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Parasites of *Diplodus puntazzo* in Western Mediterranean:

Morphological, developmental and host-parasite relationships of monogeneans.

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Tesis Doctoral
Mª de les Neus Sánchez García

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Parasites of *Diplodus puntazzo* in Western Mediterranean: Morphological, developmental and host-parasite relationships of monogeneans.

TESIS DOCTORAL POR:

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CERTIFICAN que Dª Maria de les Neus Sánchez García ha realizado bajo nuestra dirección, y con el mayor aprovechamiento, el trabajo de investigación recogido en esta memoria, y que lleva por título: "Parasites of *Diplodus puntazzo* in Western Mediterranean: Morphological, developmental and host-parasites relationships of monogenean.", 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 26 de Septiembre de 2014.

Firmado

Juan Antonio Raga Esteve

Francisco E. Montero Royo

Soren Grøve

Per a Tu,

i les teues Roses

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TABLE OF CONTENTS

Summary

World aquaculture has experienced a substantial increase related to a decrease of capture production during the last century (FAO, 2012). Europe produced in 2010 a total of 1.2 million tonnes, and Spain produced the 20% of the total European production, being the first producer of fish in the European Union (EU) (APROMAR, 2012). However, Spanish aquaculture has contracted or stagnated during the last years and a production decrease has occurred in all important cultured species (gilthead seabream, European seabass and turbot), representing this, the first reduction in the last 25 years of fish farming (APROMAR, 2011). Species diversification is one of the main objectives to improve and increase the competitiveness in this sector. This diversification entails a Research & Development (R&D) challenge: the improvement of knowledge on the pathogens that could compromise the fish farm development. Despite the reduction of emergent diseases impact, to know the causes of outbreaks and to determine the host-pathogen interactions are currently essential. The culture of "new species" has caused two main problems: i) the transmission of the endemic pathogens to the new cultured fish species, compromising their farming and ii) the introduction of new pathogens from this new fish species that could jeopardize the existent cultures (Woo, 1995). Hence, to succeed in this fish culture and to prevent endangering the consolidated production it is necessary to establish the potential pathogens of newly introduced fish.

Diplodus puntazzo (sharpsnout seabream) is a good candidate to be introduced in Spanish Mediterranean cultures as its farming features are similar to those of other intensively cultured sparids, as the gilthead seabream *Sparus aurata*. This thesis aims to provide novel data about the parasite fauna of Mediterranean *Diplodus puntazzo* in the Spanish Mediterranean, to evaluate parasite effects in culture conditions and to suggest preventive practices in aquaculture facilities. The study has been specifically focused on damages associated to monogenean attachment and morphological features of the haptor structures.

To pursue these aims, the following objectives have been undertaken:

1. To identify the parasite fauna of wild and farmed *D. puntazzo* in the Spanish Mediterranean.

2. To report the risk assessment of protists and metazoan parasites of *D. puntazzo* from the Spanish Mediterranean, reporting which parasites species could compromise the culture of this species.

3. To compare the attachment of diplectanid monogenean, gill parasites of cultured fish, and the related pathological effects, based on detailed morphological and histophatological analyses.

4. To describe and compare the post-larval morphological changes of two species of diplectanid monogeneans (*Lamellodiscus theroni* and *L. falcus*) parasitizing *D. puntazzo*.

The parasite fauna of wild *Diplodus puntazzo* from Santa Pola and Mar Menor in the Spanish Mediterranean is composed by nineteen parasite species, seven of them cited for the first time in *D. puntazzo*: *Microcotyle* sp., *Magnibursatus bartolii, Steringotrema pagelli, Galactosomum sp., Cardiocephaloides longicollis, Caligus ligusticus* and *Gnathia vorax*. We also report the first records in the wild of two parasite species previously found only in farmed *D. puntazzo*: the polyopisthocotylean monogeneans *Atrispinum seminalis* and *Sparicotyle chrysophrii*.

Most of the parasites found in the current study are monoxenous (11 species): 8 monogeneans and 3 crustaceans. The heteroxenous species consist of 7 species of trematode and one species of myxozoan.

The morphological traits of the species of the monogenean genus *Lamellodiscus* found in present study coincide with those described for *L. falcus* and *L. theroni*. However, their dimensions were mostly similar to those of *L. ignoratus* and *L. ergensi*.

The molecular results from *Lamellodiscus falcus sensu lato* and *L. theroni sensu lato* show intraspecific differences in ITS1. However, this variation lies within the range of intraspecific variation provided by previous studies for *L. ergensi* and *L. ignoratus*. Thus, despite the morphometric variation amongst the described species (*L. falcus vs. L. ignoratus* and *L. theroni vs. L. ergensi*), molecular divergences are not enough to support their separation.

Spanish Mediterranean specimens of the trematode *Peracreadium characis* analyzed in the present study are somehow smaller than previous descriptions. Therefore, the present study extends the range of the morphometric variation for this species. Molecular analyses confirm that these morphological differences are intraspecific.

Most of the ectoparasites living on skin, gills and digestive system disappear in captivity conditions with the exception of the species of the genus *Lamellodiscus*. However some endoparasites remain protected by internal organs, as *Cardiocephaloides longicollis* in the brain and *Ceratomyxa* sp. in the gall-bladder.

Three species of parasites entail a "High" risk to *D. puntazzo* in Mediterranean culture conditions: *Amyloodinium* sp., *Cryptocaryon* sp. and *Enteromyxum leei*. Moreover three species of parasites, *Sparicotyle chrisophrii*, *Caligus ligusticus* and *Gnathia vorax*, entail a "Moderate" risk to Spanish Mediterranean farms of *D. puntazzo*. Most of the parasites entailing a "Moderate" to "High" risk to *D. puntazzo* farming have been previously reported in *Sparus aurata* cultures. Cross-infections between both species must be considered.

The lamellodiscs of *Lamellodiscus theroni* and *L. falcus* are arranged in overlapped lamellae. Contrary to previous publications, lamellodiscs do not work as suckers. Each independent lamella slides posteriorly resulting in the telescopic projection and creating a pushing effect on the gill epithelium, causing the gill secondary lamellae separation. Lamellodiscs thrust force is opposite to hooks traction force, which tightens the epithelia, providing a highly efficient attachment and stability to the worms.

Furnestinia echeneis presents a split type lamellodisc, where each half lamellae slides and moves independently. Lamella halves independence allows a postero-lateral displacement (hand fan-like), resulting in a volume increasing and suction effect. The lamellodisc of *F. echeneis* is too large to fit inside the interlamellar space, and works as a large sucker that fixes the monogenean to wide and flat gill tissues. Hooks would play a secondary role strengthening the attachment.

Diplectanum aequans haptor penetrates inside the interlamellar space, establishing a tight contact with host tissues. The hooks and spines produce extensive and deep alterations in the gill epithelium surrounding the parasite attachment site. The hyperplasia and inflammatory reaction of the epithelia, including tissue oedema, passively trap the haptor of the parasite.

Dissimilar attachment mechanisms of diplectanids explain the different damage intensity provoked by each species. *Diplectanum aequans* attachment mechanism implies extensive friction of surfaces contacting the haptor, with subsequent severe alterations of gills. *D. aequans* and *Lamellodiscus* spp. are attached to the secondary gill lamellae, causing severe damages and therefore impairing the respiratory function (hyperplasia, lamellar fusion and damage of the vascular structures). In contrast, *F. echeneis* attachment does not affect respiratory structures and only causes mild damage (temporary epithelial swelling and superficial perforation of gill tissues by hooks).

Information provided about attachment of Diplectanids is useful to design specific antihelmintic treatments: *D. aequans* attachment seems to be tight and passive; therefore parasites are difficult to detach, requiring aggressive and parasiticide treatments. The attachment of *Lamellodiscus* spp. and *F. echeneis* are respectively partially or totally active, and parasites should be more easily eliminated, after a treatment with antihelmintic products as those causing spasmodic effects on parasites.

During the *L. theroni* and *L. falcus* post-larval development, six developmental phases have been identified depending on the development of the

sclerotized structures of the haptor. I) with 14 peripheral marginal hooks, II) with dorsal and ventral hooks, III and IV) with ventral and dorsal bars, V) with dorsal and ventral lamellodiscs, and finally VI) with developed the copulatory organ.

Three developmental phases are crucial for the attachment strategy of the *Lamellodiscus* species during development. In phase I, parasites are only attached by the short 14 peripheral marginal hooks, a slightly stable attachment what allow parasite free displacement on plane surfaces. In phase II, the use of the large main hooks hampers the free movement of the parasite, although parasites are more stably attached to the secondary lamellae within the interlamelar space; and finally, in phase V, the presence of dorsal and ventral lamellodiscs entails a more stable and efficient attachment.

Despite the different haptors of *L. falcus*, *L. theroni* and *L. elegans* their growth patterns were very similar. These similarities imply analogous attachment strategies along development. However some differences can be found related to the different developmental rhythm of sclerites apparently related with their final dimension in each species.

Resumen

0.1 Introducción general

En España la acuicultura ha experimentado un gran crecimiento durante el pasado siglo, sin embargo, durante los últimos años se ha producido una disminución en la producción de las especies de cultivo más importantes, como la dorada, la lubina y el rodaballo (APROMAR, 2011). Esta situación ha supuesto la primera reducción en la producción de los últimos 25 años. Por este motivo, los productores españoles apuestan por la recuperación de la productividad y el aumento de la competitividad en el mercado y, para poder llevar a cabo estas mejoras, uno de los objetivos marcados es diversificar las especies cultivadas (APROMAR, 2011). En el Mediterráneo español, una de las especies candidatas para ser introducida es el sargo picudo, *Diplodus puntazzo* (Walbaum 1792). La acuicultura está interesada en esta especie ya que sus características de crecimiento son similares a las de otros espáridos que se cultivan en la actualidad, como la dorada *Sparus aurata* L., por lo que su introducción en los sistemas de cultivo requiere menor esfuerzo (Franicevic, 1989; Caggiano *et al.*, 1993; Abellán *et al.*, 1994). Como contraste, resulta interesante el hecho de que a pesar de su reciente importancia en la industria acuícola mediterránea, el sargo picudo ha carecido de interés por parte de las pesquerías, ya que su distribución, costera y asociada a fondos rocosos, dificulta su captura (FAO, 2010).

El desarrollo de nuevos cultivos es actualmente el objetivo principal de la acuicultura española, pero no hay que olvidar los problemas que lleva asociada la introducción de nuevas especies de peces en los sistemas de producción acuícolas, principalmente por la aparición de organismos patógenos: i) la transmisión de los patógenos endémicos de una nueva especie compromete su propio cultivo y ii) los nuevos patógenos introducidos con la nueva especie ponen en peligro otros cultivos existentes. Por lo tanto, establecer cuáles son los patógenos potenciales de los peces introducidos es necesario para asegurar el éxito de estos cultivos y prevenir poner en riesgo los cultivos ya consolidados.

Respondiendo a las necesidades de la acuicultura española, la presente tesis doctoral está basada principalmente en: i) el estudio general de los parásitos del

sargo picudo en condiciones tanto de cultivo como salvaje, identificando parásitos que pueden actuar como patógenos potenciales, representado un riesgo para los cultivos y también para las otras especies cultivadas en las mismas instalaciones acuícolas; y ii) el estudio de la morfología, el desarrollo post-larval y las relaciones parásito-hospedador de los parásitos monogeneos del sargo picudo.

0.1.1 *Diplodus puntazzo*

La especie *Diplodus puntazzo* es un perciforme perteneciente a la familia Sparidae, que presenta una distribución Atlántica Suroriental. Presente en el Atlántico desde la Bahía de Vizcaya hasta Sierra Leona, es muy común en el Mar Mediterráneo, Mar Negro y el estrecho de Gibraltar. Es una especie gregaria que habita aguas costeras, sobre fondos arenosos o rocosos, entre 0 y 150 m de profundidad, pero normalmente se encuentra alrededor de los 50 m. Es omnívora, y se alimenta de algas, gusanos, moluscos y camarones (Froese y Pauly, 2007).

Los juveniles tienen líneas verticales alternas claras y oscuras, que desaparecen en los individuos de gran tamaño. En los adultos, sobre el pedúnculo caudal, se observa un anillo oscuro. Sin embargo, la característica más notable es su hocico alargado, mucho más prominente que en el resto de los miembros de su familia. Tienen una o dos filas de pequeños dientes molariformes, que desaparecen en los adultos (Froese y Pauly, 2007).

0.1.2 Conocimientos previos sobre la fauna parasita de *D. puntazzo*

A pesar de la importancia actual del sargo picudo para la acuicultura española, existen pocos estudios referidos a su fauna parásita (Álvarez-Pellitero *et al.*, 2008; Athanassopoulou *et al.*, 1999; Athanassopoulou *et al.*, 2005; Di Cave *et al.*, 2002; Katharios *el al.*, 2006; Merella *et al.*, 2005; Mladineo y Maršić-Lučić, 2007; Montero *et al.*, 2007; Toksen, 2006; Vagianou *et al.*, 2004), la mayoría de ellos referidos a condiciones de cultivo.

Hasta la fecha un total de 37 especies de parásitos han sido citadas en sargos picudos del Mediterráneo: 5 protozoos, 5 mixozoos, 8 trematodos, 13

monogeneos, 5 copépodos y 1 isópodo. Los monogeneos son el grupo más abundante y, junto a los mixozoos, constituyen los parásitos más importantes en la acuicultura mediterránea debido a las patologías que causan en sus hospedadores. El mixozoo intestinal *Enteromyxum leei* (Diamant, Lom et Dyková 1994) y el monogeneo *Sparicotyle chrysophrii* Van Beneden et Hesse 1863 son parásitos realmente patógenos que han causado importantes mortalidades y pérdidas en instalaciones acuícolas, principalmente en especies de espáridos como la dorada y en el sargo picudo (Faisal, 1990; Sanz, 1992; Di Cave, 2003; Athanassopoulou *et al.,* 2005; Katharios *et al.*, 2006; Toksen, 2006; Amine *et al.*, 2007; Montero *et al.*,, 2007; Álvarez-Pellitero *et al.*, 2008; Davey *et al.*, 2011).

0.1.3 Los Monogeneos

En esta tesis se desarrollan diferentes estudios sobre las especies de monogeneos que parasitan el sargo picudo, la dorada y la lubina, correspondientes a la familia Diplectanidae Monticelli 1903; *Lamellodiscus theroni* Amine, Euzet et Kechemir-Issad 2007 y *L. falcus* Amine, Euzet et Kechemir-Issad 2006 parásitos del sargo picudo, *Furnestinia echeneis* (Wagener 1857) Euzet et Audouin 1959 parásito de la dorada y *Diplectanum aequans* (Wagener 1857) Diesing 1858 parásito de la lubina. Los monogeneos son parásitos externos que habitan en las branquias y la piel de sus hospedadores, habitualmente peces, tanto marinos como de agua dulce (Hayward, 2005; Whittington y Chisholm, 2008). En general, estos parásitos presentan ciclos de vida directos y una elevada especificidad con respecto a sus hospedadores (Hayward, 2005).

0.1.4 El presente estudio

El presente estudio se ha llevado a cabo dentro del marco fundamentalmente de tres proyectos, uno de ellos dedicado a las enfermedades del sargo picudo como nuevo candidato para la acuicultura: i) "Parásitos patógenos en nuevas especies de la acuicultura Mediterránea: una aproximación experimental" (financiado por la Unión Europea) y dos dedicados al estudio de las enfermedades de espáridos de interés comercial: ii) "Parásitos patógenos del sargo picudo: transmisión a la dorada y riesgos" (financiado por el Gobierno de España) y iii)

"Red Valenciana de investigación y desarrollo de patologías en acuicultura" (financiado por la Generalitat Valenciana).

0.2 Justificación y Objetivos

La presente tesis tiene dos propósitos generales: i) aportar nuevos datos sobre la fauna parásita del sargo picudo en el Mediterráneo y aplicar este conocimiento en un posterior análisis de los riesgos que suponen para su cultivo, para asesorar posibles prácticas de prevención en acuicultura; y ii) examinar en detalle algunos de los parásitos monogeneos del sargo picudo y de las dos principales especies de cultivo del Mediterráneo español, la dorada y la lubina, estudiando el anclaje a los hospedadores, las patologías asociadas y su desarrollo.

Los objetivos concretos a desarrollar han sido los siguientes:

1 Identificar la fauna parásita del sargo picudo, tanto salvaje como cultivado, en el Mediterráneo español, y determinar qué parásitos pueden comprometer su cultivo.

2 Estudiar los posibles riesgos de los parásitos protozoos y metazoos a los cultivos del sargo picudo en el Mediterráneo español.

3 Comparar el anclaje de tres monogeneos diplectánidos y las patologías que causan mediante análisis morfológicos e histológicos de los parásitos.

4 Estudiar morfológicamente los cambios post-larvales de dos especies de *Lamellodiscus* parásitos del sargo picudo.

0.3 Materiales y Métodos

Se han desarrollado en cada estudio específico de los correspondientes apartados.

0.4 Análisis morfométricos, moleculares y ecológicos de los parásitos de *Diplodus puntazzo* (Walbaum 1792) (Sparidae) en el Mediterráneo español: implicaciones en acuicultura

0.4.1 Introducción

Las condiciones en acuicultura implican estrés para los peces y, a su vez, favorecen la transmisión de patógenos y un elevado número de infecciones que en ocasiones han sido relacionadas con importantes pérdidas económicas (Ogawa, 1996; Murray y Peeler, 2005). Muchos de los patógenos causantes de enfermedades en las granjas se encuentran parasitando los peces circundantes; de este modo las diferentes especies de parásitos pueden ser intercambiadas por los peces cultivados y salvajes.

