2560) and a visiting studentship to the Department of Zoology of the University of Otago in New Zealand (Ministry of Education, Culture and Sports, Spain; grant EST12/00528). The authors gratefully acknowledge Gabrielle S. van Beest and Lorenzo Miquel-Mazzetti (University of Valencia) for their help with fish examination, and the personnel of the Natural Park "Sierra Helada" and the birds sanctuaries "Centre de Recuperació de Fauna Salvatge de Torreferrussa" (Generalitat de Catalunya) and "Centre de Recuperació La Granja" (Generalitat Valenciana).

8.5. Figures and Tables

Figure 8.1. Map showing the geographical distribution of *C. longicollis* in the Mediterranean and the Black Sea and its reports from different hosts, including published data and those of present study. The silhouettes represent infection reports from fish, seagull and gastropods, with a number indicating the number of species that have been found infected in the sampling locality.

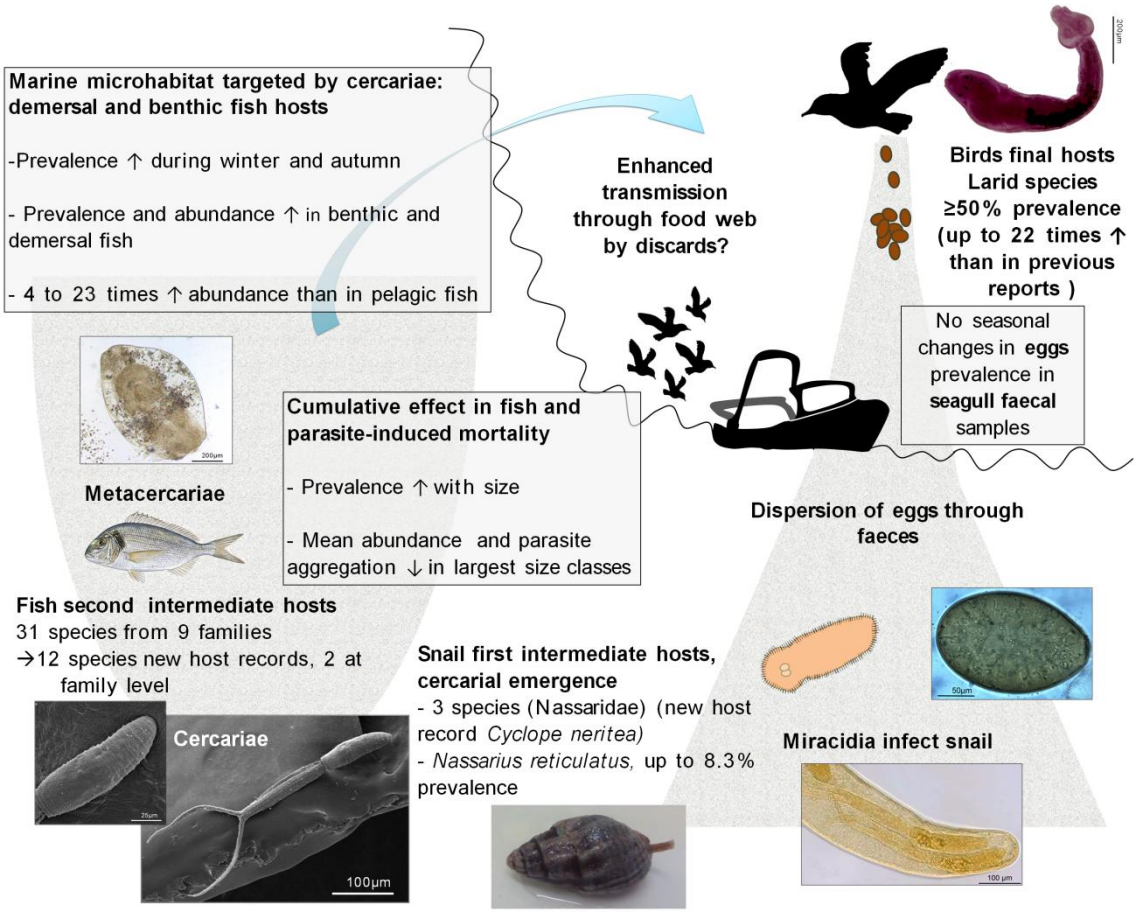

Figure 8.2. Complex life cycle of *C. longicollis* including the main factors that impact on parasite transmission, as a result of the present study.

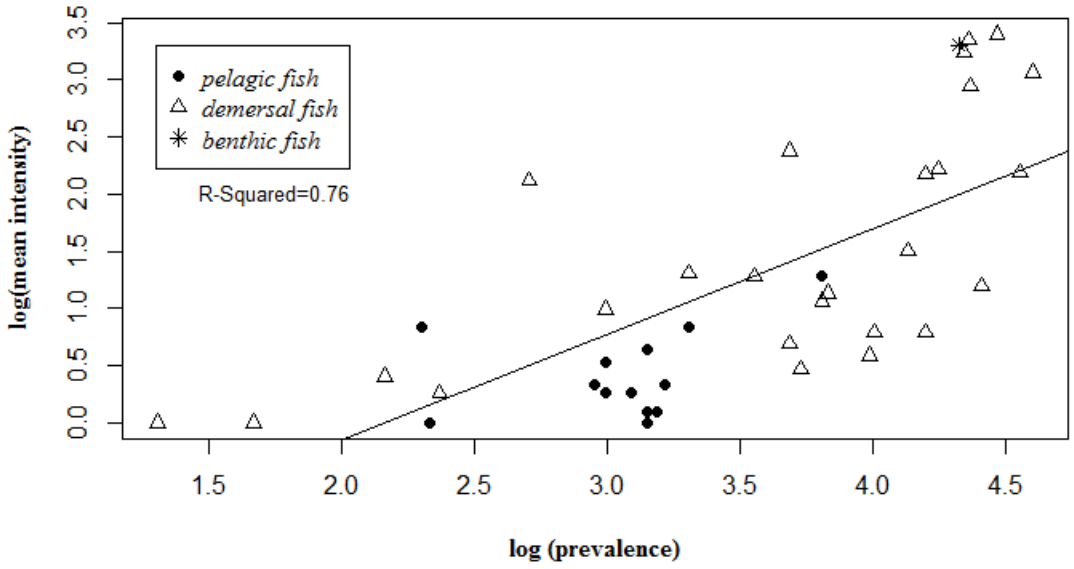

Figure 8.3. Graphical representation of the relationship between the prevalence and mean intensity of *C. longicollis* infection (log transformed data) in fish examined in this study. The regression line represents the best linear fit (y= -1.99 + 0.92x, P< 0.001; R^2=0.76). Data points were differentiated per habitat type to observe their dispersion. Only positive prevalence data with sample size larger than 10 were used.

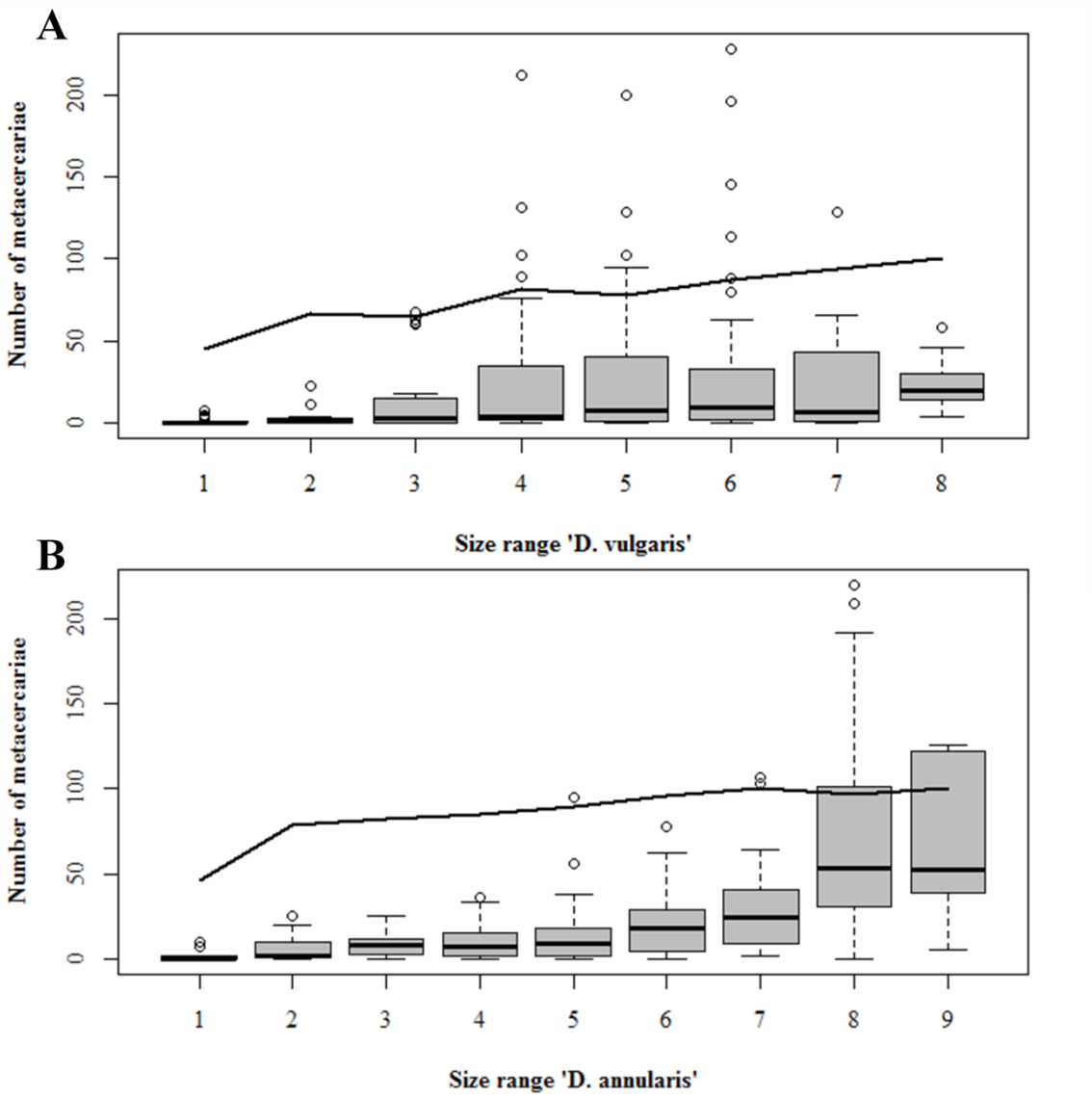

Figure 8.4. Accumulation of *C. longicollis* metacercariae with increasing fish size. Size classes of A) *D. vulgaris* (size class, standard length (mm)) (1:66-98, 2:100-110, 3:111-119, 4:120-129, 5:130-139, 6: 140-148, 7:151-159, 8:160-220) and B) *D. annularis* (1:70-79, 2:80-89, 3:90-99, 4:100-109, 5:100-119, 6:120-129, 7:130-139, 8:140-149, 9:150-162). Lines represent infection prevalence.