El sargo picudo es una de las especies con mayor potencial para ser introducida en la acuicultura mediterránea (Abellán y Basurco, 1999) debido a sus elevadas tasas de crecimiento y a su índice de transformación del alimento (Favaloro *et al.*, 2002; Hernández *et al.*, 2003). En España su cultivo se encuentra en una fase experimental (Hernández *et al.*, 2001, 2002, 2003; Pajuelo *et al.*, 2008; Nogales Mérida *et al.*, 2010; Almaida-Pagán *et al.*, 2011) y su introducción se ha visto comprometida por la presencia de patógenos (Athanassopoulou *et al.*, 2005; Merella *et al.*, 2005; Katharios *et al.*, 2006; Montero *et al.*, 2007; Álvarez-Pellitero *et al.*, 2008; Golomazou *et al.*, 2009; Rigos y Katharios, 2010; Sánchez-García *et al.*, 2011). Estos patógenos causantes de patologías pueden tener su origen de los peces salvajes que se encuentran circundantes, y el sargo picudo es una de las especies que se encuentra más frecuentemente en los alrededores de las granjas (Dempster *et al.*, 2002). Para tratar de prevenir y desarrollar controles efectivos de las posibles infecciones cruzadas es necesario un estudio en profundidad de las especies que parasitan el sargo picudo tanto en la naturaleza como en las instalaciones acuícolas (Hutson *et al.*, 2007; Rigos y Katharios, 2010). El objetivo de este trabajo es conocer las especies parásitas del sargo picudo en la naturaleza y analizar el efecto de las condiciones de cautividad sobre las mismas.

0.4.2 Materiales y Métodos

Se analizaron un total de 70 ejemplares salvajes capturados en dos localidades del Mediterráneo español: 50 procedentes del Mar Menor (San Pedro del Pinatar, Región de Murcia), y 20 de Santa Pola (Comunidad Valenciana). Treinta peces procedentes del Mar Menor fueron sacrificados y congelados inmediatamente. Para el estudio del efecto de las condiciones de cultivo en los parásitos, 20 peces procedentes del Mar Menor se transportaron vivos y se mantuvieron en las instalaciones de la planta de acuarios de la Universidad de Valencia (SCSIE). Diez peces fueron sacrificados a los 10 y 20 días de cautividad respectivamente.

Primero se examinó la superficie externa de los peces, incluyendo la piel, las aletas, la cavidad oral y la cavidad opercular, con el fin de detectar ectoparásitos. Posteriormente, se analizaron separadamente las branquias, los diferentes órganos de los sistemas digestivo y excretor, y el corazón. Por último, se analizaron el cerebro y los ojos, así como la musculatura circundante a las cinturas pélvica y escapular. Los parásitos se conservaron en etanol 70% para su examen morfológico y en etanol 100% para su estudio molecular. Los mixozoos se detectaron mediante el análisis de los órganos frescos.

El análisis morfológico se llevó a cabo mediante el análisis de muestras representativas de especímenes adultos que fueron teñidos con acetocarmín férrico (Georgiev *et al.*, 1986), se deshidrataron y se montaron en preparaciones permanentes con bálsamo de Canadá. Para el análisis de los monogeneos del género *Lamellodiscus*, sub-muestras representativas de 30 individuos se montaron en preparaciones semipermanentes de glicerogelatina. Los crustáceos se examinaron en agua destilada o en glicerogelatina. Las especies cuya identificación morfológica resultó confusa, se analizaron morfométricamente especímenes adultos, con la ayuda de un tubo de dibujo.

Para aquellas especies difíciles de clasificar morfológicamente se realizaron comparaciones genéticas. Se extrajo el ADN de tres a cuatro individuos de *L. falcus s.l.*, *L. theroni s.l.* (Monogenea) y *Peracreadium characis* (Stossich 1886) (Digenea).

Posteriormente. se amplificaron las secuencias de las regiones 18S e ITS1 en el caso de los monogeneos, y ITS1 e ITS2 de *P. characis*. Finalmente, las secuencias resultantes se alinearon usando el programa BioEdit v. 7.0.5. (Hall, 1999) y se compararon con secuencias cercanas filogenéticamente, obtenidas de la base de datos de Genbank, con ayuda de los programas BLAST (Altschul *et al.*, 1990) y MEGA 4.1 (Tamura *et al.*, 2007).

Para todas las especies de parásitos se calcularon la abundancia media y la prevalencia, con intervalos de confianza, (según Bush *et al.* 1997); excepto para los mixozoos, para los que sólo fueron calculadas las prevalencias. Para detectar diferencias significativas entre las distintas muestras de peces, se llevó a cabo la comparación de las prevalencias y abundancias entre las muestras procedentes de las dos localidades, y entre las muestras salvajes y cautivas de sargo picudo. Los análisis se realizaron con el programa Quantitative Parasitology 3.0 (Reiczigel y Rózsa, 2005).

0.4.3 Resultados

Todos los sargos picudos analizados estaban parasitados por al menos cuatro especies parásitas. En total se detectaron 19 especies, 15 de ellas en peces procedentes del Mar Menor y 11 en peces de Santa Pola. Todos los parásitos fueron identificados hasta nivel de especie, excepto *Ceratomyxa* sp., *Galactosomum* sp. y Microcotylidae gen. sp. Los peces procedentes de ambas localidades compartían un total de siete especies parásitas. Se encontraron diferencias significativas en las abundancias medias entre las muestras de peces salvajes del Mar Menor y Santa Pola. La mayor abundancia se detectó en los peces procedentes del Mar Menor, que presentaron ocho especies que no estaban presentes en los peces de Santa Pola. Los peces procedentes de Santa Pola estaban parasitados por 4 especies que no se encontraron en los peces del Mar Menor.

La carga parásita de los peces del Mar Menor, entre los que se encontraron la mayoría de las especies de monogeneos, el mixozoo y dos especies de digeneo, disminuyó gradualmente con el tiempo. La abundancia total parasitaria disminuyó significativamente después de 10 días de cautividad. A los 20 días la abundancia

total de parásitos también disminuyó significativamente: los peces continuaron parasitados por 3 especies de *Lamellodiscus*, presentando mayores abundancias, y dos especies de parásitos estrictos del medio interno, *Cardiocephaloides longicollis* (Rudolphi 1819), en cerebro, y *Ceratomyxa* sp. en vesícula biliar.

Análisis morfológicos y moleculares

El análisis morfológico de las especies de monogeneos del género *Lamellodiscus* permitió comparar los resultados obtenidos con las descripciones originales. Los morfotipos eran similares a *L. falcus* y *L. theroni* (referidos como *L. falcus sensu stricto* y *L. theroni sensu stricto*). Los resultados del análisis morfométrico de los especímenes del digeneo *Peracreadium* sp. procedentes de ambas localidades se compararon con las descripciones originales, siendo sus medidas similares entre las dos localidades.

En los análisis moleculares realizados para las especies de *Lamellodiscus* no se encontró ninguna diferencia cuando se compararon las secuencias aisladas de 18S de *L. falcus s.l.* y las obtenidas en GenBank de la especie *L. ignoratus* Palombi 1943. Cuando se compararon los aislados de 18S de *L. theroni s.l.* presentaron un porcentaje del 0% de disimilaridad y del 0.2% comparados con los de *L. ergensi* Euzet et Oliver 1966 procedentes de GenBank. Al comparar las secuencias de ITS1 obtenidos de *L. falcus s.l.* no se encontraron disimilaridades; sin embargo al comparar las mismas con las secuencias de *L. ignoratus* procedentes de GenBank, se encontraron unas disimilaridades entre 1.2 y 26.0%. Las disimilaridades entre secuencias procedentes de diferentes individuos de *L. theroni s.l.* se encontraron entre 0.0 y 0.2%, mientras que al ser comparadas con secuencias de *L. ergensi* obtenidas de GenBank estaban entre el 0.2 y el 14%. Durante el estudio molecular de los especímenes de *Peracreadium* sp. las secuencias obtenidas de 18S e ITS se compararon con las disponibles en GenBank de la especie *P. characis*. No se encontraron disimilaridades entre las secuencias de 18S aisladas de los especímenes procedentes de este estudio y las de la especie *P. characis* disponibles en GenBank. Las secuencias obtenidas de ITS no fueron disimilares entre ellas,

mientras que cuando se compararon con las de la especie *P. characis*, la disimilaridad fue de 0.3% en todos los casos.

0.4.4 Discusión

El sargo picudo presenta una comunidad de parásitos metazoos diversa en el Mediterráneo español. Algunas de las especies identificadas en el presente trabajo han sido previamente descritas en el Mediterráneo (Radujkovic y Euzet, 1989; Sasal *et al.*, 1999; Bartoli *et al.*, 2005; Mladineo, 2006; Gargouri Ben Abdallah y Maamouri, 2008) y un total de siete especies han sido citadas por primera vez en el sargo picudo en este estudio. Además, dos especies de parásitos previamente identificados en sargos picudos cultivados, se han encontrado por primera vez en peces salvajes. La mayoría de las especies parásitas encontradas son monoxenas (11 especies), ocho monogeneos y tres crustáceos; las especies heteroxenas (7 especies) corresponden principalmente a digeneos junto con una especie de mixozoo. Ningún nematodo ha sido registrado ni en este trabajo, ni en estudios anteriores.

Análisis morfológicos y moleculares

El estudio morfológico de las especies de *Lamellodiscus* muestra una clara concordancia entre la morfología de los morfotipos *L. falcus s.l.* y *L. theroni s.l.* y las descripciones de las especies *L. falcus* y *L. theroni*, siendo sin embargo sus dimensiones más similares a las proporcionadas para las especies *L. ergensi* y *L. ignoratus*. Finalmente, los resultados moleculares mostraron una variación intra-específica en las secuencias de ITS1 que se encuentra dentro del rango de variación dado por Kaci-Chaouch *et al.* (2008) para las especies *L. ergensi* y *L. ignoratus*. Por lo tanto, de acuerdo con Poisot y Desdevises (2010) y Poisot *et al.* (2011), y a pesar de las variaciones morfométricas, este tipo de divergencias moleculares en los especímenes de *Lamellodiscus* spp. no refuerza la separación de las especies (*L. falcus* vs. *L. ignoratus* and *L. theroni* vs. *L. ergensi*). Las variaciones intra-específicas debidas al hospedador combinadas con la posibilidad de la existencia de variaciones morfométricas causadas por las diferencias geográficas

podrían explicar las variaciones morfológicas existentes en las especies de *Lamellodiscus*.

La morfología de los especímenes de *Peracreadium* sp. se corresponde con la de *P. characis*, siendo sin embargo las medidas totales y parciales de los individuos españoles menores que las señaladas en su descripción (ver Bartoli *et al.*, 1989). Los resultados moleculares obtenidos confirman que corresponden a la especie *P. characis*. Las diferencias encontradas al comparar las secuencias de ITS se encuentran entre las diferencias intra-específicas dadas por Jousson *et al.* (1999). Consecuentemente, el presente estudio extiende el rango de medidas de cada órgano y las dimensiones.

Efecto de la cautividad sobre la fauna parásita

La mayoría de los ectoparásitos y parásitos digestivos desaparecen en condiciones de cautividad, excepto los monogeneos *Lamellodiscus* spp., debido a la gran susceptibilidad de los parásitos a las condiciones de cultivo (Woo, 2006), como por ejemplo la calidad del agua, el estado de salud del pez y la alimentación. La desaparición de la mayoría de los parásitos externos contrasta con la supervivencia de aquellos que parasitan órganos internos, como la metacercaria de *Cardiocephaloides longicollis*, protegida por los tejidos cerebrales, y el mixozoo *Ceratomyxa* sp., protegido por la vesícula biliar.

La información procedente de este estudio permite concluir señalando aquellos grupos de parásitos que pueden comprometer la salud del sargo picudo. Se trata principalmente de tres grupos: los mixozoos, los monogeneos microcotílidos, y los crustáceos.

0.5 Estudio de evaluación de riesgos parasitológicos del cultivo de *Diplodus puntazzo* en el Mediterráneo occidental: perspectivas de infecciones cruzadas con *Sparus aurata*

0.5.1 Introducción

La autoridad en salud alimentaria de la Unión Europea (EFSA) es la agencia encargada del análisis de riesgos relacionados con la comida y la alimentación. De acuerdo con el EFSA (2008), el análisis de riesgos es un proceso científicamente basado en la evaluación, tanto cualitativa como cuantitativa, de la probabilidad y del impacto potencial de cualquier peligro o amenaza. En la guía EFSA (2009a), se expone la necesidad de desarrollar análisis de riesgos procedentes de patógenos en las instalaciones acuícolas. Este tipo de estudios ya se han llevado a cabo en diferentes países (e.g., Akoll *et al.*, 2012; Hutson *et al.*, 2007; Nowak, 2004), sin embargo, y a pesar de las recomendaciones del EFSA, no existe ningún estudio realizado para la acuicultura Mediterránea (EFSA, 2008; EFSA, 2009a; EFSA, 2009b).

El objetivo de este estudio es llevar a cabo una evaluación de los riesgos parasitológicos para el cultivo del sargo picudo en el Mediterráneo español. Además se ha tenido en cuenta la posible existencia de infecciones cruzadas con cultivos de dorada (*Sparus aurata*). Para ello, se ha realizado un análisis cualitativo basado en los parásitos del sargo picudo y de la dorada previamente documentados.

0.5.2 Materiales y Métodos

Siguiendo la guía EFSA (2008) se identificaron las especies de parásitos que presentaban una mayor probabilidad de proliferar en las instalaciones acuícolas ("*Identificación del peligro*"). Posteriormente, se caracterizó el riesgo basándose en las especies de parásitos que pueden causar patologías comprometiendo la salud de los peces, que son difíciles de controlar y que tienen prioridad inmediata para establecer unas estrategias de manejo adecuadas ("*Caracterización del peligro y evaluación de la exposición al mismo*"). Finalmente, se consideró la posibilidad de

tratar las diferentes especies de parásitos consultando la bibliografía disponible (Schmahl y Mehlhorn, 1985, 1988; Schmahl y Taraschewsh, 1987; Schmahl *et al.*, 1988; Woo, 2006; Noga, 2009).

Los parásitos de la dorada también se estudiaron y se incluyeron en el trabajo debido a la proximidad taxonómica de ambas especies de peces y a que su cultivo se realiza en las mismas instalaciones que el sargo picudo.

0.5.3 Resultados

En la dorada y el sargo picudo, los monogeneos son el, el grupo más diverso de parásitos seguido por el de los digeneos. Dieciséis de las especies parásitas descritas en dorada se identificaron también en el sargo picudo.

Según los resultados obtenidos, ninguna especie de parásito implica una consecuencia "Extrema", ya que la mayor consecuencia, "Alta", fue asignada a las especies de parásitos *Amyloodinium* spp., *Cryptocaryon irritans* Brown 1951, una especie de ciliado no identificado, y *Enteromyxum leei*. La categoría de "Extrema" en la probabilidad de establecimiento y proliferación, fue asignada a diferentes grupos: una especie de ciliado (*C. irritans*), tres especies de mixozoos (*Ceratomyxa* sp., *Ceratomyxa puntazzi* Alama-Bermejo, Raga et Holzer 2011 y *E. leei*), tres especies de monogeneos (*Lamellodiscus bidens*, *L. hilii* y *Sparicotyle chrysophrii*) y una especie de copépodo (*Caligus ligusticus* Brian 1906). Los resultados procedentes del estudio de riesgo indican que los parásitos *Amyloodinium* spp., *Cryptocaryon* sp. y *E. leei* implican un "Alto" riesgo para el cultivo del sargo picudo en el Mediterráneo español. El monogeneo *S. chrysophrii*, el isópodo *Gnathia vorax* (Lucas 1849) y el copépodo *C. ligusticus* representan un riesgo "Moderado". La mayoría de estas especies de parásitos, a excepción del *E. leei*, pueden ser tratadas con sustancias antiparasitarias.

0.5.4 Discusión

Este es el primer análisis de riesgos llevado a cabo en la acuicultura europea. La metodología empleada es directamente aplicable para cualquier otra

especie de cultivo. Es importante incidir que el presente trabajo está basado en la información disponible actual, por lo tanto debería ser revisado en el futuro con la identificación de nuevas especies.

Tres especies de parásitos representan un riesgo "Alto" para los cultivos del sargo picudo: *Amyloodinium* spp., *C. irrintans* y *Enteromyxum leei*. *C. irritans* y *E. leei* son conocidas por ser las responsables de elevadas mortalidades en los cultivos de sargo picudo y dorada (Le Breton y Marques, 1995; Athanassolpoulou *et al.*, 1999; Golomazou *et al.*, 2004). Los resultados obtenidos nos permiten saber que *Amyloodinium* spp. también debe ser considerado como uno de los parásitos de riesgo, debido a sus efectos negativos (Noga, 1996). Adicionalmente, *Sparicotyle chrisophrii*, *Caligus ligusticus* y *Gnathia vorax* representan un riesgo "Moderado" para los cultivos de sargo picudo en el Mediterráneo. Las infecciones causadas por *S. chrisophrii* han sido responsables de mortalidades masivas en los cultivos de dorada (Paperna, 1991; Alvárez-Pellitero *et al.*, 1995) y de sargo picudo (Merella *et al.*, 2005). En lo relativo a los crustáceos no se han registrado episodios importantes en el cultivo de espáridos en el Mediterráneo, pero debido a su elevada patogeneidad descrita en otras especies de peces y a su baja especificidad, los calígidos y los isópodos deben ser controlados y gestionados para prevenir futuros problemas en las instalaciones acuícolas.