Table 8.1.Results from statistical analyses on the fish habitat targeted by cercariae of *C. longicollis*. A. Results controlling for fish species, with (a.1) LMM (Mean abundance ~ habitat + fish sample size + fish species (random) and (a.2) pairwise comparisons evaluating the effect of habitat on the abundance of *C. longicollis*. The response variable was arcsin-transformed. (a.1). The intercept value stands for the mean abundance (number of parasites per number of sampled fish), i.e. pelagic habitat, to which the other levels are compared. The estimate of a variable is added from the intercept value. Statistically significant results (at α=0.050) are indicated in bold type, with the corresponding P-value obtained with Bonferroni correction given in parentheses (a.2). LMM, random effect 'fish species', variance =0.014, Std.Dev=0.116. B. Results not controlling for fish species, evaluating the effect of the fish habitat on the response variable (i.e. prevalence or mean abundance of *C. longicollis*), obtained with different datasets. Results of LM (Response variable ~ fish habitat) are indicated in italics, followed by the pairwise comparison results in square brackets. The response variables were arcsin-transformed. The percentage indicates the increase of the response variable in the specified habitat, compared to the pelagic habitat. Only significant results (at α=0.050) are presented. Bent=benthic, Dem=demersal, Pelag=pelagic.

A. Controlling for fish species

	Estimate	SE	*t-value*	*P-value*
(a.1)				
(Intercept)	0.0667	0.0849	0.785	0.4325
Demersal	0.0985	0.0916	1.075	0.2824
Benthic	0.4017	0.1581	2.540	**0.0111**
Sample size	0.0251	0.0133	1.890	0.0588
(a.2)				
Demersal-Pelag.	0.098	0.092	1.075	0.5171(0.8467)
Benthic-Pelag.	0.402	0.158	2.540	**0.0277(0.0332)**
Benthic-Dem.	0.303	0.138	2.202	0.0667(0.0831)

B. Not controlling for fish species **Response Variable**

		Prevalence	Mean abundance
b.2) Other together with present study	i, True zero data included (n=86)	*n.s.* [n.s.]	***Demersal*** *(t=2.495, P= 0.015) 488.7%,* ***benthic*** *(t=2.426, P= 0.018) 731.4%* [dem>pel; bent>pel](t=2.495, P=0.039; t=2.426, P=0.046)
	ii, True zero data not included (n=77)	***Demersal*** *(t=2.559, P= 0.013) 38.3%* [dem>pel](t=2.559, P=0.033)	***Demersal*** *(t=2.994, P= 0.004) 697.7%,* ***benthic*** *(t=2.699, P= 0.009) 906%* [dem>pel; bent>pel](t=2.994, P=0.011; t=2.699, P=0.024)
b.3) Data present study	i, True zero data included (n=58)	*Benthic (t=2.154, P= 0.036) 74.5%* [n.s.]	***Demersal*** *(t=2.318, P= 0.024) 407.1%,* ***benthic*** *(t=3.176, P= 0.002) 1641.4%*[dem>pel](t=3.176, P=0.007)
	ii, True zero data not included (n=52)	***Demersal*** *(t=3.314, P= 0.002) 55.8%,* ***benthic*** *(t=3.485, P= 0.001) 131.6%* [dem>pel; bent>pel] (t=3.314, P=0.005; t=3.485, P=0.003)	*Demersal (t=2.946, P= 0.005) 618%, benthic (t=3.828, P< 0.001) 2373.3%* [dem>pel; bent>pel] (t=2.946, P=0.013; t=3.828, P=0.001)

8.6. Supplementary Materials

8.6.1. Detailed statistical methods

Fish

A first set of analyses included only the new data collected during this study. Fish habitat (pelagic, demersal and benthic), with information based on FishBase (Froese & Pauly 2015), and season of sampling (spring, summer, autumn, winter) were included in our dataset and used in the analyses where necessary and available. One generalized linear mixed model (GLMM) and one linear mixed model (LMM) were used (R, package lme4, version 1.1-7, Bates *et al.* 2014), including only samples where all factors were available (see Table S8.3, used samples highlighted). For the GLMM, we used prevalence (i.e. proportion of infected fish in each sample) as the response variable, fitted with a binomial error structure, with season and habitat as fixed effects and fish species as random effect. In a GLMM with binomial error structure, the response variable is a proportion or ratio, so that the effect of sample size is already accounted for. However, the built GLMM also controlled for different sample size by including it as a covariate. The LMM examined the effect of habitat on the abundance of infection (i.e. mean number of parasites per host, including uninfected ones). This response variable was arcsin-transformed, with habitat as fixed effect, sample size as covariate and fish species as random effect. Due to the lack of data (i.e. same fish species not recovered in all seasons) and because our goal was not to specifically test for differences between fish species, we did not use fish species as a fixed effect in any of the analyses. After GLMM and LMM, multiple comparisons were obtained with Tukey's all-pair comparison test (R, package multcomp, version 1.3-3, Hothorn *et al.* 2008) and the Bonferroni correction was applied to adjust the p-values of multiple comparisons.

In a second step we analysed our new data combined with previously published records of infection in the different hosts. Prevalence and abundance data were used in separated linear models as response variables, since these are frequently the only measures provided in published records. The response variables were arcsin-transformed to compare overall differences depending on the fish habitat (fixed effect). Linear models were repeated for a) the complete dataset (new data collected for this study plus published records) and b) only data from the present study to check for congruent patterns. Analyses were repeated with and without true zero prevalence data.

The relationship between the prevalence and the mean intensity of *C. longicollis* across fish samples was evaluated with a Spearman rank correlation (non-normal distribution), with log-transformed data. In a first step, data collected in the present study were used, and the analysis was then repeated including previously published data. Only positive prevalence data with sample sizes greater than 10 fish were used (see Table S8.3).

To investigate the relationship between fish length and *C. longicollis* abundanc e, two subsets of 198 *D. vulgaris* and 196 *D. annularis* were used. The data was stratified b y fish standard length into eight size classes with intervals of 10 mm. *Diplodus vulgaris* s

maller than 100 mm or larger than 160 mm were pooled in the smallest and largest classe s, respectively, resulting in eight size classes. *Diplodus annularis* larger than 150 mm were pooled in the largest class, resulting in nine size classes. A negative binomial GLM which models over-dispersed count data was fitted to evaluate the effect of size class on the nu mber of *C. longicollis* metacercariae (response variable) (Zuur *et al.* 2009) (R, package M ASS, version 7.3-33, Venables & Ripley, 2002). The degree of aggregation of the metacerca riae was calculated for each size class as the index of discrepancy (D index) with the prog ram Quantitative Parasitology (QP web, powered by R, Version 1.0.9) (Rozsa *et al.* 2000, Reiczigel *et al.* 2013).

Birds

The relationship between the prevalence and the mean intensity of *C. longicollis* adults in the digestive tract of birds was evaluated across samples with a Spearman rank correlation (non-normal distribution), with log-transformed data. All data were included, i.e. present study and previously published, since relatively few samples are available. Only positive prevalence data with sample sizes larger than 10 birds were used (see Table S8.4).

The effect of season on the mean number of *C. longicollis* eggs in bird faeces wa s evaluated in a negative binomial GLM which models over-dispersed count data due to a negative binomial distribution of data (Zuur *et al.* 2009) (R, package MASS, version 7.3-3 3, Venables & Ripley, 2002).

8.6.2. Figures and Tables

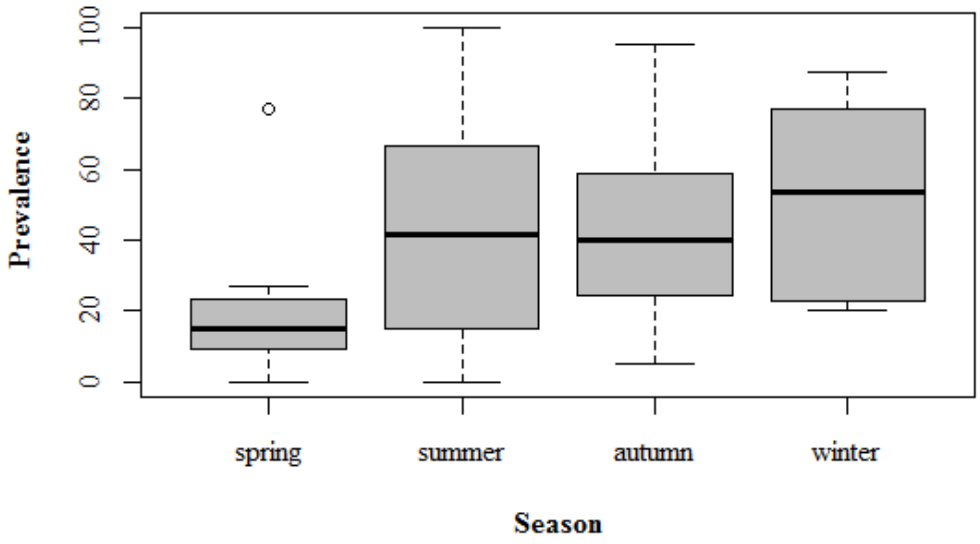

Figure S8.1. Prevalence of *C. longicollis* metacercariae in fish samples differentiated per season. Only data examined in the present study are included.

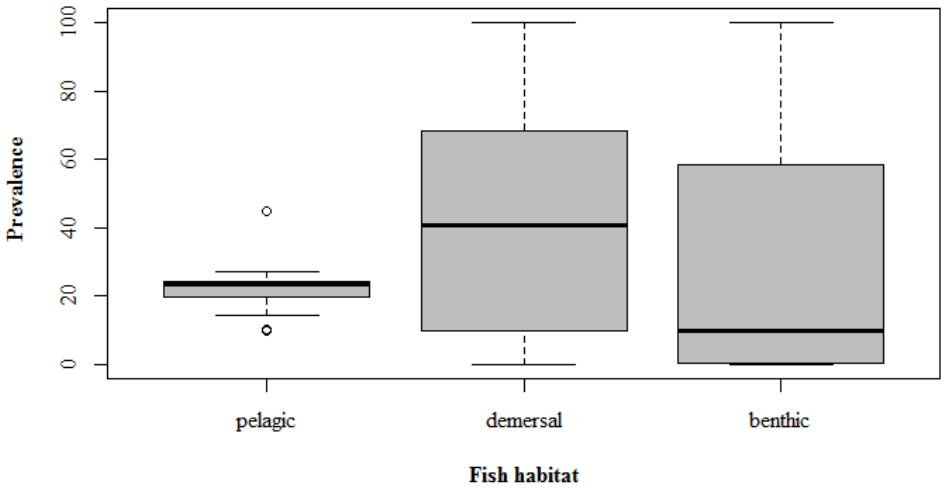

Figure S8.2. Prevalence of *C. longicollis* metacercariae in fish samples differentiated per habitat. Data examined in the present study with previous records and true zeros are included. Fish species is controlled.

Figure S8.3. Representation of the mean number of eggs present in *L. michahellis* faecal samples, differentiated per season.

Table S8.1. Specific characteristics of Mediterranean sampling sites for *C. longicollis* including comments on anthropogenic influence. All animals were obtained from the wild, only at one site, wild and cultured fish were obtained (marked with *).