La mayoría de los parásitos que conllevan un riesgo importante para el cultivo del sargo picudo, han sido previamente identificados en los cultivos de dorada (protistas, mixozoos y el monogeneo *S. chrisophrii*). Estos datos indican la posibilidad de infecciones cruzadas que aumentarían las tasas de infección y podrían comprometer los cultivos de una de las especies fundamentales en la acuicultura Mediterránea.

El presente análisis resalta la importancia de la evaluación de los riesgos en el conocimiento científico de la salud de los animales acuáticos. Los parásitos identificados como patógenos potenciales deben ser considerados en el establecimiento de nuevos cultivos, teniendo en cuenta, junto con la localización de las instalaciones, la fauna parasita de las especies circundantes, las corrientes

marinas relacionadas con las jaulas vecinas y la posibilidad de contagio por patógenos procedentes de otras especies cultivadas.

0.6 Estudio comparativo de tres mecanismos de anclaje de monogeneos diplectánidos

0.6.1 Introducción

La Familia Diplectanidae Monticelli 1903 es una familia de monogeneos monopistocotileos con especies en todo el mundo (Domingues y Boeger, 2008). Se trata de parásitos de peces que poseen un haptor característico compuesto por dos pares de ganchos grandes laterales conectados por unas barras medias, 14 ganchos marginales y, normalmente, una o dos estructuras mediales llamadas lamelodiscos (Bychowsky, 1957; Desdevises, 2001; Domingues y Boeger, 2008). Los lamelodiscos están formados por grupos de escleritos dorsales y ventrales, cuya morfología, disposición y número varía entre los diferentes grupos de diplectánidos (Domingues y Boeger, 2008; Yamaguti, 1963).

Los diplectánidos son parásitos muy comunes en el Mediterráneo, *Furnestinia echeneis*, en *Sparus aurata*, *Lamellodiscus* spp. en *Diplodus puntazzo*, y *Diplectanum aequans*, en *Dicentrarchus labrax* L. (Cruz-e-Silva *et al.*, 1997; Dezfuli *et al.*, 2007; Katharios *et al.*, 2006; Merella *et al.*, 2005; Mladineo, 2007; Toksen, 2006; Vagianou *et al.*, 2004, 2006). Las diferencias en la estructura del háptor están asociadas a la diversidad de estrategias de anclaje de las diferentes especies de monogeneos (Bychowsky, 1957; Dezfuli *et al.*, 2007; Kearn, 1997), y estas diferencias implican a su vez diferentes daños en los tejidos del hospedador.

El objetivo de este estudio es describir y comparar el mecanismo de anclaje de *F. echeneis* y de dos especies del género *Lamellodiscus* parásitos del sargo picudo. Los resultados obtenidos se compararán con el anclaje del diplectánido más patogénico *D. aequans*.

0.6.2 Materiales y Métodos

Los parásitos se aislaron de peces procedentes de dos granjas diferentes situadas en el Mediterráneo español (Burriana (Castellón) y San Pedro del Pinatar (Murcia)). Los peces fueron sacrificados y se extrajeron las branquias. Se analizaron los arcos branquiales derechos para la observación *in vivo* de los monogeneos. Los arcos izquierdos se fijaron en formalina tamponada para la realización de los estudios histológicos y de microscopia electrónica de barrido (SEM).

Para su adecuada clasificación y observación, 20 especímenes de cada una de las especies estudiadas se montaron en preparaciones semipermanentes de glicerogelatina. Los arcos branquiales fijados en formalina fueron embebidos en parafina, seccionados y teñidos con hematoxilina y eosina. Para el estudio realizado con SEM, cinco parásitos de cada especie fijados en formalina se deshidrataron, se pasaron por el punto crítico de secado y se les metalizó con oro-paladio, para ser observados posteriormente en el equipo de microscopia electrónica HITACHI S-4100.

El estudio del háptor se realizó mediante el análisis de los especímenes de las diferentes especies utilizando diferentes técnicas microscópicas: Microscopio óptico para las observaciones *in vivo*, la toma de medidas y el análisis de los cortes histológicos y microscopio electrónico, para la observación de la superficie de los monogeneos.

El estudio se completó con cortes histológicos de *F. echeneis* y *D. aequans* anclados en el tejido branquial, procedentes de la colección del Servicio de Diagnóstico de enfermedades de Peces de la Universidad Autónoma de Barcelona.

0.6.3 Resultados

Anclaje de Furnestinia echeneis

El háptor de *F. echeneis* está formado por un solo lamelodisco, cóncavo, hemi-elipsoidal y abierto ventralmente, dos pares de ganchos principales localizados a ambos lados del háptor, las barras medias dorsales y ventrales y 14

pequeños ganchos marginales. El háptor está ventralmente rodeado por un velo de tegumento donde se encuentran los ganchos marginales. El tamaño del háptor experimenta cambios *in vivo*, se comprime y extiende. Las láminas esclerotizadas que forman el lamelodisco se desplazan entre ellas, disminuyendo o aumentando el diámetro del háptor a la vez que todos los ganchos presentes en el háptor están en constante movimiento. *Furnestinia echeneis* se ancla principalmente en el lado aferente de las laminillas branquiales primarias. Los parásitos se desplazan frecuentemente: el cuerpo del parasito se expande buscando un nuevo lugar donde anclarse, el háptor se expande y se desprende del tejido y finalmente se vuelve a anclar en el tejido adyacente. Cuando el háptor se desprende de las branquias, el epitelio del área de anclaje aparece abultado y rodeado por una depresión elíptica producto de la compresión del epitelio por el margen del háptor. En los cortes histológicos no se observa ninguna reacción inflamatoria asociada al anclaje del parásito.

Anclaje de Lamellodiscus spp.

El háptor de las especies de *Lamellodiscus* está dorso-ventralmente aplanado, triangular y en forma de "T". Está formado por dos lamelodiscos, dorsal y ventral, dos pares de ganchos principales situados en los extremos laterales del háptor, una barra ventral y dos dorsales, y 14 ganchos marginales distribuidos ventral y dorsalmente. Los lamelodiscos forman un orificio dorsal y ventral en el háptor cubierto por unas extensiones de tegumento. En los especímenes *in vivo* se observó que los lamelodiscos se extienden o comprimen mediante el desplazamiento de las lamelas en un movimiento telescópico. En las branquias los especímenes de *Lamellodiscus* sp. introducen la mayor parte del háptor en el espacio que existe entre las laminillas secundarias. Los parásitos normalmente se mantienen anclados, sin embargo de forma eventual pueden desplazarse a un espacio interlamelar distinto. En las secciones histológicas se observa que los lamelodiscos desplazan las laminillas secundarias, a la vez que los ganchos principales las atraen perforando profundamente las laminillas continuas, los ganchos pueden llegar a perforar los vasos sanguíneos observándose ligeras

hiperplasias y micro hemorragias. No se detectan respuestas inflamatorias relacionadas con el anclaje de las especies de *Lamellodiscus*.

Anclaje de Diplectanum aequans

El háptor de *D. aequans* establece un contacto directo con el epitelio branquial de su hospedador *D. labrax*, con las espinas que forman el lamelodisco orientadas anteriormente y adheridas al epitelio. El tejido branquial del área de anclaje presenta una clara reacción inflamatoria junto con una hiperplasia extensa.

0.6.4 Discusión

La combinación de las diferentes técnicas microscópicas y la observación de parásitos vivos y fijados, anclados y no anclados a los tejidos del hospedador, ha permitido interpretar el papel de cada elemento del háptor en el anclaje.

Furnestinia echeneis se ancla en la zona aferente de las laminillas primarias mediante un efecto de succión ejercido por su único lamelodisco. El háptor succiona el epitelio branquial creando un abultamiento rodeado por una línea de depresión creada por los márgenes del epitelio. Los ganchos juegan un papel secundario en el anclaje, ayudando en los primeros estadios del anclaje, cuando el parásito ha cambiado de posición.

A diferencia de *F. echeneis,* el tamaño de los lamelodiscos de las especies del género *Lamellodiscus* es similar a sus ganchos principales, por lo tanto parece que ambas estructuras son fundamentales para el anclaje de los parásitos. Los 4 ganchos principales perforan el epitelio de las laminillas secundarias representando 4 puntos de anclaje que impiden que se liberen los parásitos. Las laminillas que forman los lamelodiscos realizan un movimiento telescópico, de este modo, los lamelodiscos actúan como estructuras protráctiles empujando el tejido y resultando en la separación de las laminillas secundarias contiguas. Por lo tanto, la fuerza de empuje que ejercen los lamelodiscos es contraria a la tracción ejercida por los dos pares de ganchos.

Las diferencias entre *Lamellodiscus* sp. y *F. echeneis* demuestran que estructuras similares con un origen común resultan en movimientos y estrategias de anclaje diferentes. El háptor de *F. echeneis* es demasiado grande para introducirse en el espacio interlamelar, razón por la cual necesita un anclaje basado en la succión.

El anclaje de *D. aequans* y las patologías relacionas fueron estudiadas por Dezfuli *et al.* (2007). El háptor de *D. aequans*, al igual que las especies del género *Lamelodiscus*, se introduce en el espacio interlamelar y establece un contacto estrecho con los tejidos del hospedador. El contacto del parásito parece estar relacionado con la hiperplasia y la reacción inflamatoria del epitelio, que pasivamente atrapa el háptor del parásito impidiendo su liberación.

Las principales lesiones relacionadas con los monogeneos están asociadas con su anclaje especifico (Kearn, 1997). *Diplectanum aequans* es la única especie de diplactánido que se ha relacionado con problemas en los peces (Dezfuli *et al.*, 2007), ya que la hiperplasia y la inflamación causada en las laminillas secundaria compromete la eficiencia del tejido respiratorio. Del mismo modo, los ganchos principales de *Lamelodiscus* sp. perforan el tejido branquial, pudiendo llegar hasta los vasos sanguíneos y causando pequeñas hemorragias que del mismo modo podrían llegar a afectar el intercambio gaseoso de los tejidos. Por el contrario, el anclaje *F. echeneis* no afecta a las estructuras respiratorias y sólo causa un daño superficial.

La información obtenida en el presente trabajo puede resultar útil en el diseño de tratamientos antihelmínticos considerando los diferentes modos de anclaje de cada una de las especies parásitas estudiadas.

0.7 Cambios morfológicos y de anclaje de *Lamellodiscus theroni* (Monogenea, Diplectanidae) durante su desarrollo en *Diplodus puntazo* (Sparidae)

0.7.1 Introducción

Las especies del género *Lamellodiscus* son parásitos comunes de peces salvajes y cultivados en el Mediterráneo. Principalmente parasita las branquias del sargo picudo, *Diplodus puntazzo* (Dezfuli *et al.*, 2007; Katharios *et al.*, 2006; Mladineo, 2007; Toksen, 2006; Sánchez-García *et al.*, 2013).

A pesar de la gran cantidad de trabajos relacionados con especies del genero *Lamellodiscus* (Amine *et al.*, 2007; Sánchez-García *et al.*, 2001; Poisot y Desdevises, 2010) sólo existe un estudio del desarrollo de las larvas de *Lamellodiscus* realizado por Bychowsky (1961); sin embargo no se incluyen datos de la cronología del desarrollo de los parásitos. El presente capítulo provee de información acerca del desarrollo de las piezas esclerotizadas del háptor y del órgano copulador masculino, de la especie *Lamellodiscus theroni* en el tiempo, además de estudiar los cambios que se producen en las estrategias de anclaje a lo largo del desarrollo del háptor.

0.7.2 Materiales y Métodos

Cuarenta sargos picudos infectados por *Lamellodiscus theroni* fueron capturados en el Mar Menor (Murcia) y se trasladaron a las instalaciones de la Universidad de Valencia. Estos peces actuaron como donantes de parásitos para infectar a un total de 150 sargos picudos sanos, procedentes de Grecia. La infección se realizó mediante 48h de cohabitación con los peces donantes. Después del periodo de infección, los peces se transfirieron a los tanques experimentales (de la planta de acuarios del SCIE) y se determinó ese día como el "día 0" del experimento. Desde este momento se sacrificaron diariamente 5 peces, durante 22 días. Se recolectaron los parásitos y se guardaron las branquias para posteriormente realizar análisis histológicos.

Para el análisis morfológico de los especímenes, los monogeneos se montaron en preparaciones semipermanentes en glicerogelatina y se estudiaron mediante microscopía óptica. Los análisis histológicos se llevaron a cabo mediante el análisis de secciones de tejido teñido con hematoxilina-eosina.

0.7.3 Resultados

Todos los parásitos se encontraron en las branquias. Se analizaron un total de 151 parásitos, encontrados en las branquias, que se clasificaron en diferentes fases en función de su grado de desarrollo.

Se identificaron seis fases de desarrollo en *L. theroni* de acuerdo con las primeras observaciones de las diferentes estructuras esclerotizadas que se estudiaron.

- Fase I (días 1 a 12; n=8): Estadio post-larval. Como estructuras de anclaje se observaron 14 ganchos marginales dispuestos circularmente en el háptor. Los ganchos presentaron forma de garfio. Su tamaño no varió a lo largo del desarrollo.

- Fase II (días 5 a 15; n=14, más del 50% alcanzaron esta fase el día 8): Emergen los pares de ganchos principales dorsal y ventral. Se observó un aumento de la longitud transversal del háptor.

- Fase III (días 7 a 8; n=2): Se caracterizó por la primera observación de la barra ventral.

- Fase IV (días 8 a 16; n=16, más del 50% alcanzaron esta fase el día 11): Emergencia de la barra dorsal.

- Fase V (días 9 al 16; n=20, más del 50% alcanzaron esta fase el día 14): Se observó cómo los lamelodiscos ventrales y dorsales iniciaron su desarrollo.

- Fase VI (desde el día 15 hasta el 22; n=23, más del 50% alcanzaron esta fase el día 15): Los especímenes que se observaron en esta fase correspondían a parásitos adultos, presentando el órgano copulador y las estructuras esclerotizadas con su tamaño y forma definitiva.

En el análisis de las muestras histológicas se observó que los especímenes correspondientes a la fase I utilizaban los 14 ganchos periféricos para anclarse a los tejidos del hospedador en superficies aplanadas del filamento primario o entre las laminillas secundarias. En las siguientes fases II, III y IV, que se caracterizaron por la aparición de los dos pares de ganchos y las barras ventral y dorsal, los parásitos se observaron anclados entre las laminillas secundarias mediante la acción de los ganchos principales que perforan los tejidos branquiales. En las últimas dos fases, V y VI, los especímenes desarrollan los lamelodiscos y el órgano copulador, por lo tanto se observó la acción de los lamelodiscos durante el proceso de anclaje de los parásitos adultos.

0.7.4 Discusión

Este estudio indica que el crecimiento total y parcial de *Lamellodiscus theroni* varía con el tiempo, dependiendo de las necesidades biológicas de cada periodo de vida del parásito.

Las post-larvas fase I fueron más grandes que las descritas en estudios previos (*L. elegans* 60-70 µm de longitud, Bychowsky, 1957). El desarrollo de las diferentes partes esclerotizadas del háptor de *L. theroni* siguió el mismo orden descrito previamente por Bychowsky (1957), sin embargo se observaron diferencias en los tiempos necesarios de cada estructura esclerotizada para alcanzar el tamaño y la forma definitiva. A diferencia de *L. elegans* (Bychowsky, 1957), los componentes de háptor de *L. theroni* no completan su desarrollo hasta la última fase descrita en este estudio. Estas diferencias podrían ser debidas a que los ganchos principales presentan tamaños y morfologías diferentes, siendo proporcionalmente mayores en *L. theroni*. Todas las piezas esclerotizadas del háptor fueron visibles en la fase V. Los lamelodiscos ventral y dorsal fueron observados por primera vez al mismo tiempo, de acuerdo con las observaciones de *L. elegans* hechas por Bychowsky (1957). El cuerpo de *L. theroni* creció relativamente despacio durante las primeras cinco fases, mientras que entre la fase V y VI experimentó el mayor incremento de longitud, una vez todas las piezas del háptor estuvieron presentes. Este tipo de crecimiento ya ha sido previamente

descrito en otros monogeneos (Repullés-Albelda *et al.*, 2011), invirtiendo los parásitos la mayor parte de su energía en desarrollar los órganos necesarios para garantizarse un anclaje estable y seguro. Una vez todas las estructuras de anclaje se han desarrollado, los parásitos ya pueden alimentarse, crecer y reproducirse.

El estudio histológico permitió identificar los diferentes tipos de anclaje llevados a cabo por las fases post-larvas de *L. theroni* durante el desarrollo. En la fase I, los ganchos marginales fueron observados como las únicas estructuras de anclaje, tratándose de un anclaje no selectivo que junto con el pequeño tamaño de los parásitos incrementa la diversidad de las áreas branquiales que pueden colonizar los monogeneos. Con el incremento de tamaño de *L. theroni* surge la necesidad de un anclaje más estable, que se consigue con el desarrollo de los pares de ganchos principales, las barras medias, las barras dorsales y los lamelodiscos dorsal y ventral en las fases II, III, IV, V y VI respectivamente. A partir de la fase II los parásitos se sitúan en los espacios interlamelares, entre dos laminillas secundarias branquiales, tal y como fue descrito en estudios anteriores por Sánchez-García *et al.* (2011).

El grado de lesiones causadas por los monogeneos monopistocotileos depende del tamaño y de la cantidad de ganchos. En el presente trabajo, se observó que las primeras fases post-larvas se anclan mediante los ganchos accesorios de menor tamaño que no han sido relacionados con lesiones aparentes, mientras que los ganchos principales presentes en los adultos pueden llegar a penetrar profundamente en el epitelio incrementando el número y la seriedad de la lesiones (Sánchez-García *et al.*, 2011). Por lo tanto, durante el último periodo de la infección, los daños causados en las branquias empeoran. En las granjas, el hacinamiento de peces incrementa las cargas parasitarias (Dezfuli *et al.*, 2007; Katharios *et al.*, 2006; Mladineo, 2007; Toksen, 2006; Sánchez-García *et al.*, 2013) y los peces son parasitados por monogenos correspondientes a diferentes fases larvarias con diferentes estrategias de anclaje y preferencias de hábitat, afectando áreas de las branquias más extensas.

El conocimiento del desarrollo parasitario ayuda a establecer los intervalos necesarios durante los cuales se deben administrar los tratamientos antiparasitarios

(Repullés-Albelda *et al.*, 2012). En *L. theroni* la segunda generación de parásitos aparece 20 días después del periodo infectivo, por lo que un tratamiento basado en dos dosis separadas por un mínimo de 4 días podría resultar óptimo para la desparasitación de los peces.

0.8 Comparación del desarrollo post-larvario de las especies de *Lamellodiscus*. Descripción del desarrollo de *Lamellodiscus falcus*

0.8.1 Introducción

El número de estudios relacionado con el desarrollo de los parásitos del género *Lamellodiscus* es muy escaso. Bychowsky (1966) estudió el desarrollo de *Lamellodsicus elegans* Bychowsky 1957, y más recientemente Sánchez-García *et al.* (2014b) siguió el desarrollo cronológico de las diferentes estructuras esclerotizadas de *L. theroni* Amine, Euzet et Kechemir-Issad 2007, parásito del sargo picudo *Diplodus puntazzo*. Los autores de los estudios anteriores encontraron diferencias en los momentos de desarrollo de las piezas esclerotizadas de *L. elegans* y *L. theroni*, debidas posiblemente a las diferencias morfológicas de los parásitos adultos.

Clasificaciones previas del género *Lamellodiscus* spp. dividen las especies en grupos en función de la composición del háptor, de este modo las 8 especies presentes en *D. puntazzo* se clasifican en dos tipos morfológicos (Amine y Euzet, 2005): *L. bidens* Euzet 1984, *L. elegans*, *L. falcus* Amine, Euzet et Kechemir-Issad 2006, *L. hilii* Euzet 1984, *L. ignoratus* Palombi 1943 y *L. impervius* Euzet 1984 donde cada barra dorsal del háptor está formada por una única pieza; a diferencia de *L. ergensi* y *L. theroni* donde cada barra dorsal del háptor está formada por dos piezas. Estas diferencias morfológicas implican a su vez diferencias en el anclaje y consecuentemente, diferencias en otros aspectos ecológicos y patológicos (Sánchez-García *et al.*, 2011), o incluso en el desarrollo (Sánchez-García *et al.* 2014b).

En este trabajo se estudia el desarrollo de una tercera especie de *Lamellodiscus* spp., *L. falcus* en infecciones experimentales de *D. puntazzo* para recoger y comparar con los estudios anteriores los cambios morfológicos que se producen durante el desarrollo en el tiempo.

0.8.2 Materiales y Métodos

Cuarenta sargos picudos infectados por *Lamellodiscus falcus* capturados en el Mar Menor (Murcia) se trasladaron a las instalaciones de la Universidad de Valencia. Estos peces actuaron como donantes de parásitos para infectar a un total de 120 peces sanos, procedentes de Grecia. La infección se realizó mediante 48h de cohabitación con los peces donantes. Después del periodo de infección, los peces se transfirieron a los tanques experimentales y este día se determinó como el "día 0" del experimento. Desde este momento se sacrificaron 5 peces diariamente durante 22 días. Se recolectaron los parásitos y se guardaron branquias para realizar análisis histológicos.

Para el análisis morfológico de los especímenes, los monogeneos se montaron en preparaciones semipermanentes en glicerogelatina y se estudiaron mediante microscopia óptica. Los análisis histológicos se llevaron a cabo mediante el análisis de secciones de tejido teñido con hematoxilina-eosina.

0.8.3 Resultados

- Fase I (días 1 al 12; n=8): Estadio post-larval. Como únicas estructuras de anclaje los especímenes observados de *L. falcus* presentaban 14 ganchos marginales dispuestos circularmente en el háptor. Los ganchos marginales presentaron forma de pequeños garfios.

- Fase II (días 5 al 15; n= 12, más del 50% alcanzan esta fase el día 9): Emergen los pares de ganchos principales dorsal y ventral.

- Fase III (días 7 al 8; n= 1): Se caracterizó por la observación de la barra ventral.

- Fase IV (días 8 al 16; n= 11, más del 50% alcanzan esta fase el día 14): Emergencia de la barra dorsal.

- Fase V (días 9 al 16; n=3, más del 50% alcanzan esta fase el día 14): Se observó cómo los lamelodiscos ventrales y dorsales iniciaban su desarrollo.

- Fase VI (desde el día 14; n= 12, más del 50% alcanzan esta fase el día 15): Los especímenes que se observaron en esta fase correspondían a parásitos adultos, presentando el órgano copulador y las estructuras esclerotizadas con su tamaño y forma definitiva.

En el análisis de las muestras histológicas se observó que los especímenes correspondientes a la fase I se localizaron principalmente en las zonas más basales de los filamentos branquiales, utilizando los 14 ganchos periféricos para anclarse a los tejidos del hospedador en superficies planas. En las siguientes fases II, III y IV, con la aparición de los dos pares de ganchos principales y las barras ventral y dorsal, los parásitos se observaron anclados entre las laminillas secundarias mediante la perforación de los tejidos branquiales. En las últimas dos fases, V y VI, los monogeneos desarrollaron los lamelodiscos que intervienen en el proceso de anclaje empujando las laminillas branquiales secundarias pinzadas por los ganchos principales.

0.8.4 Discusión

El presente trabajo demuestra que en *L. falcus* el crecimiento total y parcial varía con el tiempo, en función de las demandas biológicas de cada periodo de vida de los parásitos. El desarrollo de las diferentes piezas esclerotizadas sigue la misma secuencia que *L. theroni* y *L. elegans* (Bychowsky, 1961; Sánchez-García *et al.*, 2014b). Sin embargo, algunas diferencias fueron descritas en relación a los ritmos de desarrollo. La composición final y la disposición de las piezas del háptor pueden determinar el desarrollo de las especies de *Lamellodiscus* spp. Por ejemplo, y de acuerdo con la clasificación hecha por Amine y Euzet (2005) *L. theroni* corresponde al grupo de *Lamellodiscus* spp. que posee las barras dorsales formadas por dos piezas, mientras que en *L. falcus* y *L. elegans* sólo están formadas por una

pieza. *Lamellodiscus falcus* desarrolla todas sus estructuras y alcanza su tamaño definitivo un día antes que *L. theroni*, quizás en este caso la velocidad de crecimiento viene determinada por el tamaño final de los componentes del háptor (de mayor envergadura en *L. theroni*).

El tamaño de las primeras post-larvas estudiadas de *L. falcus* fue muy similar que las de *L. theroni* (Sánchez-García *et al.*, 2014b) y fue más del doble que *L. elegans* descritas por Bychowsky (1961). En Sánchez-García *et al.* (2014b) se observó que los ganchos ventrales de *L. theroni* eran más grandes que los dorsales desde el principio de su desarrollo, sin embargo en *L. falcus* al igual que en *L. elegans* (Bychowsky, 1961), ambos pares de ganchos principales presentaban un tamaño similar en sus fases iniciales. Al igual que *L. elegans*, los dos pares de ganchos principales de *L. falcus* alcanzan su tamaño definitivo en la última fase del desarrollo (fase VI) (Sánchez-García *et al.*, 2014b). Sin embargo, mientras que en *L. theroni* los ganchos principales no adquieren su morfología adulta hasta la fase VI (Sánchez-García *et al.*, 2014b) en *L. falcus* en la fase III ya se observan las protuberancias características de la especie. Esto es similar a lo que ocurre con *L. elegans*, cuyos ganchos principales mostraron la forma definitiva antes de que los lamelodiscos fueran detectados (fase IV) (Bychowsky, 1961). El mayor tamaño y robustez de los ganchos de *L. theroni* podría necesitar un mayor tiempo de esclerotización para alcanzar su longitud y forma definitiva. Respecto al incremento de la longitud total de *L. theroni* y *L. falcus* durante el desarrollo, *L. falcus* creció desde la fase I, mientras que *L. theroni* no experimentó un incremento de longitud hasta la fase II, pudiendo ser debido a que los especímenes adultos de *L. falcus* presentan una mayor longitud media.

La similitud en la secuencia de desarrollo entre *L. theroni* y *L. falcus* implica que las diferentes estrategias de anclaje a lo largo del desarrollo sean también similares, y así lo confirma el estudio histológico. Por lo tanto podemos identificar tres fases de anclaje durante el desarrollo en *L falcus: i)* primeras post-larvas ancladas a superficies planas con los 14 ganchos marginales; *ii)* fases de la II a la IV, post-larvas ancladas en los espacios interlamelares de las laminillas branquiales secundarias, los ganchos principales pinzan el tejido branquial; *iii)* fases V y VI, el

desarrollo de los lamelodiscos confiere al anclaje del parásito una mayor estabilidad y fortaleza.

Para concluir, aunque las estrategias de anclaje de los diferentes estados de desarrollo de *Lamellodiscus* spp. son similares, algunas diferencias pueden detectarse relacionadas con la diferente composición y dimensiones de los escleritos del háptor de los adultos de cada especie. El tiempo entre las diferentes fases parece ser más variable, siendo las especies mayores las que requieren de mayores tiempos de desarrollo.

0.9 Conclusiones

1. La fauna parásita de *Diplodus puntazzo* en el Mediterráneo español está compuesta por 19 especies parásitas. Siete de las especies identificadas en este trabajo han sido citadas por primera vez en *D. puntazzo*: *Microcotyle* sp., *Magnibursatus bartolii*, *Steringotrema pagelli*, *Galactosomum* sp., *Cardiocephaloides longicollis*, *Caligus ligusticus* y *Gnathia vorax*. Dos especies son nuevos hallazgos en peces salvajes que sólo habían sido registrados en granjas; los monogeneos *Atrispinum seminalis* y *Sparicotyle chrysophrii*.

2. Once de las especies parásitas encontradas fueron monoxenas, de fácil dispersión en condiciones de cautiverio y elevada densidad de peces: 8 monogeneos y 3 crustáceos. Entre las especies heteroxenas encontradas hubieron 7 trematodos y 1 especie de mixozoo.

3. Los especímenes de monogeneos del género *Lamellodiscus* encontradas en el presente presentan los rasgos taxonómicos de las especies *L. theroni* y *L. falcus*, sin embargo, sus dimensiones son más similares a las especies *L. ergensi* y *L. ignoratus* respectivamente.

4. Los estudios moleculares de las especies *Lamellodiscus falcus sensu lato* y *L. theroni sensu lato* mostraron divergencias intraespecíficas en el gen ITS1, sin embargo esta variación se encuentra dentro del rango de las variaciones

intraespecíficas indicadas en estudios previos para las especies de *L. ergensi* y *L. ignoratus*. Este tipo de divergencias moleculares en los especímenes de *Lamellodiscus* spp. no refuerza la separación de las especies a pesar de sus diferencias morfológicas.

5. Los especímenes procedentes del Mediterráneo español analizados del trematodo *Peracreadium characis* aumentan el rango de medidas característico para cada órgano, ya que las dimensiones de los individuos analizados en el presente trabajo fueron menores que las descritas anteriormente. Los estudios moleculares confirman que se trata de diferencias intraespecíficas.

6. La mayoría de las especies parásitas de la piel, branquias y sistema digestivo desaparecen en condiciones de cautividad, a excepción de los monogeneos del género *Lamellodiscus*. Sin embargo, las especies parásitas del medio interno sobreviven, como es el caso de *Cardiocephaloides longicollis* y *Ceratomyxa* sp.

7. Tres especies parásitas representan un riesgo "Alto" para los cultivos del *Diplodus puntazzo* en el Mediterráneo español: *Amyloodinium* sp., *Cryptocaryon* sp. y *Enteromyxum leei*. Adicionalmente, otras tres especies representan un riesgo "Moderado": *Sparicotyle chrisophrii*, *Caligus ligusticus* y *Gnathia vorax*. La mayor parte de las especies parásitas que representan un riesgo importante para el cultivo de *D. puntazzo* han sido previamente identificadas en cultivos de *Sparus aurata*. Es importante tener en cuenta las posibles infecciones cruzadas en los cultivos de ambas especies.

8. Los lamelodiscos de *Lamellodiscus falcus* y *L. theroni* están formados por láminas esclerotizadas superpuestas. Contrariamente a lo que las publicaciones previas indican, estos lamelodiscos no actúan como una ventosa. Cada lámina se desplaza como una única pieza describiendo un movimiento telescópico que produce un empuje de los epitelios branquiales y la subsiguiente separación de las laminillas branquiales secundarias. La fuerza de empuje ejercida por el lamelodisco durante el anclaje es contraria a la fuerza de tracción que ejercen los ganchos principales. De este modo se consigue un anclaje estable y eficiente para los parásitos.

9. *Furnestinia echeneis* posee un único lamelodisco de tipo "dividido", cada una de las mitades de las láminas esclerotizadas se desplaza independientemente, hecho que les permite deslizarse entre sí postero-lateralmente. El lamelodisco de *F. echeneis* es demasiado grande para introducirse en el espacio que existe entre las laminillas branquiales secundarias, por lo que se ancla como una ventosa en zonas de superficie amplia y lisa del filamento branquial. El lamelodisco único actúa cómo una bomba creando un vacío y fijando el monogeneo al tejido branquial. Los dos pares de ganchos ventrales y dorsales actúan como elementos secundarios en el anclaje.

10. El háptor de *Diplectanum aequans* penetra en el interior del espacio interlamelar y establece un contacto íntimo con el tejido branquial del hospedador. Los ganchos y las espinas que forman el háptor, producen alteraciones extensas en el tejido epitelial en el lugar de anclaje y en los alrededores. La hiperplasia, edema y reacción inflamatoria del epitelio atrapan pasivamente el háptor del parásito, impidiendo que se libere.

11. Los diferentes mecanismos de anclaje de los diplectánidos explican las diferentes patologías que provocan. El mecanismo de anclaje de *D. aequans* implica severas alteraciones en el epitelio branquial con inflamaciones que rodean y sustentan al háptor. *Diplectanum aequans* y *Lamellodiscus* spp. se anclan en las laminillas secundarias, causando daños y afectando la función respiratoria debido a la hiperplasia, la fusión lamelar y el daño causado a las estructuras vasculares. Por el contrario, *F. echeneis* al anclarse fuera de las laminillas secundarias, no afecta a las estructuras respiratorias y sólo causa daños leves relacionados con las perforaciones superficiales causadas por los ganchos.

12. La información dada acerca del anclaje de las diferentes especies de diplectánidos es útil para diseñar tratamientos orientados efectivos: el anclaje *D. aequans* es pasivo, por lo que los parásitos no se desprenden fácilmente, con lo que sólo tratamientos agresivos y parasiticidas pueden resultar efectivos. El anclaje de *F. echeneis* y *Lamellodiscus* spp. es total o parcialmente activo, por lo que pueden desprenderse más fácilmente con tratamientos con productos antihelmínticos, como los que tienen efectos espasmódicos sobre los parásitos.

13. Durante el desarrollo postlarvario de las especies *Lamellodiscus theroni* y *L. falcus* han sido identificadas seis fases de desarrollo, en función del desarrollo de las piezas esclerotizadas que forman el háptor y el órgano copulador: I, con 14 ganchos periféricos; II, con ganchos dorsales y ventrales; III y IV, con barras ventral y dorsales, respectivamente; V, con lamelodiscos dorsales y ventrales; y VI, con órgano copulador desarrollado.

14. Tres fases resultan cruciales para la estrategia de anclaje de los parásitos durante el desarrollo postlarval de las especies de *Lamellodiscus,*: fase I, anclaje realizado únicamente por los 14 ganchos marginales, que permiten al parásito anclarse sobre superficies planas, con mayor capacidad de desplazamiento aunque menos estables; fase II, el uso de los ganchos dorsales y ventrales confiere menor movilidad al tiempo que anclan con mayor firmeza a las laminillas secundarias en el espacio interlamelar; y por último, en la fase V, el desarrollo de los lamelodisco implica la presencia de una nueva estructura de anclaje que ejerce una fuerza contraria a la de los ganchos principales incrementando la eficiencia del anclaje.