Locality, zone	**Anthropogenic influence and habitat**	**Hosts**
Barcelona, Spain	Moderate to high fishing activities, sandy areas and open water.	Fish
Ebro Delta, Spain	Near a protected area, supports permanent and temporary bird communities. Bird sanctuary "Centre de Recuperació de Fauna salvatge de Torreferrussa". The Ebro Delta lagoon, with muddy soft sediment, and its surroundings support high fishing activity as well as aquaculture facilities (netpens).	Birds, molluscs
Valencia including Albufera, Spain	Near the Natural Park La Albufera, littoral lagoon, sandy areas. A preferred bird habitat, with a Bird sanctuary "Centre de Recuperació La Granja". High fishing activities.	Birds
Burriana/Sagunto/ Cullera, Spain	Moderate to high fishing activities, sandy areas and open water.	Fish
Jávea/Dénia, Spain	Close to a protected area, with sandy as well as rocky zones, and open sea with moderate fishing activity in the surroundings.	Fish, molluscs
Carboneras, Spain	High fishing activity as well as many aquaculture facilities (netpens), some rocky areas and open water.	Fish*, molluscs
Guardamar/Santa Pola/San Pedro del Pinatar, Spain	High fishing activity as well as many aquaculture facilities (net pens), rocky areas and open water	Fish
Benidorm Island, Spain	Uninhabited, small rocky island (<0.01 km²), with high ecological value (Natural Park "Sierra Helada"), different seagull populations clearly separated.	Bird faeces
Barbate, Spain	Intermediate zone between Atlantic and Mediterranean. High fishing activity, especially tuna; open sea.	Fish
San Giovanni/ Santa Gilla/Porto Pino (South Sardinia), Italy	Three lagoons close to protected areas, but with very high fishing activity in the surroundings, and also to catch fish migrating into the sea.	Fish

Table S8.2. Mollusc samples examined for infections with *C. longicollis* (sporocysts and cercariae) during present and previously published studies. Taxonomic information with P=Polyplacophora, B=Bivalvia, G=Gastropoda, number of hosts screened, Prev=Infection prevalence, locality and date are specified. New host records are marked with §.

Family	Species	No	Prev (%)	Locality	Date	Study
Anomiidae (B)	*Anomia ephippium* L.	25	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
Arcidae (B)	*Arca noae* L.	23	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
	Barbatia barbata (L.)	16	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
Buccinidae (G)	*Pisania striata* (Gmelin, 1791)	12	0	Dénia, Spain, Mediterranean	Jun 2008	Present study
	Pollia dorbignyi (Payraudeau, 1826)	17	0	Dénia, Spain, Mediterranean	Jun 2008	Present study
Cerithiidae (G)	*Bittium reticulatum* (Costa, 1778)	41	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
	Cerithium vulgatum Bruguière, 1792	29	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
	Cerithium vulgatum	160	0	Ebro Delta, Spain, Mediterranean	March -Jun 2010	Present study
Chitonidae (P)	*Chiton (Rhyssoplax) olivaceus* Spengler, 1797	41	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
Chromodoridae (G)	*Felimare picta* (Schultz in Philippi, 1836)	6	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
Conidae (G)	*Conus ventricosus* Gmelin, 1791	14	0	Dénia, Spain, Mediterranean	Jun 2008	Present study
Fissurelidae (G)	*Diodora* spp.	25	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
Haliotidae (G)	*Haliotis tuberculata* L.	17	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
Haminoeoidea (G)	*Haminoea hydatis* (L.)	34	0	Ebro Delta, Spain, Mediterranean	April 2009	Present study
Hydrobiidae (G)	*Peringia ulvae* (Pennant, 1777)	70	0	Ebro Delta, Spain, Mediterranean	April 2011	Present study
Table S8.2. Continued						
Limidae (B)	*Lima lima* (L.)	12	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
Littorinidae (G)	*Littorina litorea* (L.)	42	0	Dénia, Spain, Mediterranean	Oct 2006	Present study
Muricidae (G)	*Hexaplex trunculus* (L)	20	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
	Stramonita haemastoma (L.)	8	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study

Table S8.2. Continued.

Family	Species	No	Prev (%)	Locality	Date	Study
Mytilidae (B)	*Mytilus edulis* L.	32	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
	Musculus subpictus (Cantraine, 1835)	19	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
Nassaridae (G)	*Cyclope neritea* (L.)	354	0	Ebro Delta, Spain, Mediterranean	Jun-Aug 2011	Present study
	Cyclope neritea §	2	1 of 2	Carboneras, Spain, Mediterranean	Jul 2006	Present study
	Cyclope neritea	104	0	Ebro Delta, Spain, Mediterranean	Sept 2008	Present study
	Cyclope neritea §	309	0.65	Ebro Delta, Spain, Mediterranean	April-Jun 2009	Present study
	Nassarius reticulatus (L.)	12	8.3	Ebro Delta, Spain, Mediterranean	April 2009	Present study
	Nassarius reticulatus	316	0.32	Ebro Delta, Spain, Mediterranean	Jun 2010	Present study
	Nassarius reticulatus	40	0	Ebro Delta, Spain, Mediterranean	Oct 2011	Present study
	Nassarius reticulatus	294	4.76	Ebro Delta, Spain, Mediterranean	Oct 2012	Present study
	Nassarius reticulatus		3(2.5-4.35)	Aveiro Estuary and South Portugal, Atlantic	Jun-Sep 2006	Rato *et al.* 2009
	Nassarius reticulatus	1419	3.57	Aveiro Estuary, Portugal, Atlantic		Russell-Pinto *et al.* 2006
	Nassarius corniculum (Olivi, 1792)		1.0-6.0	Brusc lagoon, France, Mediterranean		Prévot & Bartoli 1980
Opisthobranchia (G)	*Opisthobranchia* (various spp.)	22	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
Ostreidae (B)	*Ostrea edulis* L.	24	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
Patellidae (G)	*Patella vulgata* L.	35	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
Tellinidae (G)	*Tellina incarnata* L.	35	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
Trochidae (G)	*Calliostoma* spp.	15	0	Carboneras, Spain, Mediterranean	Jul 2006	Present study
	Gibbula spp.	10	0	Dénia, Spain, Mediterranean	Oct 2006	Present study
	Gibbula adansonii (Payraudeau, 1826)	27	0	Ebro Delta, Spain, Mediterranean	April 2009	Present study
	Gibbula adansonii	526	0	Ebro Delta, Spain, Mediterranean	March 2010	Present study
	Gibbula adansonii	563	0	Ebro Delta, Spain, Mediterranean	April-May 2011	Present study

Table S8.3. Fish species screened for *C. longicollis* metacercarial infection in the brain, together with information present in the literature. Host taxonomy, fishing locality, season (Spr=spring, Su=summer, Au= autumn, Win=winter), and fish habitat are specified. Records include fish screened but found negative for infection. Samples used in GLMM (N>10 and all factors specified) are highlighted with [1], new host records with §, and host species where *C. longicollis* has never been found with [2]. The fish species *G. niger*, *P. pungitius* and *S. spinachia* were treated as not infected by *C. longicollis* despite an existing record (found by Zander *et al.* 1999) since the parasite identified as *C. longicollis* was located in the eye instead of the brain and likely represents other diplostomids with this particular site of infection (e.g., Karvonen *et al.* 2003, Seppälä *et al.* 2004). These samples are marked with [3]. Samples examined in pool with chloropeptic digestion are marked with **; one single data, partially published by Pérez-del-Olmo *et al.* 2007 is marked with [a]. Records were infection was not quantified, but was present are referred as "pres". Prevalence (%), mean intensity (range), mean abundance (± SD), mean total length of fish (TL), mean standard length (ST) and mean weight (MW) are provided if available.

Family	Species	Locality	Season	Habitat	No fish	Prev %	Mean Int (range)	Mean Abund ±SD	TL	SL	MW	Study
Anguillidae	*Anguilla anguilla* (L.) [2]	Santa Gilla, Italy, Mediterranean		benthic	103	0	0	0				Culurgioni *et al.* 2014
Atherinidae	*Atherina boyeri* Risso, 1810 [2]	Carboneras, Spain, Mediterranean	Su	pelagic	4	0	0	0				Present study
	Atherina boyeri [2]	Santa Gilla, Italy, Mediterranean		pelagic	90	0	0	0				Curlugioni *et al.* 2014
Blenniidae	*Parablennius sanguinolentus* (Pallas, 1814)	Carboneras, Spain, Mediterranean	Su	benthic	4	0	0	0				Present study
	Parablennius sanguinolentus	Sebastopol, Russia, Black Sea		benthic	43			3.63				Korniychuk 1997
	Parablennius pilicornis (Cuvier, 1829) [2]	Carboneras, Spain, Mediterranean	Su	benthic	6	0	0	0				Present study
	Parablennius tentacularis (Brünnich, 1768)	Sebastopol, Russia, Black Sea		benthic	7			4.17				Korniychuk 1997
	Salaria pavo (Risso, 1810)	Sebastopol, Russia, Black Sea		benthic	39			1.14				Korniychuk 1997
	Aidablennius sphynx (Valenciennes, 1836)	Sebastopol, Russia, Black Sea		benthic	110			0.39				Korniychuk 1997
Belonidae	*Belone belone* (L.)	Brusc lagoon, France, Mediterranean				pres						Prévot & Bartoli 1980
Bothidae	*Bothus podas* (Delaroche, 1809) [2]	Carboneras, Spain, Mediterranean	Su	benthic	16	0	0	0				Present study
Carangidae	*Trachinotus ovatus* (L.) [2]	Carboneras, Spain, Mediterranean	Su	pelagic	12	0	0	0				Present study
Clupeidae	*Clupea harengus* L. [2]	Riga Bight, Latvia, Baltic Sea		pelagic		0	0	0				Vismanis *et al.* 1980

Table S8.3. Continued

Family	Species	Locality	Season	Habitat	No fish	Prev %	Mean Int (range)	Mean Abund ±SD	TL	SL	MW	Study
Cyprinodontidae	*Aphanius fasciatus* (Valenciennes, 1821) [2]	Santa Gilla, Italy, Mediterranean		demersal	89	0	0	0				Culurgioni *et al.* 2014
Gasterosteidae	*Gasterosteus aculeatus* L.[3]	Salzhaff, Germany, Baltic Sea		benthic	7	0.29	1.5					Zander *et al.* 1999
	Gasterosteus aculeatus	Salzhaff, Germany, Baltic Sea		benthic	71	0.21	4.5	0.9				Zander *et al.* 1999, 2000
	Pungitius pungitius (L.) [2,3]	Salzhaff, Germany, Baltic Sea		benthic	115	0.12	1.6					Zander *et al.* 1999
	Pungitius pungitius [2]	Salzhaff, Germany, Baltic Sea		benthic		0	0	0				Zander *et al.* 2000
	Spinachia spinachia (L.) [2,3]	Salzhaff, Germany, Baltic Sea		benthic	11	<0.1	2					Zander *et al.* 1999
	Spinachia spinachia [2]	Salzhaff, Germany, Baltic Sea		benthic	35	0	0	0				Zander *et al.* 2000
Gobiidae	*Zosterisessor ophiocephalus* (Pallas, 1814) [1]§	Porto Pino, Italy, Mediterranean	Win	benthic	41	75.60	27.19(3-64)	20.56± 19.70	15.6		55.1	Present study
	Zosterisessor ophiocephalus (as *G. ophiocephalus*)	Egorlytsky Bay in Lake Donuzlav, Russia, Black Sea		benthic	38	0	0	0				Machkevsky *et al.* 1990
	Gobius bucchichi Steindachner, 1870 [2]	Carboneras, Spain, Mediterranean	Su	benthic	4	0	0	0				Present study
	Gobius niger L. [2,3]	Salzhaff, Germany, Baltic Sea		benthic	79	<0.1	18.5					Zander *et al.* 1999
	Gobius niger [2]	Dahmeshöved, Germany, Baltic Sea		benthic	71	0	0	0				Zander 2003, 2004