15. A pesar de las diferencias entre los háptores de *L. falcus*, *L. theroni* y *L. elegans*, las tres especies siguen un patrón de desarrollo muy similar. La similitud en la secuencia de desarrollo de los escleritos del háptor en *L. falcus* y *L. theroni* implica que la secuencia ontogenética de estrategias de anclaje serán también similares. Se encontraron algunas diferencias en relación a los ritmos de desarrollo de algunos escleritos aparentemente relacionadas con las dimensiones definitivas específicas de cada especie.

CHAPTER 1

General introduction

In the last half century, world aquaculture has experienced a remarkable increase, related to the decrease of capture production. In 2010 the aquaculture contribution to world fish production was 40.3%, compared to 20.9% in 1995. Global production of farmed food fish was 59.9 million tonnes in 2010, increasing by 7.5% compared to 2009 (55.7 million tonnes)[Food and Agriculture Organization of the United Nations (FAO), 2012]. Europe produced in 2010 a total of 1.2 million tonnes fish, specifically Spain producing the 20% of the total European production, being thus the first producer in the European Union (EU) [Asociación Empresarial de Productores de Cultivos Marinos de España (APROMAR), 2012].

The third main fish species produced in the EU aquaculture is the gilthead seabream (*Sparus aurata* L.), with 87.596 tonnes in 2010. This species, together with the European seabass (*Dicentrarchus labrax* L.), constitutes the bulk of the Mediterranean aquaculture production (APROMAR, 2012). Both species have also experienced a big increase in their production in Spain, until 2009 when the Spanish Economic crisis affected the aquaculture sector (APROMAR, 2011). Currently, the Spanish aquaculture is contracted or stagnated and, in 2010, the production suffered a reduction of 9.4% compared to the previous year (APROMAR, 2011). Decrease has occurred in all important cultured species [also including others than the one of the turbot *Scophthalmus maximus* (L.)], meaning the first reduction in the last 25 years. APROMAR (2011) pointed to a loss of competitiveness, and identified endogenous challenges to be more competitive in the European market, as diversification of production, innovation and increase in R&D investment. Therefore, two of the main objectives of the Mediterranean aquaculture is the species diversification as well as to enhance the traditional fish culture with technological research. Nowadays there is a tendency to develop new farm species in order to spread the aquaculture industry, expand the markets and develop new cultures [Junta Asesora de Cultivos Marinos (JACUMAR), 2011]. In this way, providing solutions for an adequate culture of well-established and new fish species becomes crucial.

Concerning species diversification in the Mediterranean aquaculture, the sparid sharpsnout seabream *Diplodus puntazzo* (Walbaum 1792) has been considered a good candidate to be introduced in Spanish cultures. Features and growth patterns of this species are similar to those of other intensively cultured sparids with great acceptance in markets, as the gilthead seabream *S. aurata* (Franicevic, 1989; Caggiano *et al.*, 1993; Abellán *et al.*, 1994). Previous experiences of culture of *D. puntazzo* in inland farms and sea facilities usually have satisfactory results (Bermúdez *et al.*, 1989; Divanach *et al.*, 1993; Abellán and García Alcázar, 1995). Studies on larval development have been carried out in Spain to evaluate the feasibility of this culture, showing promising results (Nogales-Mérida *et al.*, 2010, 2011; García and García, 2010; García *et al.*, 2011).

The species diversification entails a consequent R&D challenge, the knowledge about the pathogens that could compromise the farming of these "new species". In general, the intensive aquaculture involves the increase of frequency and intensity of fish diseases (Woo *et al.*, 1995; Karagouni *et al.*, 2005; Fioravanti *et al.*, 2006; Palenzuela, 2006). Despite breeding systems have become more controlled and the impact by diseases has been reduced, knowing the particular causes of fish outbreaks and understanding the host-pathogen interactions is essential, especially in new cultures. The culture of new species has provoked two main problems: i) the transmission of the endemic pathogens to the new fish species cultured, compromising their farming and ii) the introduction of new pathogens from this new fish species which could jeopardize the existent cultures (Woo *et al.*, 1995). Hence, to succeed in this fish culture and to prevent endangering the consolidated productions it is necessary to establish the potential pathogens of newly introduced fish species.

The knowledge of the diversity of parasites that can parasitize a particular fish species and knowing their pathogenic potential is essential to predict possible future diseases. Some of the main parasitic problems in Mediterranean aquaculture have been related to monogeneans, ectoparasite platyhelminths commonly found in gills and skin of fish (Sitjà-Bobadilla *et al.,* 2006). Monogeneans are parasites with complex attachment systems, mostly based on hooks and clamps structures

that imply gill tissue damages, dealing in respiratory malfunction, secondary infections or/and anemia. Most serious monogenosis have been related to the monopisthocotylean *Diplectanum aequans* (Wagener 1857) in *D. labrax* and the polyopisthocotylean *Sparicotyle chrisophrii* (Van Beneden et Hesse 1863) in *S. aurata*. Other monogeneans apparently more harmless are also very frequently detected, as the monopisthoctyleans *Lamellodiscus* spp. and *Furnestinia echeneis* (Euzet et Audouin 1959). These monogeneans are not usually related to severe pathologies, but their role as synergic pathogens needs further study. We consider indispensable the morphological study of the haptor of monogenean species parasitizing the *D. puntazzo* in the Mediterranean, as *Lamellodiscus* spp., including the attachment strategies and the pathological consequences, as well as their development. This knowledge will help to understand the pathologies and also to design adequate treatments and management protocols for controlling monogeneans in the aquaculture facilities.

This introductory section includes the main features of *D. puntazzo* as well as a summary of the previous knowledge of its parasite fauna. Moreover, information about the main parasite group studied, the monogeneans, is also included.

1.1 Morphological and biological features of *Diplodus puntazzo* (Walbaum 1792, Sparidae)

The sharpsnout seabream is a sparid perciform teleost very common in the Mediterranean. The family Sparidae includes about 115 species of mainly marine coastal fish of high economic value, exploited and farmed for human consumption as well as on recreational purposes (Pavlidis and Mylonas, 2011). *Diplodus puntazzo* is a gregarious fish with an Eastern-Atlantic distribution, from the African Congo region to the Bay of Biscay in Spain, going deep into the Mediterranean, where it is commonly found (Fig. 1.1).

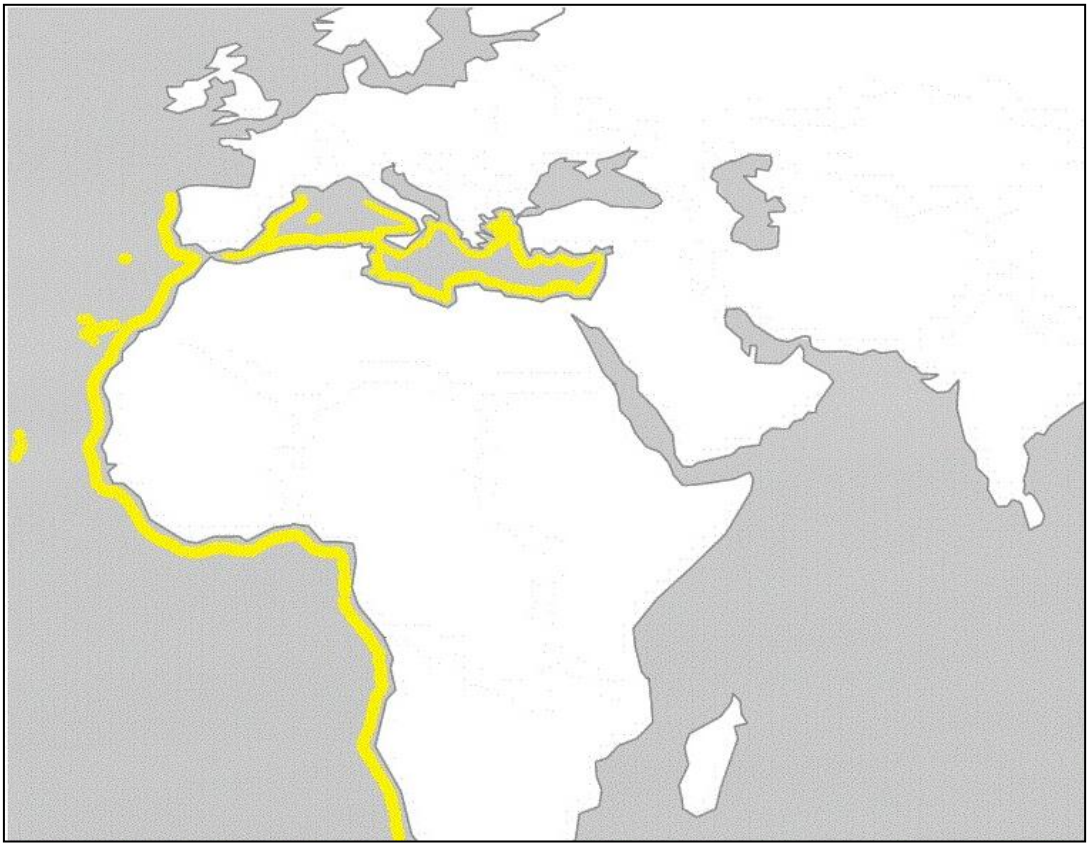

Figure 1.1 Distribution of the sharpsnout seabream *Diplodus puntazzo*. Obtained from Froese and Pauly (2007).

This species presents the typical morphology of the sparids (Fig 1.2): body oval and laterally flattened, with superior head profile markedly rounded. The specific feature to differentiate this species within the genus *Diplodus* Rafinesque, 1810 is a pointed and long snout ending in a small mouth, frontally orientated and located in the lower half body. It shows a grey silver characteristic coloration, with dark and bright transversal bands, and a big saddle form black spot in the tail axis. The distal extremes of the dorsal and anal fins radius and caudal fin posterior edge show a black edging. The maximum length of *D. puntazzo* is 45 cm (Froese and Pauly, 2007).

Diplodus puntazzo habitat changes from pelagic to benthic along their development. Young specimens can be found in brackish waters and may live in littoral pools, while the adults occur in coastal waters on rocky or sandy bottoms,

up to 150 m (only occasionally over 50 m) (Froese and Pauly, 2007). *Diplodus puntazzo* specimens are omnivorous and their diet mainly consists of algae and sponges (Sala and Ballesteros, 1997); their sharp teeth pull off algae and molar ones grind the crustaceans, snails and molluscs located among the plants (Bauchot and Hureau, 1986).

Figure 1.2 Sharpsnout seabream *Diplodus puntazzo* (Cetti, 1777). (Extracted from http://delvaneo.ru)

This is a permanent hermaphrodite species with some protandric episodes. The breeding period goes from September to October. Reproduction takes place in deep waters (Divanach, 1985). Eggs and larvae remain in the plankton (egg size 0.85 mm, larval length at hatching 1.7 mm) and, after a month of larval life, they settle in very shallow benthic habitats, where they stay for several months in distinct nursery areas under a high degree of site-fidelity. When the juveniles reach a length between 4.5 and 5.5 cm, they leave the nursery areas spreading to join the adult population; nevertheless, this settlement intensity to near-shore habitats in the northwest Mediterranean, exhibits high annual variations at both local and regional scales in all *Diplodus* species (Macpherson, 1998). During the first period of larval development and settlement, juveniles formed small monoespecific shoals, exhibiting markedly clumped distribution, and as the individuals grow, the shoals

gradually become fragmented. In contrast to other sparids, *D. puntazzo* adults use to be solitaire and did not join to form big shoals (Pavlidis and Mylonas, 2011).

1.2 *Diplodus puntazzo* parasite fauna

In spite of the potential importance of *D. puntazzo* for the Mediterranean aquaculture, there are few studies referred to its parasites (Álvarez-Pellitero *et al.*, 2008; Athanassopoulou *et al.*, 1999; Athanassopoulou et al., 2005; Di Cave *et al.*, 2002; Katharios el al., 2006; Merella *et al.*, 2005; Madlineo and Maršić-Lučić, 2007; Montero *et al.*, 2007; Toksen, 2006; Vagianou *et al.*, 2004). Most of them are monospecific parasite studies or based on a particular taxonomic group, with special interest on those pathogens of farmed *D. puntazzo*.

Studies to date have been cited a total of 37 parasites species of Mediterranean *D. puntazzo* (see Table 1 in Chapter 5): 5 protozoan species, 5 myxozoans, 8 trematodes, 13 monogeneans, 5 copepods and 1 isopod. Monogenean species are the most numerous and abundant, with several publications. Something similar happens in myxozoans (Athanassapoulou *et al.*, 1999; Katharios *et al.*, 2006; Toksen, 2006; Álvarez-Pellitero and Sitjà-Bobadilla, 1993; Amine *et al.*, 2007; Montero *et al.*, 2007; Muñoz *et al.*, 2007). Both groups (myxozoans and monogeneans) are important in farmed Mediterranean sparids as their associated pathologies are quite well-known. The myxozoan *Enteromyxum leei* (Diamant, Lom et Dyková 1994), an intestinal parasite related with serious outbreaks in Mediterranean farms (Álvarez-Pellitero *et al.*, 2008; Davey *et al.*, 2011); and the monogenean *Sparicotyle chrysophrii* (Van Beneden et Hesse 1863), a gill parasite related with trickled mortalities, anaemia and bad health fish conditions aquaculture facilities (Faisal, 1990; Sanz, 1992; Di Cave, 2003; Athanassopoulou *et al.* 2005).

Interestingly, most of the parasites cited in *D. puntazzo* have been previously cited in cultures of *S. aurata*, thus meaning that exist an elevated risk of rapid and successful switching of parasites between the two sympatric and phylogenetically related species (Madlineo and Maršič-Lučić, 2007). Therefore, this

situation could result in severe infestations and possible losses in the culture of new hosts and also in well-established cultures, as the ones of *S. aurata*.

1.3 The Monogenea Carus 1863

(Summarized and modified from Yamaguti, 1963 and Rohde, 2005)

The class Monogenea belongs to Phylum Platyhelminthes. Monogeneans are hermaphroditic flatworm parasites of aquatic or amphibious vertebrates, occasionally of aquatic invertebrates. They are typically ectoparasites of skin and gills, feeding on mucus, epithelial cells and blood. Most species have a high degree of host specificity, i.e. they infect only a single host species or few related ones. Effects on their hosts are usually insignificant, mostly in the wild, where monogenean loads are not extremely high. However, occasionally important mortalities caused by monogeneans are reported, particularly in aquaculture. For this reason, the economic importance of this group is high, and a considerable amount of work has been devoted to its study.

Monogeneans are divided in two major groups: the Polyopisthocotylea Odhner 1912 and the Monopisthocotylea Odhner 1912, that can be easily distinguished by the structure of the posterior attachment organ, the haptor. The haptor of the Polyopisthocotylea is complex, consisting in a number of suckers or clamps supported by sclerites. The haptor of the Monopisthocotylea is formed by either of a large sucker bearing various types of hooks, or entirely of small and large hooks.

Monogeneans are thus characterized by the haptor, a unique adhesive organ. During their development the early haptor develops from a more or less discoid, muscular organ to large suckers, or more often, to more complex organs which may be subdivided, provided with hooks, clamps or suckers, sometimes on digitiform tentacles. The haptor may be symmetrical or asymmetrical, sessile or pedunculated. The mouth is terminal or sub terminal, often surrounded by the anterior adhesive organs, which can be formed by paired or unpaired suckers, occasionally with glandular structures. One or two pairs of eyes are present in the

larvae, and often disappear during the parasite's development. The excretory system is paired, opening dorsally to two symmetrical pores near the anterior end. The digestive system includes a strong pharynx, followed by caeca, usually bifurcated and ramified. Testes can consist of one or sometimes numerous follicles, forming a closely packed mass or divided into groups. In contrast, the ovary is single, globular or tubular (last type often referred as germarium). Male and female genital pores are usually common.

Monogeneans have direct life-cycles, i.e. without intermediate hosts. The adult worm, infecting a vertebrate host, lays its eggs from which ciliated larvae (oncomiracidia) will hatch and freely swim in water to infect other vertebrates, usually of the same species (see Fig. 1.3). Each egg develops to a single adult worm, but an adult produces many eggs during its life, resulting in a very effective transmission way.

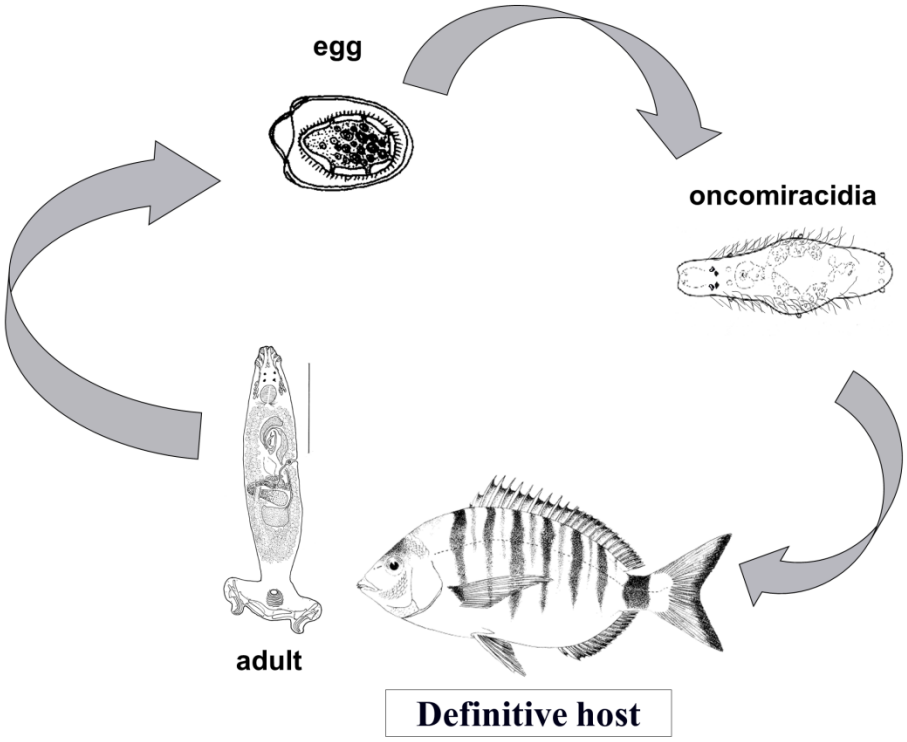

Figure 1.3 Direct life cycle of a monogenea, *Lamellodiscus theroni*, parasitizing *Diplodus puntazzo* gills.