Table S8.3. Continued

Family	Species	Locality	Season	Habitat	No fish	Prev %	Mean Int (range)	Mean Abund ±SD	TL	SL	MW	Study
	Gobius niger [2]	Salzhaff, Germany, Baltic Sea		benthic		0	0	0				Zander *et al.* 2000
	Gobius niger (*as N. niger*) [2]	Egorlytsky Bay in Lake Donuzlav, Russia, Black Sea		benthic	27	0	0	0				Machkevsky *et al.* 1990
	Gobius niger [2]	Santa Gilla, Italy, Mediterranean		benthic	86	0	0	0				Culurgioni *et al.* 2014
	Gobius paganellus L. [2]	Santa Gilla, Italy, Mediterranean		benthic	3	0	0	0				Culurgioni *et al.* 2014
	Pomatoschistus microps (Krøyer, 1838)	Salzhaff, Germany, Baltic Sea	Spr	benthic	63	1.6	1		31.59			Zander 2003, 2004
	Pomatoschistus microps [2]	Salzhaff, Germany, Baltic Sea	Spr	benthic	34	2.9	1		38.43			Zander 2003, 2004
	Pomatoschistus microps	Salzhaff, Germany, Baltic Sea		benthic	171	0	0	0				Zander *et al.* 1999, 2000
	Pomatoschistus minutus (Pallas, 1770) [2]	Dahmeshöved Germany, Baltic Sea		benthic	293	0	0	0				Zander 2003, 2004
	Pomatoschistus minutus [2]	Salzhaff, Germany, Baltic Sea		benthic	41	0	0	0				Zander *et al.* 1999, 2000
	Pomatoschistus pictus (Malm, 1865) [2]	Dahmeshöved Germany, Baltic Sea		benthic	201	0	0	0				Zander 2003, 2004
	Pomatoschistus marmoratus (Risso, 1810) (as *P. microps leopardinus*) [2]	Egorlytsky Bay in Lake Donuzlav, Russia, Black Sea		benthic	46	0	0	0				Machkevsky *et al.* 1990

Table S8.3. Continued

Family	Species	Locality	Season	Habitat	No fish	Prev %	Mean Int (range)	Mean Abund ±SD	TL	SL	MW	Study
	Gobiusculus flavescens (Fabricius, 1779) [2]	Salzhaff, Germany, Baltic Sea		benthic	62	0	0	0				Zander *et al.* 1999, 2000
	Gobiusculus flavescens [2]	Dahmeshöved, Germany, Baltic Sea		benthic	234	0	0	0				Zander 2003, 2004
	Neogobius melanostomus (Pallas, 1814)	Kerch Peninsula, Russia, Black Sea		benthic	119	2.5	(1-3)					Machkevsky et al 1990
	Neogobius melanostomus	Egorlytsky Bay in Lake Donuzlav, Russia, Black Sea		benthic	67	58.2	(1-84)					Machkevsky *et al.* 1990
	Neogobius fluviatilis (Pallas, 1814)	Egorlytsky Bay in Lake Donuzlav, Russia, Black Sea		benthic	51	0	0	0				Machkevsky *et al.* 1990
	Proterorhinus marmoratus (Pallas, 1814) [2]	Egorlytsky Bay in Lake Donuzlav, Russia, Black Sea		benthic	14	0	0	0				Machkevsky *et al.* 1990
Labridae	*Coris julis* (L.)[1]§	Carboneras, Spain, Mediterranean	Su	demersal	24	41.70	1.60 (1-3)	0.70 ± 1.00				Present study
	Ctenolabrus rupestris (L.)	Sebastopol, Russia, Black Sea		demersal	3	33.3	1					Korniychuk 2001
	Symphodus ocellatus (L.)	Sebastopol, Russia, Black Sea		demersal	93	2.15	1					Korniychuk 2001
	Symphodus ocellatus (as *Crenilabrus ocellatus*)	Egorlytsky Bay in Lake Donuzlav, Russia, Black Sea		demersal		16.6	(1-3)					Machkevsky 1990

Table S8.3. Continued

Family	Species	Locality	Season	Habitat	No fish	Prev %	Mean Int (range)	Mean Abund ±SD	TL	SL	MW	Study
	Symphodus tinca (L.) (as *Crenilabrus tinca*) [2]	Egorlytsky Bay in Lake Donuzlav, Russia, Black Sea		demersal		0	0	0				Machkevsky 1990
	Symphodus tinca [2]	Sebastopol, Russia, Black Sea		demersal	143	0	0	0				Korniychuk 2001
	Symphodus cinereus (Bonnaterre, 1788)	Sebastopol, Russia, Black Sea		demersal	51	0	0	0				Korniychuk 2001
	Symphodus cinereus (as *Crenilabrus griseus*)	Egorlytsky Bay in Lake Donuzlav, Russia, Black Sea		demersal		6.7	Max. 2					Machkevsky 1990
	Symphodus roissali (Risso, 1810) (as *Crenilabrus quinquemaculatus*)[2]	Egorlytsky Bay in Lake Donuzlav, Russia, Black Sea		demersal		0	0	0				Machkevsky 1990
Moronidae	*Dicentrarchus labrax* (L.) [2]	Santa Gilla, Italy, Mediterranean		demersal	56	0	0	0				Culurgioni *et al.* 2014
Mugilidae	*Chelon labrosus* (Risso, 1827) [2]	Santa Gilla, Italy, Mediterranean		benthic	6	0	0	0				Culurgioni *et al.* 2014
	Liza aurata (Risso, 1810) [2]	Santa Gilla, Italy, Mediterranean		benthic	15	0	0	0				Culurgioni *et al.* 2014
	Liza ramada (Risso, 1827) [2]	Santa Gilla, Italy, Mediterranean		benthic	12	0	0	0				Culurgioni *et al.* 2014
	Liza saliens (Risso, 1810) [2]	Santa Gilla, Italy, Mediterranean		benthic	9	0	0	0				Culurgioni *et al.* 2014
	Mugil cephalus L.[2]	Santa Gilla, Italy, Mediterranean		benthic	10	0	0	0				Culurgioni *et al.* 2014
Petromyzontidae	*Lampetra fluviatilis* (L.) [2]	Riga Bight, Latvia, Baltic Sea		benthic		0	0	0				Vismanis *et al.* 1980

Table S8.3. Continued

Family	Species	Locality	Season	Habitat	No fish	Prev %	Mean Int (range)	Mean Abund ±SD	TL	SL	MW	Study
Pomacentridae	*Chromis chromis* (L.) [1§]	Carboneras, Spain, Mediterranean	Su	demersal	34	82.40	3.30(1-13)	2.70± 3.30				Present study
Serranidae	*Serranus scriba* (L.) §	Carboneras, Spain, Mediterranean	Su	benthic	8	75.0	6.70(3-14)	5				Present study
Soleidae	*Solea solea* (L.) [2]	Carboneras, Spain, Mediterranean	Su	benthic	2	0	0	0				Present study
	Solea solea (L.)[2]	Santa Gilla, Italy, Mediterranean		benthic	10	0	0	0				Culurgioni *et al.* 2014
Sparidae	*Sparus aurata* L.[1]	Carboneras, Spain, Mediterranean	Win	demersal	26	53.85	1.79 (1-4)	0.96± 1.15				Present study
	Sparus aurata[1]	Guardamar, Spain, Mediterranean	Spr	demersal	31	0	0	0	12.1	11.1	20.9	Present study
	Sparus aurata	Valencia, Spain, Mediterranean	Au	demersal	4	0	0	0	21.4	17.3		Present study
	Sparus aurata [1]	Sagunto, Spain, Mediterranean	Spr	demersal	22	0	0	0	14.6	12.2		Present study
	Sparus aurata [1]	Guardamar, Spain, Mediterranean	Su	demersal	34	0	0	0	20.7	17.5		Present study
	Sparus aurata [1]	Santa Gilla, Italy, Mediterranean	Au	demersal	26	46.20	3.08 (1-10)	1.42 ± 2.34	19.8		119.2	Present study
	Sparus aurata [1]	Porto Pino, Italy, Mediterranean	Au	demersal	32	62.50	4.50(1-13)	2.81± 3.50	24.5		201.2	Present study
	Sparus aurata	San Giovanni, Italy, Mediterranean		demersal	30	40.0	10.75(1-29)	4.30± 8.35				Present study
	Sparus aurata	Santa Gilla, Italy, Mediterranean		demersal	26	46.2	3.1					Culurgioni *et al.* 2014
	Diplodus puntazzo (Walbaum, 1792) [1]	San Pedro del Pinatar, Spain, Mediterranean	Spr	demersal	69	8.70	1.50 (1-3)	0.13± 0.48	9.3	7.7	15.3	Present study

Table S8.3. Continued

Family	Species	Locality	Season	Habitat	No fish	Prev %	Mean Int (range)	Mean Abund ±SD	TL	SL	MW	Study
	Diplodus puntazzo	San Pedro del Pinatar, Spain, Mediterranean	Spr	demersal	5	20.0	2	0.40±0.89	7.7	7.3	8.63	Present study
	Diplodus puntazzo (Walbaum, 1792) [1]	Jávea, Spain, Mediterranean	Su	demersal	20	15.0	8.33(4-18)	1.25± 4.09	11.7	9.8		Present study
	Diplodus puntazzo[1]	Santa Pola, Spain, Mediterranean	Spr	demersal	11	27.27	3.67(1-7)	1.00± 2.19	24.5	19.6	213.4	Present study
	Diplodus puntazzo	Santa Pola, Spain, Mediterranean	Au	demersal	9	55.56	1.6 (1-4)	0.89± 1.27	27.3	23.6	282.5	Present study
	Diplodus puntazzo	San Pedro del Pinatar, Spain, Mediterranean		demersal	30	70	4.6	3.2	252.1		300	Sánchez-García *et al.* 2013
	Diplodus puntazzo	Santa Pola, Spain, Mediterranean		demersal	20	20	5	1	188.5		111.95	Sánchez-García *et al.* 2013
	Diplodus sargus (L.) §	Valencia, Spain, Mediterranean	Au	demersal	8	0	0	0	21.6	17.3		Present study
	Diplodus sargus [2]§	Porto Pino, Italy, Mediterranean	Su	demersal	24			2.83				Present study**
	Diplodus annularis[1]	Santa Pola, Spain, Mediterranean	Au	demersal	19	78.95	19 (1-63)	15.00± 20.16	16.1	13.3	76.1	Present study
	Diplodus annularis [1]	Santa Pola, Spain, Mediterranean	Spr	demersal	13	76.92	25.50(4-55)	19.62± 19.06	89.1	13.9	89.1	Present study
	Diplodus annularis [1]	Valencia, Spain, Mediterranean	Win	demersal	196	87.24	29.94(1-220)	26.12± 38.56		114.4		Present study
	Diplodus annularis	Gulf of Cagliari, south Sardinia, Italy, Mediterranean	Win	demersal	182	3.3	8	0.3				D'Amico *et al.* 2006
	Diplodus annularis	Gulf of Valencia, Spain, Mediterranean	Au	demersal	259	99.6	50.3	50.1				Osset *et al.* 2005

Table S8.3. Continued

Family	Species	Locality	Season	Habitat	No fish	Prev %	Mean Int (range)	Mean Abund ±SD	TL	SL	MW	Study
	Diplodus annularis	Gulf of Marseille, France, Mediterranean		demersal	53	17	3.4	3.4				Rebecq & Leray 1960b
	Diplodus annularis	Bonifacio (Corse) France, Mediterranean	Su	demersal	5	3/5						Rebecq & Leray 1960a
	Diplodus annularis, *D. vulgaris* (Geoffroy Saint-Hilaire, 1817)	Brusc lagoon, France, Mediterranean		demersal		60	7					Prévot & Bartoli 1980
	Diplodus vulgaris	Santa Pola, Spain, Mediterranean	Spr	demersal	4	50.0	6.00(1-11)	3.00± 5.35		23.4		Present study
	Diplodus vulgaris	Porto Pino, Italy, Mediterranean	Au	demersal	8	87.50	49.86(7-159)	43.63±49.90	16.5		78.3	Present study
	Diplodus vulgaris	San Giovanni, Italy, Mediterranean	Au	demersal	7	100.0	19.86(7-54)	19.86± 19.61	15.2		58.6	Present study
	Diplodus vulgaris [1]	Valencia, Spain, Mediterranean	Win	demersal	198	78.28	28.64(1-228)	22.42± 39.66		131.3		Present study
	Dentex dentex (L.)[1]§	Santa Pola, Spain, Mediterranean	Su	demersal	15	66.67	2.20(1-5)	1.47± 1.46	25.6	20.1	208.2	Present study
	Dentex dentex [1] §	Burriana, Spain, Mediterranean	Au	demersal	20	55.0	2.18 (1-8)	1.2± 1.85	21.8	18.04	146.15	Present study
	Spicara maena (L.) §	Valencia, Spain, Mediterranean	Spr	pelagic	7	14.29	1	0.14± 0.38	21.9	18.6		Present study
	Spicara maena [1] §	Burriana, Spain, Mediterranean	Au	pelagic	20	45.0	3.56(1-21)	1.6± 4.64	19.235	16.3	81.5	Present study
	Spicara maena [1]§	Santa Pola, Spain, Mediterranean	Spr	pelagic	30	10.0	2.33 (1-3)	0.23± 0.77	18	15.1	62.6	Present study
	Spicara maena [1]§	Santa Pola, Spain, Mediterranean	Au	pelagic	33	27.27	2.33 (1-2)	0.39± 0.70	17.3	14.6	57.9	Present study

Table S8.3. Continued

Family	Species	Locality	Season	Habitat	No fish	Prev %	Mean Int (range)	Mean Abund ±SD	TL	SL	MW	Study
	Lithognathus mormyrus (L.)[1]	Valencia, Spain, Mediterranean	Su	demersal	16	100.0	21.38(2-120)	21.38± 29.38	25.3	21.1		Present study
	Lithognathus mormyrus	Valencia, Spain, Mediterranean	Au	demersal	2	100.0	10.0(4-16)	10.0± 8.49	18.4	15.2		Present study
	Lithognathus mormyrus [2]	Porto Pino, Italy, Mediterranean		demersal	30			2.3	20		140	Present study**
	Lithognathus mormyrus	San Giovanni, Italy, Mediterranean		demersal	18	66.67	8.83(1-26)	5.89± 7.95	19.5		110	Present study
	Lithognathus mormyrus[1]	Burriana, Spain, Mediterranean	Au	demersal	20	95.0	8.89(2-23)	8.45± 5.80	34.6	19.6	160.1	Present study
	Lithognathus mormyrus	Carboneras, Spain, Mediterranean	Su	demersal	6	100.0	26.00(3-41)	26.0± 20.2				Present study
	Lithognathus mormyrus	Brusc lagoon, France, Mediterranean				pres						Prévot & Bartoli 1980
	Pagellus acarne (Risso, 1827) [1]§	Santa Pola, Spain, Mediterranean	Su	demersal	31	45.16	2.93 (1-9)	1.32± 2.18	21.1	19.9	146	Present study
	Pagellus erythrinus (L.)[1]§	Santa Pola, Spain, Mediterranean	Su	demersal	27	3.70	1	0.04± 0.19	20.6	18.6	136.9	Present study
	Pagellus erythrinus [1]§	Santa Pola, Spain, Mediterranean	Spr	demersal	28	10.71	1.33 (1-2)	0.14± 0.45	19.9	16	101	Present study
	Pagellus erythrinus [1]§	Carboneras, Spain, Mediterranean	Su	demersal	10	40.0	2.0(1-3)	0.80± 1.10				Present study
	Pagellus bogaraveo (Brünnich, 1768) [1]§	Santa Pola, Spain, Mediterranean	Au	demersal	20	35.0	3.57(1-18)	1.25± 3.99	19.8	16.5	113.1	Present study
	Oblada melanura (L.)§	Carboneras, Spain, Mediterranean	Su	demersal	8	25.0	1	0.25± 0.50				Present study
	Oblada melanura [1] §	Santa Pola, Spain, Mediterranean	Au	demersal	19	5.26	1	0.05± 0.23	24.9	20.3	224.6	Present study

Table S8.3. Continued

Family	Species	Locality	Season	Habitat	No fish	Prev %	Mean Int (range)	Mean Abund ±SD	TL	SL	MW	Study
	Sarpa salpa (L.)	Brusc lagoon, France, Mediterranean				pres						Prévot & Bartoli 1980
	Spondyliosoma cantharus (L.)[1]§	Cullera, Spain, Mediterranean	Spr	demersal	15	20.0	2.67(2-3)	0.53± 1.13	22.5	18.3	208.4	Present study
	Boops boops (L.)[1]	Barbate, Spain, Atlantic-Mediterranean	Spr	pelagic	30	23.33	1	0.23± 0.43	26	22.4	183.3	Present study
	Boops boops [1]	Santa Pola, Spain, Mediterranean	Spr	pelagic	131	19.08	1.40 (1-3)	0.27± 0.62	20.5	17.9	103.7	Present study
	Boops boops [1]	Barcelona, Spain, Mediterranean	Spr	pelagic	30	23.33	1.86 (1-4)	0.43± 0.94	25.7	22.2	189.6	Present study
	Boops boops [1]	Barbate, Spain, Atlantic-Mediterranean	Win	pelagic	30	23.33	1.14 (1-2)	0.27± 0.52	23.9	20.8	163.2	Present study
	Boops boops [1]	Santa Pola, Spain, Mediterranean	Win	pelagic	35	20.0	1.29 (1-3)	0.26± 0.61	25.6	22.2	201.4	Present study
	Boops boops [1]	Barcelona, Spain, Mediterranean	Au	pelagic	30	20.0	1.67 (1-2)	0.33± 0.71	26.2	22.4	184	Present study
	Boops boops [1]	Santa Pola, Spain, Mediterranean	Spr	pelagic	29	10.34	1	0.10± 0.31	25.8	22.2	178.4	Present study
	Boops boops [1]	Santa Pola, Spain, Mediterranean	Win	pelagic	50	22.0	1.27 (1-3)	0.28± 0.61	25.7	22.2	158.9	Present study
	Boops boops [1]	Valencia, Spain, Mediterranean [a]	Au	pelagic	33	24.24 [a]	1.13 (1-2)	0.27 [a] ± 0.52	23	19.5	116.9	Present study
	Boops boops [1]	Burriana, Spain, Mediterranean	Au	pelagic	20	25.0	1.4 (1-2)	0.35± 0.67	24.6	20.97	144.45	Present study
	Boops boops	Valencia, Spain, Mediterranean	Su	Pelagic		24.2		0.3				Pérez-del-Olmo *et al.* 2007

Table S8.3. Continued

Family	Species	Locality	Season	Habitat	No fish	Prev %	Mean Int (range)	Mean Abund ±SD	TL	SL	MW	Study
Syngnathidae	*Syngnathus typhle* L.[2]	Salzhaff, Germany, Baltic Sea		demersal	83	0	0	0				Zander *et al.* 1999, 2000
Syngnathidae	*Nerophis ophidion* (L.) [2]	Salzhaff, Germany, Baltic Sea		demersal	73	0	0	0				Zander *et al.* 1999, 2000
Zoarcidae	*Zoarces viviparus* (L.)	Salzhaff, Germany, Baltic Sea		benthic	191	11						Reimer & Walter 1998
	Zoarces viviparus	Riga Bight, Latvia, Baltic Sea		benthic	191	10						Vismanis *et al.* 1980
	Zoarces viviparus	Salzhaff, Germany, Baltic Sea		benthic	12	0.25	1	0.3				Zander *et al.* 1999, 2000

Table S8.4. Bird species screened for the presence of *C. longicollis* adults in the digestive tract, data from present study and published records. Host taxonomy, locality, number, mean infection intensity (Int) and mean abundance (Abund± SD). Records were infection was not quantified, but was present are referred as "pres". Samples used in statistical analyses are marked with *. Dubois (1968) and Szidat (1928) did not provide locality data. Under current taxonomy, *Larus cachinnans michahellis* is synonymised with *L. michahellis*, thus their records are treated under the latter name

Family	Species	Locality	N	Prev	Mean Int (range)	Mean Abun ± SD	Study
Ardeidae	*Egretta garzetta* (L.)	Valencia, Spain, Mediterranean	2	0	0	0	Present study
	Ardea cinerea L.	Valencia, Spain, Mediterranean	1	0	0	0	Present study
	Ardea cinerea	Ebro Delta, Spain, Mediterranean	5	0	0	0	Present study
	Ardea alba L.			pres			Szidat 1928
	Ixobrychus minutus (L.)	Ebro Delta, Spain, Mediterranean	2	0	0	0	Present study
	Botaurus stellaris (Linnaeus, 1758) (as *Ardea stellaris)*			pres			Szidat 1928
Laridae	*Larus argentatus* Pontoppidan, 1763	Ebro Delta, Spain, Mediterranean	3	66.67	50 (42-58)	33.33± 29.96	Present study*
	Larus argentatus	Skomer and Skokholm Islands (Wales), North Atlantic	45	2			Gysels & Rabaey 1964*
	Larus argentatus	Selenga Delta, Lake Baikal, Russia	890	1.1	0.2-2.5	0.04	Nekrasov *et al.* 1999*
	Larus argentatus	Small Sea Strait, Lake Baikal, Russia	890	2.2	0.5-4.5	0.09	Nekrasov *et al.* 1999*
	Larus argentatus	Bulgarian Black Sea Coast		7.7	(2-78)		Kostadinova 1997*
	Larus argentatus	North Lake Baikal, Russia	890	2	0-8	0.12	Nekrasov *et al.* 1999*

Table S8.4. Continued

Family	Species	Locality	N	Prev	Mean Int (range)	Mean Abun ± SD	Study
	Larus argentatus	Near Cambridge, UK		pres			Jennings & Soulsby 1957
	Larus argentatus	Port Said, Egypt, Mediterranean		>100 indiv.			Abdel-Aal *et al.* 2004
	Larus argentatus			pres			Dubois 1968
	Larus argentatus; *Larus atricilla* L.			pres			Szidat 1928
	Larus audouinii Payraudeau, 1826	Valencia, Spain, Mediterranean	4	50	3(1-5)	1.5 ± 2.38	Present study*
	Larus audouinii	Chafarinas Islands, off Marrocco, Mediterranean	58	22.4	4.8 (1-19)	1.1 ± 3.5	Roca *et al.*1999*
	Larus genei Brème, 1839 §	Valencia, Spain, Mediterranean	11	27.27	10.67 (3-19)	2.91 ± 6.14	Present study*
	Larus genei	Bulgarian Black Sea Coast	7	4/7	(1-61)		Kostadinova 1997*
	Larus michahellis J.F. Naumann, 1840	Valencia, Spain, Mediterranean	65	69.23	13.76(1-119)	9.52± 18.3	Present study*
	Larus michahellis	Ebro Delta, Spain, Mediterranean	13	61.54	24.25(1-77)	14.92± 24.54	Present study*
	Larus michahellis (as *Larus cachinnans michahellis* Pallas, 1811)	Corsica, Mediterranean	67	10.4	6.3(max 31)	0.66	Bartoli 1989*
	Larus michahellis	Campania, South Italy, Mediterranean	20	55			Dipineto *et al.* 2013
	Chroicocephalus ridibundus (L.)	Ebro Delta, Spain, Mediterranean	6	0	0	0	Present study
	Chroicocephalus ridibundus	Valencia, Spain, Mediterranean	13	0	0	0	Present study
	Chroicocephalus ridibundus	Lake Burano, North Italy, Mediterranean	46	56.5	6		Stancampiano *et al.* 1994*
	Chroicocephalus ridibundus	Ferrara, North Italy, Mediterranean	38	13.2	3		Stancampiano *et al.* 1994*