The taxonomy of monogeneans, from subclasses to species, is mainly based on the morphology of the haptor. This organ is essential for parasite attachment, developing different attachment strategies and, subsequently their habitat, the pathologic mechanisms and, the expected response to the different available treatments. Moreover, in spite of their apparently simple direct life cycle, along monogenean development, the parasites suffer deep morphological changes, reflected in different post-larval stages with different haptor structures and functionality. These changes imply different habitat selection and also different pathologies in the host tissues during the development that must be studied.

This thesis has been mainly focused on a family of monopisthocotylean monogeneans, the Diplectanidae Monticelli 1903. Next section summarizes the biological features of this family with several species infecting *D. puntazzo*.

1.3.1 Monopisthocotylea: Family Diplectanidae Monticelli 1903

The Diplectanidae Monticelli 1903 is represented by approximately 250 species occurring primarily on the gills of marine perciform fish. Diplectanidae species are historically diagnosed by the combination of the following characters: an attachment structure formed by accessory adhesive organs (squamodiscs or lamellodiscs) and three transversal bars connected to two pairs of hooks (dorsal, ventral); pretesticular germarium; and looping right intestinal caecum. The morphology of the sclerites of the lamellodisc/squamodisc largely varies among the diplectanid genera, and subsequently its role in the attachment (Domingues and Boeguer, 2008).

In the Spanish Mediterranean, the genera *Diplectanum* Diesing 1858, *Furnestinia* Euzet et Audouin 1959 and *Lamellodiscus* are the most important diplectanid parasites of wild and farmed fish species. *Lamellodiscus* spp. are parasites of sparid fish as *S. aurata* or *Diplodus* spp., while *Furnestinia echeneis* is specific of *S. aurata* (Antonelli *et al.*, 2010). *Diplectanum aequans* parasitize the gills of *D. labrax* (González-Lanza *et al.*, 1991), the second most important species for the Spanish aquaculture. Previous studies have indicated the presence of pathologies related to *D. aequans* and *Lamellodiscus* spp. in *D. labrax* and *D.*

puntazzo respectively (Oliver, 1977; Dezfuli *et al.*, 2007; Katharios *et al.*, 2006). In contrast, no important pathologies have been related with the presence of *F. echeneis* in *S. aurata* cultures (Antonelli *et al.*, 2010). Pathological differences should be related to the diverse haptor structures and consequently, to their attachment system. Haptor and attachment strategies of *D. aequans* have been previously studied (Dezfuli *et al.*, 2007; González-Lanza *et al.*, 1991), however several aspects remain unknown. The attachment and pathologies related of *Lamellodiscus* spp. and *F. echeneis* have not been described in detail to date.

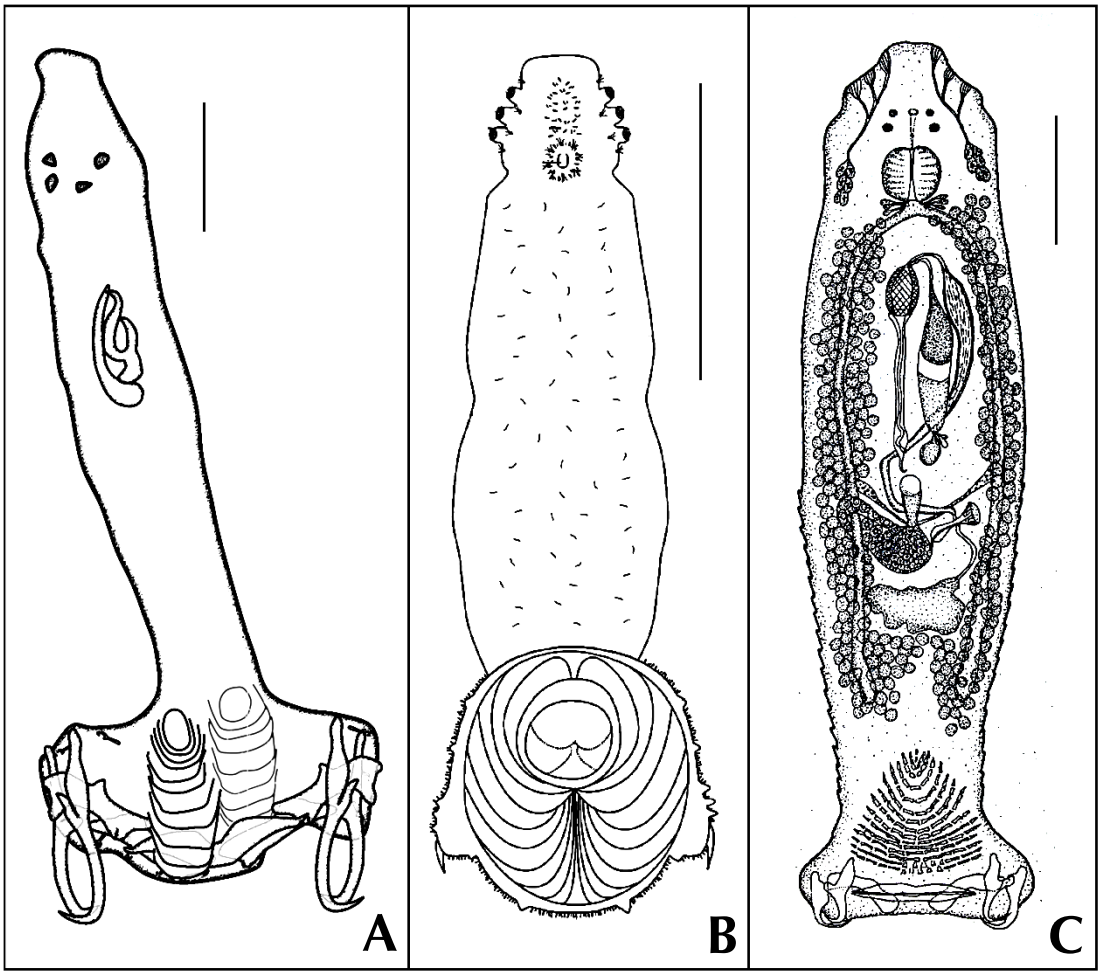

Figure 1.4 A, *Lamellodiscus falcus,* parasite of *Diplodus puntazzo*. B, *Furnestinia echeneis,* parasite of *Sparus aurata*. C, *Diplectanum aequans,* parasite of *Dicentrarchus labrax*. Scale bar 100µm. Drawings of *F. echeneis* and *D. aequans* extracted from Antonelli *et al.,* 2010 and Radujkovic and Euzet, 1989, respectively.

1.4 This study

This study has been carried out within the framework of three projects, two of them dedicated to the study of the diseases affecting *Diplodus puntazzo* as a new candidate for aquaculture: i) 'Parasite pathogens in new species of Mediterranean aquaculture: an experimental approach' (funded by the European Union) and ii) 'Parasite pathogens of the sharpsnout seabream: transmission to the gilthead seabream and risks' (funded by Spanish Government). A third project is dedicated to the study of the diseases of all fish of commercial interest in the Valencian Community: iii) '*REVIDPAQUA,* Valencian network of research and development of pathologies in aquaculture' (funded by Valencian Government).

The aim of the first project was to determine the pathogenic species infecting *D. puntazzo* in the Mediterranean. To attain this purpose, a parasite taxonomical dataset from Mediterranean has been compiled, and their potential pathogenic effects have been evaluated. Moreover the description of the development of monogenean parasites recurrently found in cultures has been experimentally completed. In the frame of the second project, novel risk assessment analyses for *D. puntazzo* Mediterranean Spanish cultures have been completed, considering all cited parasites in the Mediterranean and their related pathologies. *Sparus aurata* parasites have also been considered due the closeness of their cages in aquaculture facilities and their phylogenetic proximity. Finally, the third project was focused on the obtaining of biological data on parasites causing losses in cultures of sparids (gilthead and sharpsnout seabreams) in order to avoid crossed infections and to develop efficient treatments against them. For this purpose, the biological features of parasites shared by these two host species and the relationships between host and parasites have been analysed.

This thesis is devoted to the study of the parasites of concern for *D. puntazzo* cultures and the pathological effects of diplectanid monogeneans recurrent in Mediterranean cultures by addressing the following questions:

(i) What species constitute the parasite community of *Diplodus puntazzo* in the Mediterranean?

(ii) Which are the potentially pathogenic parasite species for *Diplodus puntazzo* cultures in the Spanish Mediterranean? Thus, which are the moderate to high risky parasites for *D. puntazzo* Mediterranean Spanish cultures?

(iii) How are the attachment systems and the pathologies associated to the different diplectanid species?

(iv) What is the chronology of development of two species of *Lamellodiscus* (*L. theroni* and *L. falcus*), parasites of *Diplodus puntazzo*? How do attachment and damage change along the parasite's development?

CHAPTER 2

Aim and objectives

AIM

The aim of the present study is two-fold: I) providing novel data on the parasitic fauna of *Diplodus puntazzo* (Walbaum 1792) in the Spanish Mediterranean, in order to evaluate the effects in culture conditions and to suggest preventive practices in aquaculture facilities; and II) describing the morphological features of the haptor and the attachment mechanisms of diplectanid monogeneans, as well as the connotations in the biology of parasites and related damage.

OBJECTIVES

The following objectives have been undertaken:

1. To identify the parasitie fauna of wild and farmed *D. puntazzo* in the Spanish Mediterranean.

2. To report a risk assessment of protists and metazoan parasites of *D. puntazzo* from the Spanish Mediterranean, reporting which parasites species could compromise the culture of this fish species.

3. To compare the attachment of diplectanid monogeneans, gill parasites of cultured fish, and the related pathological effects, based on detailed morphological and histophatological analyses.

4. To describe and compare the post-larval morphological changes of two species of diplectanid monogeneans (*Lamellodiscus theroni* and *L. falcus*) parasitizing *D. puntazzo*.

CHAPTER 3

General Materials and Methods

3.1 Fish sampling

To achieve the objectives of the present thesis, parasites of Mediterranean specimens of sharpsnout seabream *Diplodus puntazzo* were studied. Some specimens of gilt-head seabream *Sparus aurata* were also obtained for additional studies and comparisons.

Depending on the study (see sections 3.2 to 3.4), fish were collected from extractive fishing or captured alive. For *in vivo* challenges fish were transported alive and reared in the facilities of the Central Service for the Support to Experimental Research (Servei de Suport a la Investigació Experimental (SCSIE) of the University of Valencia). They were maintained in marine water (37‰ salinity, 20°C temperature, and 8:16 light:dark photoperiod) and fed with commercial pellets. Fishes were killed by fast medullar section before being processed. Anaesthetic overdose was not used as ectoparasites may be affected.

Particular details of the samplings and analyses of each group of fish and parasite are indicated in their respective chapters.

3.1.1 Samplings of *Diplodus puntazzo* and *Sparus aurata*

Three different groups of *D. puntazzo* and one of *S. aurata* were sampled:

1. To study the parasite fauna, 70 wild fish were captured from two localities from the Spanish Mediterranean: 50 fish from Mar Menor (off San Pedro del Pinatar), Murcia (37°41′N, 0°44′W); and 20 fish off Santa Pola (Valencian Community; 38°11′N, 0°33′W). Fish were killed, immediately frozen and transported to the laboratory of the Marine Zoology Unit.

2. To study the attachment strategy and the development of *Lamellodiscus* spp., 100 *D. puntazzo* infected with *Lamellodiscus* spp. were acquired and used as a donor fish. Fish were captured alive in Mar Menor and reared in the installations of Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA) at

San Pedro de Pinatar (Murcia). Thereafter they were transported and maintained alive in the facilities of the SCSIE.

3. To complete the development study of *Lamellodiscus* spp., 80 naïve fish were obtained from a Greek fish hatchery (38°17'N, 21° 47'E), and transported alive to the facilities of the SCSIE.

4. To study the attachment mechanisms of the diplectanid *Furnestinia echeneis*, 10 infected *S. aurata* were obtained from sea cages in Burriana (Valencian Community; 39°53'N, 0°5'W), and transported alive to the facilities of the SCSIE.

3.2 Fish analyses and parasite collection and study

In order to study the morphological and molecular features of the parasites, fish were dissected and analysed looking for parasites. Organs were isolated and examined in sea water or saline using a stereomicroscope up to 100X magnification. Parasites were removed, pre-classified and preserved in 70% ethanol for morphological examination. Some specimens were preserved in 100% ethanol for molecular studies. Additionally, some parasites and fish gills were fixed in 10% buffered formalin for histological studies. In some studies, morphological features and behaviour of diplectanid monogeneans were observed *in vivo* in sea water under stereomicroscope up to 100X magnification.

3.3 Parasites description and identification

3.3.1 Morphological analyses

Platyhelminthes were mostly studied with stereomicroscope (LEICA DMZ-APO) or with light microscope with the aid of differential interference contrast (DIC) (LEICA DMR). Most of the specimens were fixed in 70% ethanol, stained with iron acetocarmine (Georgiev *et al.*, 1986), dehydrated in a graded ethanol series, cleared in dimethyl phthalate, and examined on permanent mounts in Canada

balsam under the light microscope up to 1000X magnification. Diplectanid monogenean specimens were washed in distilled water and examined on semi-permanent preparations in glycerol gelatine under the light microscope for their identification and detailed morphological examination. Arthropods were examined under the light microscope up to 400X, in distilled water or in glycerine.

To complete the morphological studies, morphometric data were taken under the light microscope at 400-1000X magnification with a drawing tube (OPTIPHOT-2). During the developmental study of the haptor of *Lamellodiscus* spp. esclerotised elements were drawn and analysed using Image Tool for Windows 3.00. Co. 1995-2002, UTHSCSA. Measurements are given in micrometers as mean ± standard deviation (SD) followed by ranges in parentheses.

Movement sequence of the haptors of *Lamellodiscus* spp. and *F. echeneis* was recorded *in vivo*, free as well as attached to the gill, by means of an analogical camera JAI CV-S3200 coupled to the stereomicroscope.

For the SEM study, samples fixed in 10% buffered formalin were dehydrated in ethanol series and critical point-dried with liquid CO_2. Specimens were mounted on stubs with conductive carbon paint, coated with gold–palladium to a thickness of 25–30 µm in a Bio Rad–Sc 500 coating unit, and examined with the aid of a HITACHI S-4100 scanning electron microscope at 5 kV.

3.3.2 Molecular analyses

Identification of some parasite species (*Lamellodiscus* spp. and *Peracreadium* sp.) required molecular analyses. Three to four specimens of each morphotype, previously fixed in 100% ethanol, were individually transferred into 300 µL TNES urea [10 mM Tris-HCl (pH 8), 125 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulphate (SDS), 4 M urea]. Samples were digested with 100 $\mu g mL^{-1}$ Proteinase K overnight at 55 °C, following thereafter a phenol–chloroform standard procedure for genomic DNA (gDNA) extraction. The extracted DNA was resuspended in 20 µL of RNAse/DNAse free water and left to

dissolve overnight in the fridge. Polymerase chain reactions were performed with a programmable thermal cycler (Techne, TC-512, GMI) in a final volume of 30 µL containing 0.3 U of BioLabs Taq DNA polymerase and the related 10X Standard Taq Reaction Buffer with 1.5 mM MgCl2, 200 µM of each dNTP, 10 mM of each PCR primer and 20–70 ng template.

DNA fragments were amplified and sequenced for each species (see Chapter 4 for details on used primers and gene amplification profiles). After checking and detecting the presence of DNA in a 1% agarose gel with sodium acetate buffer following GelRedTM Nucleic Acid Gel Stain staining and ultraviolet transillumination (VWR, Genoview), PCR products were purified using the GFX PCR DNA and Gel Band purification Kit (GE Healthcare UK Ltd). Cycle sequencing was conducted in a 48 capillary ABI 3730 sequencer (Applied Biosystems) using the BIG Dye terminator v 3.1 Ready Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions with the same primers used for the PCR. The contiguous sequences were aligned using BioEdit v7.0.5. (©1997–2005, Hall, 1999) and compared for similarity with sequences lodged in GenBank (see details in Chapter 4), using BLAST (Altschul et al., 1990) and MEGA 4.1. (Tamura *et al.*, 2007).