Table S8.4. Continued

Family	Species	Locality	N	Prev	Mean Int (range)	Mean Abun ± SD	Study
	Chroicocephalus ridibundus	Bulgarian Black Sea Coast	6	3/6	(4-34)		Kostadinova 1997*
	Chroicocephalus ridibundus	Diff. localities, Czech Republic	1292	pres			Sitko 1993
	Chroicocephalus ridibundus	Kherson Region, Ukraine, Black Sea		pres			Olson *et al.* 2003
	Chroicocephalus ridibundus			1/10			Dubois 1968
	Chroicocephalus ridibundus	Near Venecia, Italy		pres			Ninni 1900
	Larus fuscus L.	Valencia, Spain, Mediterranean	2	0	0	0	Present study
	Larus fuscus			pres			Dubois 1968
	Larus fuscus	Skomer and Skokholm Islands (Wales), North Atlantic	35	3			Gysels & Rabaey 1964*
	Larus cachinnans Pallas, 1811	Galicia, Spain, Atlantic coast	324	4	3.6 (1-10)	0.15 ± 0.94	Sanmartín *et al.*, 2005*
	Larus cachinnans	Medes Island, Spain, Mediterranean	122	11.5	16.2		Bosch 2000*
	Larus cachinnans	Comacchio lagoon, North Italy, Mediterranean	55	7.3			Defranceschi *et al.* 2003*
	Larus cachinnans (as *Larus argentatus cachinnans Pall.*)			pres (in 5)			Dubois 1968
	Larus melanocephalus Temminck, 1820			pres			Dubois 1968
	Larus melanocephalus	Gizzeria, South Italy, Mediterranean	34	15	1.5 (1-3)	0.2 ± 0.6	Santoro *et al.* 2011*
	Larus melanocephalus	3 localities, Bulgaria, Black Sea coast		2	2		Kostadinova 1997*
	Larus canus L.	Hiddensee Island, Germany, Baltic Sea coast		7.5			Reimer & Pav 1973*
	Larus canus	Germany, Baltic Sea coast		pres			Reimer 2002

Table S8.4. Continued

Family	Species	Locality	N	Prev	Mean Int (range)	Mean Abun ± SD	Study
	Larus canus			pres			Dubois 1968
	Larus canus	Norway	201	pres			Bakke 1972
	Larus crassirostris Vieillot, 1818			11			Dubois 1968*
	Larus hyperboreus Gunnerus, 1767			pres			Dubois 1968
	Larus marinus L.			pres			Dubois 1968
	Larus spp.	Diff. localities, Greece	6	16.7			Papazahariadou *et al.* 2008*
Phalacrocoracidae	*Phalacrocorax aristotelis* (L.)	Valencia, Spain, Mediterranean	1	0	0	0	Present study
	Phalacrocorax aristotelis	Ebro Delta, Spain, Mediterranean	1	0	0	0	Present study
	Phalacrocorax carbo (L.)	Valencia, Spain, Mediterranean	7	0	0	0	Present study
Sternidae	*Sternula albifrons* (Pallas, 1764)	Valencia, Spain, Mediterranean	2	0	0	0	Present study
	Sterna hirundo L.	Valencia, Spain, Mediterranean	4	0	0	0	Present study
	Sterna sandvicensis Latham, 1787	Valencia, Spain, Mediterranean	7	0	0	0	Present study
	Sterna sandvicensis	Ebro Delta, Spain, Mediterranean	1	0	0	0	Present study
	Sterna sandvicencis			pres			Dubois 1968
	Gelochelidon nilotica (Gmelin, 1789)	Valencia, Spain, Mediterranean	1	0	0	0	Present study
	Chlidonias hybrida (Pallas, 1811)	Valencia, Spain, Mediterranean	1	0	0	0	Present study
	Hydroprogne caspia (Pallas, 1770)	Valencia, Spain, Mediterranean	2	0	0	0	Present study
	Sterna spp.	Greece	2	50			Papazahariadou *et al.* 2008*

Table S8.5. Summary of infection data for *L. michahellis* faecal samples examined in the present study (all samples from Benidorm Island, Spain). Season, prevalence (%) and the number of eggs are specified.

Season	No faeces	Prev (%)	Mean No eggs ± SD (range)
Spring	42	42.86	71.90 ± 170.65 (8 -780)
Summer	40	35	45.30 ± 125.64 (8-750)
Autumn	40	27.5	41.88 ± 234.30 (8- 1485)
Winter	36	22.2	150.19 ± 533.96 (8-2665)

Table S8.6 Results of (a) GLMM (Prevalence ~ season + habitat + fish sample size + fish species (random) and (b) pairwise comparisons evaluating the influence of fixed effects on the prevalence of *C. longicollis*. (a) The intercept value stands for the mean prevalence (proportion of infected fish) on the logit scale, i.e. spring and pelagic habitat, to which the other levels are compared. The estimate of a variable is added or subtracted from the intercept value. Statistically significant results (at α=0.050) are indicated in bold type, with the corresponding P-value obtained with Bonferroni correction given in parentheses (b). Random effect 'fish species', variance =1.717, Std.Dev=1.31.

	Estimate	SE	*z-value*	*P-value*
(a)				
(Intercept)(=spring/pelagic)	-1.9091	0.9477	-2.014	0.044
Summer	0.1311	0.3233	0.406	0.685
Autumn	1.1077	0.2253	4.917	**<0.001**
Winter	0.9088	0.2239	4.059	**<0.001**
Demersal	1.2379	1.0192	1.215	0.2245
Benthic	2.1495	1.6620	1.293	0.1959
Sample Size	0.1321	0.0783	1.687	0.0915
(b)				
Summer-spring	0.1311	0.3233	0.406	0.9767(1.000)
Autumn-spring	1.1077	0.2253	4.917	**<0.001(<0.001)**
Winter-Spring	0.9088	0.2239	4.059	**<0.001(<0.001)**
Autumn-Summer	0.9766	0.3188	3.063	**0.011(0.013)**
Winter-Summer	0.7777	0.3513	2.214	0.115(0.161)
Winter-Autumn	-0.1989	0.2455	-0.810	0.845(1.000)
Demersal-Pelag.	1.2379	1.0192	1.215	0.433(0.674)
Benthic-Pelag.	2.1495	1.6620	1.293	0.388(0.5888)
Benthic-Dem.	0.9116	1.4286	0.638	0.793(1.000)

Table S8.7. Prevalence, mean abundance and mean intensity, as well as the discrepancy index (D index), for *C. longicollis* metacercariae in the sample of *D. vulgaris* stratified by fish standard length into eight size classes with intervals of 10mm. Specimens smaller than 100 mm or larger than 160 mm were pooled in the smallest and largest classes, respectively.

Size class	1	2	3	4	5	6	7	8
No fish	20	12	20	32	41	39	15	19
Standard length								
Range	66-98	100-110	111-119	120-129	130-139	140-148	151-159	160-220
Mean	82.10	106.08	115.85	123.69	134.32	143.59	155.40	177.05
SD	6.03	3.80	1.90	2.78	2.91	2.71	2.32	14.15
Total No metacercariae	24	43	304	899	1071	1281	382	435
Prevalence (%)	45.00	66.67	65.00	81.25	78.05	87.18	93.33	100
Intensity								
Mean	2.67	5.34	23.39	34.58	33.47	37.68	27.29	22.9
Range	1 - 7	1 - 22	1 - 67	1 - 212	1 - 200	1 - 228	1 - 128	4 - 58
Abundance								
Mean	1.20	3.58	15.20	28.09	26.12	32.85	25.47	22.90
SD	1.99	6.57	24.83	48.20	42.30	54.25	35.90	14.53
D index	0.698	0.678	0.691	0.715	0.693	0.695	0.614	0.324

Table S8.8. Prevalence, mean abundance and mean intensity, as well as the discrepancy index (D index), for *C. longicollis* metacercariae in the sam ple of *D. annularis* stratified by fish standard length into nine size classes with intervals of 10mm. Specimens larger than 150 mm were pooled in th e largest class.

Size class	1	2	3	4	5	6	7	8	9
No fish	13	19	17	39	29	23	13	33	10
Standard length									
Range	70-79	80-89	90-99	100-109	110-119	120-129	130-139	140-149	150-162
Mean	74.85	84.05	94.41	103.77	113.76	124.30	134.69	143.24	155.80
SD	2.97	2.84	2.98	3.17	2.79	2.93	3.25	2.61	3.94
Total No metacercariae	23	105	148	449	460	501	447	2322	665
Prevalence (%)	46.15	78.95	82.35	84.62	89.66	95.65	100.00	96.97	100.00
Intensity									
Mean	3.83	7.00	10.57	13.61	17.69	22.77	34.38	72.56	66.50
Range	1 - 10	1 - 25	1 - 25	2 - 36	1 - 95	2 - 78	2 - 107	3 - 220	5 - 126
Abundance									
Mean	1.77	5.53	8.71	11.51	15.86	21.78	34.38	70.36	66.50
SD	3.14	7.45	7.40	11.49	20.60	21.18	36.30	59.79	42.76
D index	0.696	0.61	0.435	0.516	0.571	0.483	0.495	0.429	0.305

Table S8.9. Results of negative binomial GLM (Mean number eggs ~ season) evaluating the effect of season on the number of *C. longicollis* eggs in *L. michahellis* faecal samples. The intercept value stands for the mean number of eggs in spring, to which the other levels are compared. The estimate of a variable is added or subtracted from the intercept value. Statistically significant results (at α=0.050) are indicated in bold type. The theta value was equal to 0.05782.

	Estimate	SE	*z-value*	*P-value*
(Intercept)	4.2753	0.6420	6.660	**<0.001**
Summer	-0.4620	0.9192	-0.503	0.615
Autumn	-0.5407	0.9193	-0.588	0.556
Winter	0.7366	0.9448	0.780	0.436

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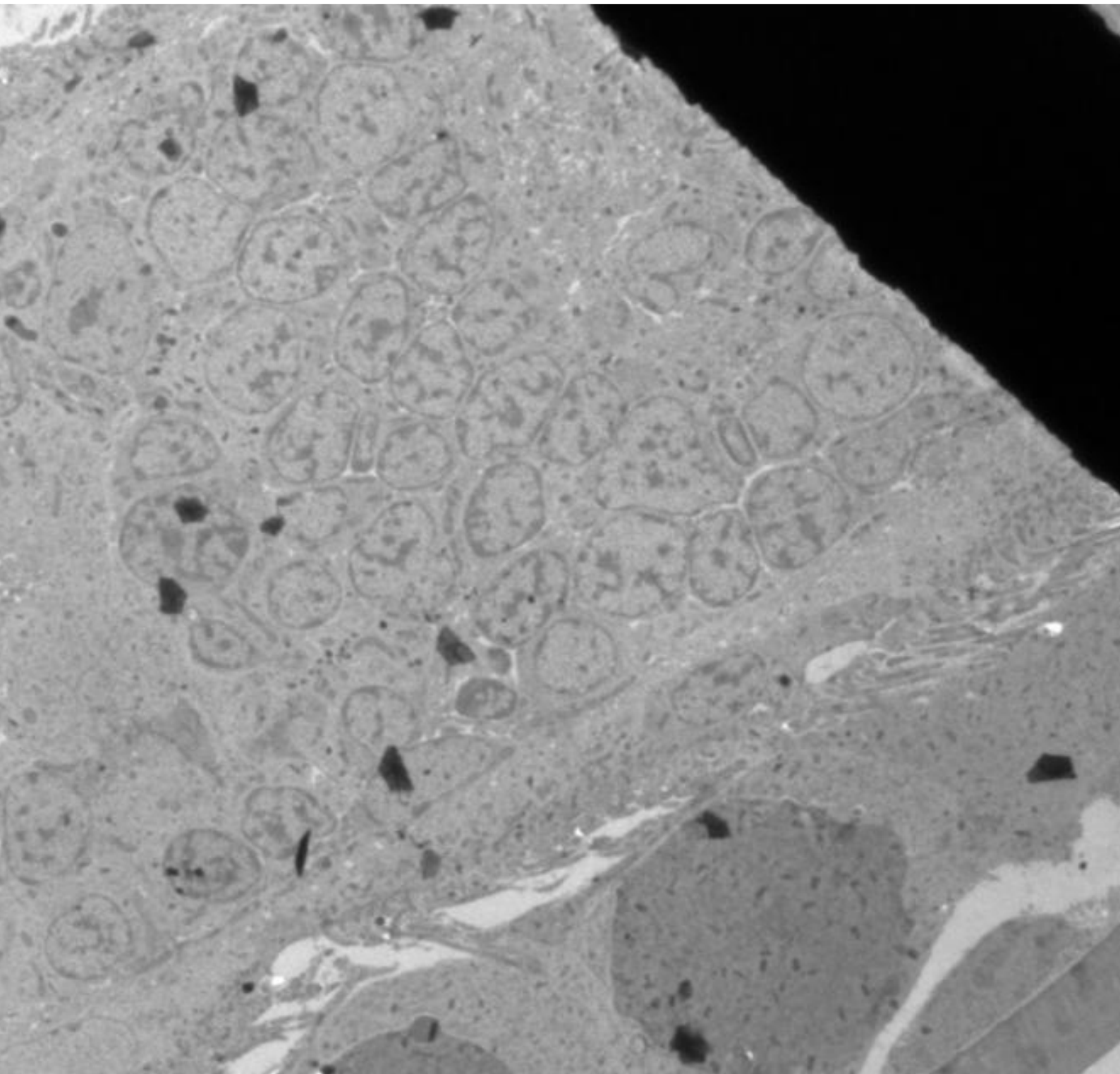
~General Conclusions~



As a result of this study the following conclusions were drawn:

1. Cercariae and metacercariae infecting the gastropods *G. adansonii* and *C. neritea*, common in Mediterranean lagoons, were described, compared with literature records and identified as the larval stages of *Cainocreadium labracis* (Dujardin, 1845) and *Macvicaria obovata* (Molin, 1859), trematodes belonging to the family Opecoelidae. Both cercariae were found at high prevalences of cercarial infections (22.04% and 23.1%, respectively) in *G. adansonii* and as metacercarial infections of *M. obovata* in *C. neritea* (reaching 100% prevalence), and thus indicating the successful transmission and life cycle completion of these species in Mediterranean lagoons.

2. The cercariae of *C. labracis* and *M. obovata* are of cotylocercous-type and morphologically very similar, however, ITS rDNA sequences ascribed them to two different trematode species, for which DNA sequences are published and available on GenBank. Thereby, the discovery of the cercaria of *M. obovata* helped to elucidate a new trematode life cycle, since its larval stages as well as second intermediate host were unknown.

3. Due to the problem of comparing morphological descriptions of trematode life cycle stages, which were produced by different authors and often different methods of fixation, staining etc., it has to be stressed that a complete characterization and/or identification of such life cycle stages relies on a combination of morphological description with a DNA sequence of the unique genotype. This is especially important in the case of morphologically similar stages such as the cercariae of *M. obovata* and *C. labracis*.

4. An experimental approach allowed describing the abiotic factors that impact on the emergence of the cercariae of *C. labracis* and *M. obovata* from the snail *G. adansonii*. Digeneans, and particularly free-living stages such as cercariae, are amongst the parasites most exposed to natural environmental variations, however, there is a lack of information of the adaption of trematode transmission strategies to the extreme environmental variations in lagoons, so that this study extends our knowledge of the specific emergence rhythms from digeneans inhabiting this habitat, and more specifically regarding cercariae with limited dispersal abilities.

5. The larval emergence patterns of these two species, which share the same habitat, the same snail host and the same downstream host-finding strategy, are contrasting and demonstrate the complex exogenous and endogenous control mechanisms that are in

place and aimed at maximising the encounter with their specific downstream hosts. Increased temperature, low water level and darkness seem to trigger the emergence of *M. obovata* so that cercariae encounter the nocturnal mollusc *C. neritea*, probably when tidal pools are formed and cercariae concentrate in a small water volume. On the contrary, the increased emergence during daylight hours and early summer (12-h light: 12-h dark photoperiod) permits *C. labracis* to coincide with small benthic gobiids in shallow zones with increased salinity.

6. The correct estimation of larval trematode infection prevalence is essential for understanding parasite community structure and the dynamics of multiple infections. The design and application of a duplex PCR for two different host-parasite systems with mixed infections demonstrated the higher accuracy of molecular methods for detection of infected snails than that of the classical method of observing the emergence of cercariae. To the best of our knowledge, this is the first time infection prevalences determined by both techniques are compared. The duplex PCR detected between 0,6% and 54,2% more single infections and between 2,4% and 9,5% more double infections than cercarial emission, thus evidencing the presence of immature infection that escape detection by the classical method.

7. The designed duplex PCR assay furthermore aids the identification of morphologically similar species co-infecting single snail hosts, such as *M. obovata* and *C. labracis*, as well as resolving immature infections, which is of particular importance in double infections, where interspecific competition may affect cercarial development and release. The prevalence detected by duplex PCR suggests that double infections are more frequent than expected, which may point to a lower competition within the host than previously thought. Nevertheless, possible early infections of one species might be eliminated by the previous established competitive species, hence both species co-exist only briefly; in such a case duplex PCR could overestimate the frequency of fully established double infections leading to cercarial emission and transmission, thus these results have to be interpreted with caution.

8. The findings of this thesis challenge previous conclusions based on cercarial emission alone in relation to interspecific competition, parasite population parameters and seasonal fluctuations of infections in snail hosts. However, while generally providing a more exact account of infection levels, the specific duplex PCR assay does not account for the maturity of infections and thus should be considered as an additional tool in a

combined approach of emission observation and molecular analyses that would both determine prevalences and maturity levels of larval trematodes in snails with the highest accuracy.

9. The ecological, physiological and biological peculiarities of the *C. labracis*/*M. obovata*-*G. adansonii* host-parasite system were further scrutinized by determining developmental patterns inside sporocysts, located in the gonads and digestive glands of the snail. *Gibbula adansonii* is found in the intertidal habitat of the Ebro Delta lagoon only from March to May, due to a spawning-related upward migration and reproduction-associated residency in the uphore zone of the lagoon. Due to the sessility of both cercariae, they need to emerge from the snail host in close vicinity to their next intermediate hosts. Mature cercariae were found to be released continuously and in high numbers during the spring months, with a maximum sporocyst maturity in April, thus guaranteeing the completion of both life cycles as second intermediate hosts of both species are present at this time in the intertidal habitat.

10. A link between larval maturation and snail upshore residency can be hypothesised given the synchronization of host and parasite maturation in time and space. Moreover, manipulation of the host behaviour to increase larval transmission probabilities to next hosts is extensive among trematodes, with parasitized snail specimens changing their vertical distribution and thus facilitating cercarial transmission. The potential exploitation of the reproduction-related snail migration by both cercariae opens a door to further studies on parasite-induced behaviour changes as a true adaptation for transmission.

11. The large-scale study on host spectra and infection levels of *C. longicollis* during its life cycle demonstrated new host records at the first and especially second intermediate host level, with 2 snail taxa, 31 fish taxa and 15 bird taxa known as hosts to date (including present results). *Cardiocephaloides longicollis* shows low host specificity especially at the second intermediate host level. This is the first time infection levels of *C. longicollis* in fish from different habitats have been compared. Higher infection rates were found in demersal fish compared to pelagic and benthic fish, confirming that this habitat is the one targeted by cercariae after their emergence from the snail.

12. The accumulation of *C. longicollis* metacercariae in the fish brain over time was demonstrated in two sparid host species. Increasing numbers of parasites induce behaviours that make infected fish more susceptible to predation by the definitive host,

thus enhancing their transmission to seagulls. In addition, the decline of parasite aggregation in the largest fish size classes indicates a "parasite-induced mortality" by which the most infected fish are eliminated from the population.

13. The prevalence of *C. longicollis* in snail and bird hosts from Mediterranean areas where extensive fishing activities are common, was higher than reported in any previous study. Bycatch attracts scavengers such as seagulls, which feed on discarded demersal and benthic fish that would otherwise be less accessible to them. Thus, the higher infection rates found in seagulls can be explained by consumption of discards of demersal fish that carry the highest parasite loads. This suggests an enhanced transmission of *C. longicollis* by anthropogenic factors. Moreover, metacercariae of this parasite have been found for the first time in a fish from aquaculture installations, i.e. the gilthead sea bream *Sparus aurata* with about 54% prevalence, so that, although still difficult to estimate, aquaculture activities may further impact on the *C. longicollis* life cycle increasing its transmission.

14. As a trophically-transmitted generalist species with a complex life cycle affecting a high number of host species, *C. longicollis* may have a strong effect on the energy flow through the food web, even under natural conditions, thus being an ideal candidate to compare future food web structures in natural communities with those impacted by fisheries.

~Appendix~

Appendix-Table 1. Mollusc samples examined during this study for different purposes. Taxonomic information with P= Polyplacophora, B=Bivalvia, G=Gastropoda. Number of hosts screened, locality and aim of the sampling are specified. Aims are referred as: (1) Description of emerging cercariae or metacercariae, (2) Comparison of emergence rates by changing abiotic factors, (3) Comparison of infection levels using visual emission and PCR, (4) Study of developmental stages and maturity, (5) Examination for *C. longicollis* infections.

Family	**Species**	**Locality**	**No molluscs**	**Aim**
Nassaridae (G)	*Cyclope neritea* (L.)	Ebro Delta, Spain	354	(1, 5)
	Cyclope neritea	Carboneras, Spain	2	(1, 5)
	Cyclope neritea	Ebro Delta, Spain	104	(1, 5)
	Cyclope neritea	Ebro Delta, Spain	309	(1, 5)
	Nassarius reticulatus (L.)	Ebro Delta, Spain	12	(1, 5)
	Nassarius reticulatus	Ebro Delta, Spain	316	(1, 5)
	Nassarius reticulatus	Ebro Delta, Spain	40	(1, 5)
	Nassarius reticulatus	Ebro Delta, Spain	294	(1, 5)
Trochidae (G)	*Gibbula. adansonii* (Payraudeau, 1826)	Ebro Delta, Spain	27	(1, 2, 3, 4)
	Gibbula. adansonii	Ebro Delta, Spain	526	(1, 2, 3, 4)
	Gibbula. adansonii	Ebro Delta, Spain	563	(1, 2, 3, 4)
	Calliostoma spp.	Carboneras, Spain	15	(5)
	Gibbula spp.	Dénia, Spain	10	(5)
Batillariidae (G)	*Zeacumantus subcarinatus* (Sowerby, 1855)	Lower Portobello Bay, New Zealand	1571	(3)
	Zeacumantus subcarinatus	Oyster Bay, Bay, New Zealand	1248	(3)
Anomiidae (B)	*Anomia ephippium* L.	Carboneras, Spain	25	(5)
Arcidae (B)	*Arca noae* L.	Carboneras, Spain	23	(5)
	Barbatia barbata (L.)	Carboneras, Spain	16	(5)
Buccinidae (G)	*Pisania striata* (Gmelin, 1791)	Dénia, Spain	12	(5)
	Pollia dorbignyi (Payraudeau, 1826)	Dénia, Spain	17	(5)

Appendix-Table 1. Continued.