3.4 Histological studies

Attachment mechanisms and related pathologies caused by diplectanids were studied using histological techniques. For the study of adult and juvenile specimens of Lamellodiscus spp., infected gills, previously fixed in 10% buffered formalin, were placed in cassettes, dehydrated in graded ethanol series and transferred through xylene into paraffin. Thereafter, samples were serially sectioned (4µm) with a rotator microtome, stained with haematoxylin and eosin (H-E) and mounted in Entellan™ (Merk). Specimens were observed using the light microscope (Leica DMR HC).

The study of the diplectanid attachment mechanisms was complemented with additional paraffin H-E stained histological sections of *F. echeneis* and *D. aequans* attached to gills of *S. aurata* and *Dicentrarchus labrax* respectively, from paraffin blocks of the sample collection of the Fish Diseases Diagnostic Service of the Autonomous University of Barcelona, obtained from different aquaculture facilities.

CHAPTER 4

Morphometric, molecular and ecological analyses of the parasites of the sharpsnout seabream *Diplodus puntazzo* Cetti (Sparidae) from the Spanish Mediterranean: implications for aquaculture

Journal of Helminthology, page 1 of 15
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Morphometric, molecular and ecological analyses of the parasites of the sharpsnout seabream *Diplodus puntazzo* Cetti (Sparidae) from the Spanish Mediterranean: implications for aquaculture

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Abstract

One of the fish species with the highest potential for aquaculture is the sharpsnout seabream, *Diplodus puntazzo* Cetti. Among other aspects, the development of new fish cultures requires studies of potential pathogens that may compromise survival of the fish in captivity. Moreover, both cultured and wild fish can act as sources or reservoirs of pathogens which may negatively affect other well-established cultures. We have studied the parasite fauna of the wild sharpsnout seabream, and monitored the survival of the parasites in culture conditions. The sharpsnout seabream was sampled from two different Spanish localities and examined for parasites. Additionally, 20 fish were maintained in captivity. Ten of them were examined for parasites after a period of 10 days and a further ten fish after another 10 days. All fish were parasitized with at least four species, with 19 parasite species being identified, seven of which were recorded for the first time in the sharpsnout seabream. These included *Microcotyle* sp., *Magnibursatus bartolii*, *Steringotrema pagelli*, *Galactosomum* sp., *Cardiocephaloides longicollis*, *Caligus ligusticus* and *Gnathia vorax*. We also report the first records of two parasite species in the wild sharpsnout seabream, the polyopisthocotylean monogeneans *Atrispinum seminalis* and *Sparicotyle chrysophrii*. Previously, these parasites had only been recorded in farmed sharpsnout seabream. Most parasites in the skin, gills and alimentary tract disappeared under the conditions of captivity, with the exception of the monogeneans of the genus *Lamellodiscus*. The information provided about the sharpsnout seabream parasite fauna will be useful to prevent possible problems in fish farms due to some parasite species. Many parasites of the sharpsnout seabream recorded in the present study are shared by the main fish species in Mediterranean aquaculture, the gilthead seabream, thus suggesting the possibility of cross-infections.

Introduction

Despite the recommendations of European governments and institutions to diversify fish cultures (Abellán & Basurco, 1999; Anonymous, 2012), Mediterranean aquaculture is still focused predominantly on two

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species, the gilthead seabream, *Sparus aurata* L., and the European seabass, *Dicentrarchus labrax* L. One of the species with higher potential for aquaculture is the sharpsnout seabream, *Diplodus puntazzo* Cetti (see Abellán & Basurco, 1999). Although catches of the sharpsnout seabream are low in Spanish fisheries (Food and Agriculture Organization,2010), this species has good aquaculture prospects because of its easy adaptation to conditions of captivity, high growth rate and food conversion efficiency (Favaloro *et al.*, 2002; Hernández *et al.*, 2003). The culture of this sparid is still under development, with a limited number of larvae produced in Italy, Greece and Portugal (Federation of European Aquaculture Producers, 2008; Vinagre *et al.*, 2010). In Spain this culture has been mostly experimental (Hernández *et al.*, 2001, 2002, 2003; Pajuelo *et al.*, 2008; Nogales Mérida *et al.*, 2010; Almaida-Pagán *et al.*, 2011). However, the introduction of the sharpsnout seabream into aquaculture has been compromised by the presence of many pathogens, often producing severe pathologies (Athanassopoulou *et al.*, 2005; Merella *et al.*, 2005; Katharios *et al.*, 2006; Montero *et al.*, 2007; Álvarez-Pellitero *et al.*, 2008; Golomazou *et al.*, 2009; Rigos & Katharios, 2010; Sánchez-García *et al.*, 2011).

Aquaculture conditions imply fish stress which favours pathogen transmission and, therefore, abnormally high infection levels (Ogawa, 1996). Fish parasite diseases are often associated with important economic losses in aquaculture because of fish mortalities, production decrease or increased costs of antiparasitic treatments (Murray & Peeler, 2005; Rhode, 2005). Often infections appear in farms because many of the pathogens causing diseases are also associated with sea cages. Considerable aggregations of wild fish are usually associated with sea cages in coastal areas (Sánchez-Jerez *et al.*, 2007), and parasite species infecting these neighbouring fish can be transmitted, resulting in wild fish acting as reservoirs of parasite infections in farmed fish (Kent, 2000; Mladineo *et al.*, 2009) or parasites in fish farms spreading to the surrounding environment (Rohde, 2005; Krkošek *et al.*, 2007). Thirdly, different fish species farmed in neighbouring installations can experience cross-infections (Di Cave *et al.*, 2002). These situations should be considered with special attention on new cultures with a short farming history, since their pathogens are unknown or poorly known.

This situation requires the development of effective control of cross-infections, based on considerable knowledge of pathogens living in farmed and wild fish and identification of potential harmful species (Hutson *et al.*, 2007; Rigos & Katharios, 2010). The sharpsnout seabream is, in fact, one of the fish species reported by Dempster *et al.* (2002) associated with sea cages in the Mediterranean and, therefore, wild populations of this species could be a source for pathogen transmission to farmed fish of the same or other species. Although a number of studies referring to parasites of sharpsnout seabream in the Mediterranean Sea exist (Athanassopoulou *et al.*, 1999, 2005; Di Cave *et al.*, 2002; Vagianou *et al.*, 2004; Merella *et al.*, 2005; Katharios *et al.*, 2006; Toksen, 2006; Mladineo & Maršić-Lučić, 2007; Montero *et al.*, 2007; Álvarez-Pellitero *et al.*, 2008), these are focused on pathogens of farmed fish and no data on the parasites of sharpsnout seabream in the wild are available. From an epizootiological point of view, the description of parasite communities of this species is indispensable to prevent economic and pathological impacts of certain parasitoses.

The aim of the present study was to identify the parasites infecting wild and farmed sharpsnout seabream. The parasite fauna of wild fish from two locations in the Spanish Mediterranean are described. Furthermore, we tested the effect of culture conditions on these parasites, in order to find those species that can survive and proliferate in farms.

Materials and methods

Collection and examination of fish

Seventy sharpsnout seabream aged 1 year or more were collected alive from two different locations in the Spanish Mediterranean in 2007: 50 fish with a total length (mean ± standard deviation) of 252.1 ± 11 mm, weight 300 ± 37.7 g were collected in Mar Menor off the coast of San Pedro del Pinatar, Region of Murcia (37°41′14″ N, 0°44′10″W); and 20 fish with a total length of 188.5 ± 18 mm, weight 111.95 ± 27.73 g were collected off the coast of Santa Pola, Valencian Community (38°11′23″N, 0°33′20″W). No more fish could be obtained since, as previously stated, the sharpsnout seabream is fished in low numbers in Spain. Thirty fish from Mar Menor and all the individuals from Santa Pola were killed by medullar section and immediately frozen. In order to study the effect of culture conditions on parasites, the other 20 specimens from Mar Menor were transported alive and reared in the aquaculture facilities of the Central Service for the Support to Experimental Research of the University of Valencia (SCSIE). Fish were maintained in marine water (salinity 37‰, temperature 20°C, 8:16 h light:dark photoperiod) and fed with commercial gilthead seabream pellets. After 10 days, ten fish were killed by medullar section; the remaining ten fish were killed after 20 days of captivity.

Fish were first examined for external parasites on the skin, fins and eyes, and following a post-mortem examination internal organs, such as digestive tract, gonads, liver, gills, kidney and brain, were examined in saline solution under a stereomicroscope (100× magnification). Parasites were removed and preserved in either 70% ethanol for morphological examination or in 100% ethanol for molecular study. Myxozoans were detected by examination of wet preparations of squeezed fresh organs, using a light microscope at magnifications of up to 1000× with differential interference contrast (DIC).

Morphometrics

Platyhelminths fixed in 70% ethanol were stained with iron acetocarmine (Georgiev *et al.*, 1986), dehydrated through a graded ethanol series, cleared in dimethyl phthalate, and examined as permanent mounts in Canada balsam under a light microscope. In the case of the diplectanid monogeneans *Lamellodiscus* spp., representative subsamples of 30 specimens from each fish were randomly selected to be identified and counted. For those species of *Lamellodiscus* with no more than 30 specimens,

all parasites were examined. Monogeneans were washed in distilled water and examined on semi-permanent preparations in glycerol–gelatine under a light microscope for their detailed morphological examination and identification. Crustaceans were examined under a light microscope in distilled water or in glycerine.

For some species, when the morphological identification was particularly confusing, detailed specific morphometric studies were performed. Fifteen mounted adult specimens of each of these species (i.e. *Lamellodiscus* spp. and *Peracreadium* sp.) from each location were selected for their detailed examination. Morphometric data of specimens of *Lamellodiscus* spp. and *Peracreadium* sp. were obtained by using a light microscope with a drawing tube. Haptoral parts of the diplectanids were measured according to Amine & Euzet (2005) and the resulting measurements were compared with the available published data. All measurements are given in micrometres.

Molecular analysis

Those species difficult to classify morphologically (*Lamellodiscus* spp. and *Peracreadium* sp.) were also studied through genetic comparisons. Three to four specimens of these species (see table 1), previously fixed in 100% ethanol, were transferred into 300 µl TNES urea (10 mM Tris–HCl (pH 8), 125 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium dodecyl sulphate (SDS), 4 M urea). Genomic DNA (gDNA) was extracted from single specimens using a phenol–chloroform standard procedure. The extracted DNA was resuspended in 20 µl of RNAse/DNAse-free water and left to dissolve overnight in the fridge. Polymerase chain reactions (PCRs) were performed with a programmable thermal cycler (Techne, TC-512, GMI, Ramsey, Minnesota, USA) in a final volume of 30 µl containing 0.3 U *Taq* DNA polymerase (BioLabs, Madrid, Spain) and the related 10 × Standard *Taq* Reaction Buffer with 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (dNTP), 10 mM of each PCR primer and 20–70 ng of template.

Partial 18S and entire internal transcribed spacer 1 (ITS1) of *Lamellodiscus* spp. were amplified and sequenced using the primers L7 (5′-TGA TTT GTC TGG TTT ATT CCG AT-3′ (Verneau *et al.*, 1997)) and IR8 (5′-GCT AGC TGC GTT CTT CAT CGA-3′ (Kaci-Chaouch *et al.*, 2008)) that anneal to the 18S and 5.8S rRNA genes, respectively. It has been shown that ITS1 is highly variable and not useful for inferring evolutionary relationships among *Lamellodiscus* spp., but it can be used to differentiate species (Desdevises *et al.*, 2000). Complete sequences of ITS1, 5.8S and ITS2 of *Peracreadium* sp. were amplified and sequenced using primers 18dF (5′-CAC ACC GCC CGT CGC TAC TAC CGA TTG-3′ (Hillis & Dixon, 1991)) and ITS2.2 (5′-CCT GGT TAG TTT CTT TTC CTC CGC-3′ (Anderson & Barker, 1993)). The thermocycling profile used for the amplification of sequences of *Lamellodiscus* spp. consisted of denaturation of DNA (95°C for 3 min); 38 cycles of amplification (94°C for 50 s, 50°C for 50 s and 72°C for 1 min 20 s); and 4 min extension hold at 72°C. The same profile was used for gene amplification of

Table 1. The range of monogenean and digenean species occurring in *Diplodus puntazzo* with accession numbers of the ITS and 18S rDNA sequences.

Species	Source	GenBank accession numbers
Lamellodiscus falcus s.l.	Present study	KC470292[a]
		KC470293[a]
		KC470294[a]
		KC470298[b]
		KC470299[b]
		KC470300[b]
Lamellodiscus ignoratus	Desdevises (2001)	AF294957[b]
	Kaci-Chaouch *et al.* (2008)	EU259026[a]
		EU259027[a]
		EU259029[a]
		EU259031[a]
Lamellodiscus ergensi	Desdevises *et al.* (2002)	AY038190[b]
	Kaci-Chaouch *et al.* (2008)	EU259056[a]
		EU259057[a]
		EU259058[a]
		EU259059[a]
Lamellodiscus theroni s.l.	Present study	KC470295[a]
		KC470296[a]
		KC470297[a]
		KC470301[b]
		KC470302[b]
		KC470303[b]
Peracreadium characis	Jousson *et al.* (1999)	AJ241796[a,b]
		KC470304[a,b]
		KC470305[a,b]
	Present study	KC470306[a,b]
		KC470307[a,b]

[a]ITS; [b]18S.

Peracreadium sp. but with an annealing temperature of 55°C. After checking the presence of DNA in a 1% agarose gel in sodium acetate buffer and detection following GelRed™ Nucleic Acid Gel Stain staining and ultraviolet transillumination (VWR, Genoview, Barcelona, Spain), the PCR products were purified for sequencing using the GFX PCR DNA and Gel Band purification Kit (GE Healthcare UK Ltd, Pollards Wood, Bucks, UK). Cycle sequencing was conducted in a 48 capillary ABI 3730 sequencer (Applied Biosystems, Madrid, Spain) using the BIG Dye terminator v 3.1 Ready Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions, using the same primers as those used for the PCR. The contiguous sequences were aligned using BioEdit v. 7.0.5. (Hall, 1999) and compared for similarities with sequences lodged in GenBank (detailed in table 1) using BLAST (Altschul *et al.*, 1990) and MEGA 4.1. (Tamura *et al.*, 2007).

In the case of the species of *Lamelodiscus*, not all sequences were available in GenBank. In particular, no information on *L. falcus* and *L. theroni* was found, and the sequences of *L. falcus s.l.* and *L. theroni s.l.* obtained in the present study were aligned and compared with the morphologically similar and phylogenetically closest species with available sequences, i.e. *L. ignoratus* Palombi, 1943 and *L. ergensi* Euzet et Oliver, 1966, respectively (Amine & Euzet, 2005).

Data analysis

For all parasite species, mean abundance and prevalence (P%) were calculated as defined by Bush *et al.* (1997), except for the myxozoans for which only prevalence was determined. Confidence intervals for the prevalence were also calculated. A comparison of prevalences and abundances between the samples of the two locations studied, and between wild and captive sharpsnout seabream, were calculated to identify significant differences. Statistical boostrap *t*-test for abundance and Fisher's exact test for prevalence were carried out with Quantitative Parasitology 3.0 (Rózsa *et al.*, 2000).

Results

All sharpsnout seabream analysed were infected by at least four parasite species. In total, 19 parasite species were found, 15 in the fish sampled in Mar Menor and 11 in the fish sampled off Santa Pola (table 2). Seven species of the parasites were shared by fish from the two localities. All parasites were identified to species level, except for the myxozoan *Ceratomyxa* sp., the digenean brain parasite *Galactosomum* sp. met. and the monogenean Microcotylidae gen. sp. The first two of these parasites were identified only up to genus level as their species identification requires further molecular or morphological analyses. Regarding Microcotylidae gen. sp., one single specimen was found and its morphological traits were not assignable to any known microcotylid species: (1) genital atrium armed with three types of spines (14 long peripheral, 9 small posterior and 4 long central); (2) vaginal pore dorsal and single, with narrow and sinuous conspicuous vaginal duct; and (3) 104 symmetrical clamps in the haptor.

Significant differences in the total mean abundance were found between wild fish from Mar Menor and Santa Pola (bootstrap *t*-test, $P < 0.01$), although the only parasites with significantly different mean abundance were the monogeneans *L. falcus* and *L. hilii* (bootstrap *t*-test, $P < 0.05$). Fish from both localities also showed significant differences in the prevalence of these monogenean species and of the digeneans *Cardiocephaloides longicollis* (Rudolphi, 1819) and *Peracreadium characis* (Stossich, 1886) (Fisher tests, $P < 0.05$). A greater variety of species was found in the fish from Mar Menor, which harboured eight parasite species not found in the fish from off Santa Pola. These include one myxozoan (*Ceratomyxa* sp.), two monogeneans (Microcotylidae gen. sp. and *Atrispinum seminalis* Euzet et Maillard, 1973), three digeneans (*Proctoeces maculatus* Looss, 1901; *Steringotrema pagelli* (van Beneden, 1871); and *Monorchis monorchis* Stossich, 1890) and two copepods (*Caligus ligusticus* Brian, 1906 and *Lernanthropus vorax* Richiardi, 1880). Fish from Santa Pola also harboured four parasite species not found in fish from Mar Menor, including two monogeneans (*Lamellodiscus bidens* Euzet, 1984 and *Sparicotyle chrysophrii* (van Beneden et Hesse, 1863)), one digenean (*Galactosomum* sp.) and one isopod (*Gnathia vorax* Lucas, 1849).