Family	Species	Locality	No molluscs	Aim
Cerithiidae (G)	*Bittium reticulatum* (Costa, 1778)	Carboneras, Spain	41	(5)
	Cerithium vulgatum Bruguière, 1792	Carboneras, Spain	29	(5)
	C. vulgatum Bruguière, 1792	Ebro Delta, Spain	160	(5)
Chitonidae (P)	*Chiton (Rhyssoplax) olivaceus* Spengler, 1797	Carboneras, Spain	41	(5)
Chromodorididae (G)	*Felimare picta* (Schultz in Philippi, 1836)	Carboneras, Spain	6	(5)
Conidae (G)	*Conus ventricosus* Gmelin, 1791	Dénia, Spain	14	(5)
Fissurelidae (G)	*Diodora* spp.	Carboneras, Spain	25	(5)
Haliotidae (G)	*Haliotis tuberculata* L.	Carboneras, Spain	17	(5)
Haminoeoidea (G)	*Haminoea hydatis* (L.)	Ebro Delta, Spain	34	(5)
Hydrobiidae (G)	*Peringia ulvae* (Pennant, 1777)	Ebro Delta, Spain	70	(5)
Limidae (B)	*Lima lima* (L.)	Carboneras, Spain	12	(5)
Littorinidae (G)	*Littorina litorea* (L.)	Dénia, Spain	42	(5)
Muricidae (G)	*Hexaplex trunculus* (L)	Carboneras, Spain	20	(5)
	Stramonita haemastoma (L.)	Carboneras, Spain	8	(5)
Mytilidae (B)	*Mytilus edulis* L.	Carboneras, Spain	32	(5)
	Musculus subpictus (Cantraine, 1835)	Carboneras, Spain	19	(5)
Opisthobranchia (G)	*Opisthobranchia* (various spp.)	Carboneras, Spain	22	(5)
Ostreidae (B)	*Ostrea edulis* L.	Carboneras, Spain	24	(5)
Patellidae (G)	*Patella vulgata* L.	Carboneras, Spain	35	(5)
Tellinidae (G)	*Tellina incarnata* L.	Carboneras, Spain	35	(5)

Appendix-Table 2. Fish samples examined during this study for their examination for *C. longicollis* infections. Number of hosts screened and locality are specified.

Family	Species	Locality	No fish
Atherinidae	*Atherina boyeri* Risso, 1810	Carboneras, Spain	4
Blenniidae	*Parablennius sanguinolentus* (Pallas, 1814)	Carboneras, Spain	4
	P. pilicornis (Cuvier, 1829)	Carboneras, Spain	6
Bothidae	*Bothus podas* (Delaroche, 1809)	Carboneras, Spain	16
Carangidae	*Trachinotus ovatus* (L.)	Carboneras, Spain	12
Gobiidae	*Gobius bucchichi* Steindachner, 1870	Carboneras, Spain	4
Labridae	*Coris julis* (L.)	Carboneras, Spain	24
Pomacentridae	*Chromis chromis* (L.)	Carboneras, Spain	34
Serranidae	*Serranus scriba* (L.)	Carboneras, Spain	8
Soleidae	*Solea solea* (L.)	Carboneras, Spain	2
Sparidae	*Sparus aurata* L.	Carboneras, Spain	26
	Sparus aurata	Guardamar, Spain	31
	Sparus aurata	Valencia, Spain	4
	Sparus aurata	Sagunto, Spain	22
	Sparus aurata	Guardamar, Spain	34
	Diplodus puntazzo (Walbaum, 1792)	San Pedro del Pinatar, Spain	69
	Diplodus puntazzo	San Pedro del Pinatar, Spain	5
	Diplodus puntazzo	Jávea, Spain	20
	Diplodus puntazzo	Santa Pola, Spain	11
	Diplodus puntazzo	Santa Pola, Spain	9
	Diplodus sargus (L.)	Valencia, Spain	8
	Diplodus annularis (L.)	Santa Pola, Spain	19
	Diplodus annularis	Santa Pola, Spain	13
	Diplodus annularis	Valencia, Spain	196
	Diplodus vulgaris (Geoffroy Saint-Hilaire, 1817)	Santa Pola, Spain	4
	Diplodus vulgaris (Geoffroy Saint-Hilaire, 1817)	Valencia, Spain	198
	Dentex dentex (L.)	Santa Pola, Spain	15
	Dentex dentex	Burriana, Spain	20
	Spicara maena (L.)	Valencia, Spain	7

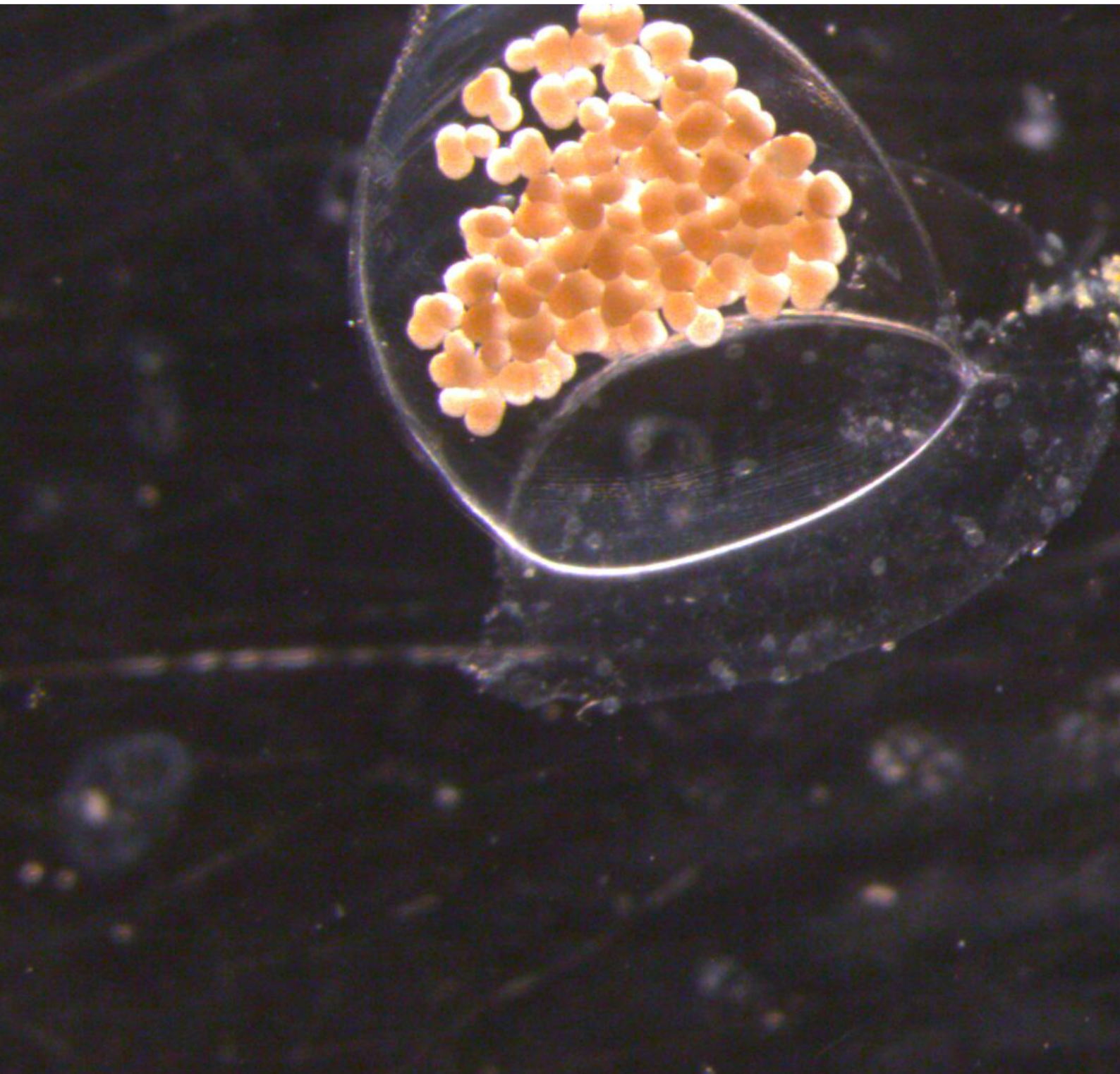
Appendix-Table 2. Continued.

Family	Species	Locality	No fish
Sparidae			
	Spicara maena	Burriana, Spain	20
	Spicara maena	Santa Pola, Spain	30
	Spicara maena	Santa Pola, Spain	33
	Lithognathus mormyrus (L.)	Valencia, Spain	16
	Lithognathus mormyrus	Valencia, Spain	2
	Lithognathus mormyrus	Burriana, Spain	20
	Lithognathus mormyrus	Carboneras, Spain	6
	Pagellus acarne (Risso, 1827)	Santa Pola, Spain	31
	Pagellus erythrinus (L.)	Santa Pola, Spain	27
	Pagellus erythrinus	Santa Pola, Spain	28
	Pagellus erythrinus	Carboneras, Spain	10
	P. bogaraveo (Brünnich, 1768)	Santa Pola, Spain	20
	Oblada melanura (L.)	Carboneras, Spain	8
	Oblada melanura	Santa Pola, Spain	19
	Spondyliosoma cantharus (L.)	Cullera, Spain	15
	Boops.boops (L.)	Burriana, Spain	20

Appendix-Table 3. Bird samples examined during this study for their examination for *C. longicollis* infections. Number of hosts screened and locality are specified.

Family	Species	Locality	No birds
Ardeidae	*Egretta garzetta* (L.)	Valencia, Spain	2
	Ardea cinerea L.	Valencia, Spain	1
	Ardea cinerea	Ebro Delta, Spain	5
	Ixobrychus minutus (L.)	Ebro Delta, Spain	2
Laridae	*Larus argentatus* Pontoppidan, 1763	Ebro Delta, Spain	3
	Larus audouinii Payraudeau, 1826	Valencia, Spain	4
	Larus genei Brème, 1839	Valencia, Spain	11
	Larus michahellis J.F. Naumann, 1840	Valencia, Spain	65
	Larus michahellis	Ebro Delta, Spain	13
	Chroicocephalus ridibundus L.	Ebro Delta, Spain	6
	Chroicocephalus ridibundus	Valencia, Spain	13
	Larus fuscus L.	Valencia, Spain	2
Phalacrocoracidae	*Phalacrocorax aristotelis* (L.)	Valencia, Spain	1
	Phalacrocorax aristotelis	Ebro Delta, Spain	1
	Phalacrocorax carbo (L.)	Valencia, Spain	7
Sternidae	*Sterna albifrons* (Pallas, 1764)	Valencia, Spain	2
	Sterna hirundo L.	Valencia, Spain	4
	Sterna sandvicensis Latham, 1787	Valencia, Spain	7
	Sterna sandvicensis	Ebro Delta, Spain	1
	Gelochelidon nilotica (Gmelin, 1789)	Valencia, Spain	1
	Chlidonias hybrida (Pallas, 1811)	Valencia, Spain	1
	Hydroprogne caspia (Pallas, 1770)	Valencia, Spain	2

~General References~



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