Parasite species richness and total abundance in the fish samples from Mar Menor gradually decreased in captivity conditions. Total parasite abundance was significantly lowered after 10 days of captivity (bootstrap *t*-test, $P < 0.01$), and reached even lower levels after 10 more days (bootstrap *t*-test, $P < 0.01$). In 10 days, many monogenean species (*A. seminalis*, *L. falcus s.l.*, *L. hilii* Euzet, 1984 and *L. theroni s.l.*) and the myxozoan *Ceratomyxa* sp. were still observed. In contrast, most digenean species had disappeared, and only two species were found: *P. maculatus* and the metacercariae of *C. longicollis*. The last 10 fish examined after 20 days in captivity were parasitized by the same three species of *Lamellodiscus*, the abundance of these species being higher (although not significantly different) than in the fish examined immediately after capture. Digeneans of the digestive tract were not found at this time, whereas parasites in strictly internal microhabitats (*C. longicollis* in the brain and *Ceratomyxa* sp. in the gall bladder) were still present.

Morphometrics

Table 3 summarizes the metrical data for the diplectanid monogeneans found in this study identified to the species level, compared with the original descriptions of *Lamellodiscus bidens* Euzet, 1984 and *L. hilii*. The identification of the other two morphotypes of '*Lamellodiscus*' was controversial (morphometric data in table 4). These morphotypes were similar to *L. falcus* or to *L. theroni* (further referred respectively to as *L. falcus sensu stricto* (*s.s.*) and *L. theroni sensu stricto*, for the original descriptions, and *L. falcus sensu lato* (*s.l.*) and *L. theroni sensu lato*, for the specimens of the present study). Both morphotypes in sharpsnout seabream had similar morphology and body length, but the haptor of *L. falcus s.l.* was, in general, narrower, with sclerotized structures thinner than those of *L. theroni s.l.* (fig. 1a). Moreover, the

Table 2. The prevalence (%) and mean abundance (% MA) of myxozoan, heminth and crustacean parasites from *Diplodus puntazzo* in two localities of the Spanish Mediterranean, and from fish maintained in captivity for 10 and 20 days; *n* = number of fish examined, CI (95% confidence intervals) given in parentheses.

		Mar Menor (Murcia) *n* = 30		Santa Pola (Alicante) *n* = 20		Mar Menor (10 days in captivity) *n* = 10		Mar Menor (20 days in captivity) *n* = 10	
Taxon	Microhabitat	%	% MA	%	% MA	%	% MA	%	% MA
Cnidaria, Myxozoa									
Ceratomyxidae									
Ceratomyxa sp.[b]	Gall bladder	10.0 (0.3–44.5)	–	–	–	20.0 (7.1–38.5)	–	10.0 (0.3–44.5)	–
Platyhelminthes, Monogenea									
Capsalidae									
Encotyllabe vallei	Gills	3.3 (0.1–17.2)	0.0	40.0 (19.1–63.9)		–	–	–	–
Diplectanidae									
Lamellodiscus bidens	Gills	–	–	40.0 (19.1–63.9)	0.4	–	–	–	–
Lamellodiscus falcus	Gills	100 (88.4–100)	107.9	80.0 (56.3–94.7)	42.2	100 (69.1–100)	135.0	100 (69.1–100)	156.9
Lamellodiscus hilii	Gills	100 (88.4–100)	66.2	80.0 (56.3–94.7)	3.6	100 (69.1–100)	82.2	100 (69.1–100)	91.0
Lamellodiscus theroni	Gills	90.0 (73.5–97.9)	26.7	80.0 (56.3–94.7)	40.8	100 (69.1–100)	38.9	100 (69.1–100)	42.0
Microcotylidae									
Atrispinum seminalis	Gills	20.0 (7.1–38.5)	0.3	–	–	10.0 (0.3–44.5)	0.2	–	–
Microcotylidae gen. sp.[a]	Gills	3.3 (0.1–22.3)		–	–	–	–	–	–
Sparicotyle chrysophrii[b]	Gills	–	–	40.0 (19.1–63.9)	1.2	–	–	–	–
Platyhelminthes, Digenea									
Derogenidae									
Magnibursatus bartolii[a]	Gills/oesophagus	23.3 (9.9–42.2)	0.4	40.0 (19.1–63.9)		–	–	–	–
Fellodistomidae									
Proctoeces maculatus	Intestine/caeca	3.3 (0.1–17.2)	0.1	–	–	20.0 (7.1–38.5)	0.5	–	–
Steringotrema pagelli[a]	Intestine/caeca	6.7 (8.1–22.0)	0.1	–	–	–	–	–	–
Heterophyidae									
Galactosomum sp.[a]	Brain	–	–	20.0 (5.7–43.7)	0.2	–	–		
Monorchiidae									
Monorchis monorchis	Intestine/caeca	26.7 (12.2–45.8)	1.1	–	–	–	–	–	–
Opecoelidae									
Peracreadium characis	Intestine/caeca	80.0 (61.4–92.2)	4.5	40.0 (19.1–63.9)	3.6	–	–	–	–
Strigeidae									
Cardiocephaloides longicollis[a]	Brain	70.0 (50.6–85.2)	3.2	20.0 (5.7–43.7)	1.0	40.0 (12.1–73.8)	0.8	60.0 (26.2–87.8)	1.7
Crustacea, Copepoda				–					
Caligidae									
Caligus ligusticus[a]	Gills	6.7 (8.1–22.0)	0.1	–		30.0 (6.7–65.2)	0.6	–	–
Lernanthropidae									
Lernanthropus vorax	Gills	26.7 (14.7–49.4)	0.3	–	–	–	–	–	–
Crustacea, Isopoda									
Gnathidae									
Gnathia vorax[a]	Gills	–	–	100.0 (83.1–100.0)	13.4	–	–	–	–

[a] New records for *D. puntazzo*.
[b] New records for wild *D. puntazzo*.

Table 3. Comparative morphometrics (μm) of the monogenean species *Lamellodiscus hilii* and *L. bidens* infecting *Diplodus puntazzo*; *n* = number of specimens examined and range in size given in parentheses.

	L. hilii			*L. bidens*	
Morphometrics	Present study, Mar Menor, *n* = 10	Present study, Santa Pola, *n* = 7	Euzet (1984)	Present study, Santa Pola, *n* = 2	Euzet (1984)
Body length	833 ± 182 (640–1159)	790 ± 236 (500–1169)	800–1000	788 ± 243 (616–959)	700–800
Haptor width	201 ± 18 (181–225)	163 ± 75 (166–223)	250	201 ± 2 (200–203)	
Ventral bar (total length)	121 ± 7 (111–128)	114 ± 18 (104–147)	100–120	94 ± 10 (87–101)	95–105
Lateral bar (total length)	84 ± 6 (78–97)	81 ± 6 (76–90)	80–90	75 ± 2 (73–76)	65–80
Lamellodisc diameter	61 ± 9 (50–78)	61 ± 7 (51–68)	75	58 ± 10 (51–65)	60
Dorsal anchor (total length)	62 ± 4 (56–69)	60 ± 4 (54–65)	55–67	59 ± 4 (56–62)	52
Ventral anchor (total length)	68 ± 6 (61–78)	69 ± 6 (62–76)	70–80	63 ± 6 (59–68)	64
Male copulatory organ (total length)	77 ± 20 (52–108)	92 ± 16 (65–104)	100	66 ± 2 (65–68)	74

dorsal bar of *L. falcus s.l.* was undivided while that of *L. theroni s.l.* was divided in two pieces (fig. 1a, b). The copulatory organs of both species were both lyre-shaped, although the single piece of the copulatory organ of the specimens of *L. falcus s.l.* was observed to be markedly hooked (fig. 1c), and that of *L. theroni s.l.* was more straightened (fig. 1d).

The morphometric measurements of the specimens of the digenean *Peracreadium* sp. found in both localities are given in table 5.

Molecular analysis

For molecular markers, ITS1 and 18S, the sequences of *L. falcus s.l.* and *L. theroni s.l.* obtained here were aligned and compared with the available sequences in GenBank for *L. ignoratus* and *L. ergensi*, respectively (accession numbers in table 1). There was no variation in the length of the 18S sequences (525 bp). The 18S sequences for isolates of *L. falcus s.l.* were identical with the sequences of *L. ignoratus* retrieved from GenBank. The 18S sequences for the isolates of *L. theroni s.l.* were identical and exhibited 0.2% divergence from the sequence for *L. ergensi* retrieved from GenBank. In case of the ITS1 region, the sequences varied in length from 441 to 563 bp. ITS1 sequences obtained in the present study were also aligned and sequence divergences (% of p-distances and number of differences) computed are given in tables 6 and 7.

The ITS1 isolates of *L. falcus s.l.* were identical, but exhibited 1.2–26.0% divergence with the four sequences for *L. ignoratus* retrieved from GenBank (table 6). The divergences between the ITS1 sequences for isolates of *L. theroni s.l.* ranged between 0 and 0.2% whereas dissimilarities between them and the sequence for *L. ergensi* from GenBank ranged between 0.2 and 14.0% (table 7). The mean inter-individual uncorrected genetic distances for ITS1 sequences (± SD and range in parentheses) of '*L. ignoratus* complex' (i.e. the new sequences for *L. falcus s.l.* obtained in the present study together with the sequence for *L. ignoratus* from GenBank) was 10.5 ± 10.0 (0.0–26), and of '*L. ergensi* complex' (i.e. the new sequences for *L. theroni s.l.* obtained here together with the sequence for *L. ergensi* from GenBank) was 6.6 ± 6.9 (0.0–14.2). The mean intraspecific distances for each species separately were: *L. falcus s.l.*, 0; *L. theroni s.l.*, 0.1 ± 0.1 (0.0–0.2); *L. ignoratus*, 15.4 ± 8.2 (9.1–26.0); *L. ergensi*, 16.1 ± 5.7 (0.0–14.0).

Three specimens from Santa Pola and one from Mar Menor identified as *Peracreadium* sp. were sequenced (see GenBank accession numbers in table 1). The sequences obtained were aligned and compared with sequences of the partial 18S rDNA and complete ITS1–5.8S–ITS2 for *P. characis* from GenBank (AJ241896). The length of the 18S sequences was 120 bp in all sequences examined. The length of the complete ITS1–5.8S–ITS2 sequences was 1081 bp. The 18S sequences for isolates of *Peracreadium* sp. were identical with the GenBank sequence for *P. characis*. The ITS sequences for the isolates of *Peracreadium* sp. were identical, whereas the divergence between these and the sequence for *P. characis* in GenBank was 0.3% in all cases.

Discussion

We present here the first survey on the parasite fauna of the sharpsnout seabream in the wild, finding 19 different parasites species. The sharpsnout seabream harboured a diverse community of metazoan parasites, many of them previously reported in this species in the Mediterranean Sea (Radujkovic & Euzet, 1989; Sasal *et al.*, 1999; Bartoli *et al.*, 2005; Mladineo, 2006; Gargouri Ben Abdallah & Maamouri, 2008). The current study also provides new parasite records in the wild fish populations. The sharpsnout seabream is a new host record for seven species, including the polyopisthocotylean monogenean Microcotylidae gen. sp., the digeneans *Magnibursatus bartolii* Kostadinova, Power, Fernández, Balbuena, Raga et Gibson, 2003, *Steringotrema pagelli*, *Cardiocephaloides longicollis* and *Galactosomum* sp., the copepod *Caligus ligusticus* and the isopod *Gnathia vorax*. In addition, two parasite species, the polyopisthocotyleans, *Atrispinum seminalis* and *Sparicotyle chrysophrii*, which have already been reported in farmed fish, were found for the first time in wild populations of sharpsnout seabream. Most of these species were reported previously in other sparid fish which cohabit with sharpsnout seabream. Among these new records, ten parasite species were common in this fish, as they were noticeably frequent (prevalence ≥40%, in at least one locality, see table 2). The remaining

Table 4. Comparative morphometrics (µm) of the monogenean species *Lamellodiscus ignoratus* and *L. ergensi* groups infecting *Diplodus puntazzo*; *n* = number of specimens examined and range in size given in parentheses.

	L. ignoratus group				*L. ergensi* group			
Morphometrics	*L. falcus s.l.* Present study, Mar Menor, $n = 15$	*L. falcus s.l.* Present study, Santa Pola, $n = 15$	*L. ignoratus* Amine *et al.* (2006)	*L. falcus* Amine *et al.* (2006), $n = 34$	*L. theroni s.l.* Present study, Mar Menor, $n = 15$	*L. theroni s.l.* Present study, Santa Pola, $n = 15$	*L. ergensi* Amine & Euzet (2005)	*L. theroni* Amine *et al.* (2007), $n = 22$
Body length	635 ± 103 (470–854)	511 ± 207 (465–715)	550 ± 61	394 ± 50	613 ± 71 (512–695)	597 ± 99 (413–773)	400–550	715 ± 77
Haptor width	115 ± 7 (106–129)	96 ± 37 (87–128)	173 ± 13		151 ± 14 (129–179)	138 ± 54 (111–193)		201 ± 29
Medial bar (total length)	53 ± 5 (45–58)	52 ± 9 (27–60)	71 ± 8	34 ± 1	85 ± 7 (78–99)	77 ± 4 (70–80)	90–95	94 ± 12
Lateral bar (total length)	43 ± 5 (38–51)	46 ± 8 (26–54)	51 ± 4	37 ± 1	43 ± 3 (38–47)	42 ± 4 (37–49)	46–52	54 ± 6
					44 ± 4 (40–52)	42 ± 6 (31–51)	46–51	55 ± 6
Lamellodiscus diameter	44 ± 6 (33–54)	42 ± 8 (21–49)	43 ± 4	29 ± 1	34 ± 3 (30–38)	33 ± 2 (30–37)	30–35	35 ± 3
Dorsal hook (total length)	34 ± 2 (31–37)	35 ± 1 (33–37)	34 ± 2	30 ± 1	56 ± 3 (48–58)	55 ± 3 (50–59)	51–57	70 ± 9
Ventral hook (total length)	38 ± 2 (33–40)	37 ± 2 (34–40)	41 ± 3	31 ± 1	68 ± 5 (62–72)	64 ± 4 (59–69)	63–69	82 ± 10
Male copulatory organ (total length)	40 ± 1 (41–52)	49 ± 1 (47–54)	46 ± 3	40 ± 1	45 ± 5 (35–50)	46 ± 3 (40–49)	44–47	53 ± 6

parasite species were infrequent (prevalence ≤ 20%) and, therefore, they could be more specific to other hosts in the area (mainly in other sparids, see Álvarez-Pellitero *et al.*, 1995; Golomazou *et al.*, 2006).

Most parasites were monoxenous (11 species, 8 monogeneans and 3 crustaceans). The heteroxenous species were mainly digeneans (7 species), together with the only species of myxozoan. Interestingly, no nematodes have been recorded in the current study or in previous studies of sharpsnout seabream (Gibson *et al.*, 2005). This fact is surprising, especially considering that the species of genera *Hysterothylacium* Ward et Magath, 1917 and *Contracaecum* Railliet et Henry, 1912 exhibit very low host specificity and have been reported frequently in many fish species in the Mediterranean (e.g. Petter & Maillard, 1987; Petter & Radujkovic, 1989; Fernández *et al.*, 2005; Pérez-del Olmo *et al.*, 2007).

Morphological and molecular identification

The identification of the species of *Lamellodiscus* is particularly complicated, as species taxonomy is often based on subtle morphological differences. *Lamellodiscus falcus* and *L. theroni* belong to two species groups ('*ignoratus*' and '*ergensi*' group, respectively) composed of species with very similar morphological traits; hooks, lateral bars and copulatory organs of the species within each group are highly similar (Amine *et al.*, 2007). In fact, *L. falcus* differs from *L. ignoratus* only in the smaller size of all sclerotized pieces and by slight differences in the morphology of the single piece of the male copulatory organ (referred as to 'impair piece' in Amine *et al.*, 2006). The material of *L. falcus s.l.* examined in the present study showed mixed morphological features of both *L. falcus* and *L. ignoratus* (table 4). The shape of the sclerotized structures was clearly similar to that described for *L. falcus* by Amine *et al.* (2006), especially in relation to the hooked shape of the single piece of the copulatory organ (see fig. 1). However, the range of total body lengths and the lengths of the medial and lateral haptor bars overlapped the ranges described for *L. falcus* and *L. ignoratus* (table 4). In contrast, the hooks in the Mediterranean material were larger than those described for *L. falcus*, and more similar to those of *L. ignoratus* (see Amine *et al.*, 2006). A similar situation occurred in the case of the specimens of *L. theroni s.l.* compared with its original description (Amine *et al.*, 2007). *Lamellodiscus theroni* differs from *L. ergensi* in the size of the body and the sclerotized structures, particularly those of the medial bar. In the case of the specimens of *L. theroni s.l.* found in the present study, the medial bar morphology was closer to that described for *L. theroni* and the total body length fell within the range described by Amine *et al.* (2007). However, the dimensions of the sclerotized haptor structures were smaller than those of *L. theroni*, and more similar to those of *L. ergensi* (table 4). In summary, these results show a distinct similarity between the morphology of the species of *Lamellodiscus* found in the present study and the descriptions of *L. falcus* and *L. theroni* but, in contrast, their dimensions were mostly similar to those of *L. ignoratus* and *L. ergensi*, respectively.

Finally, although the comparative sequence analysis showed intraspecific differences in ITS1, this variation was within the range of the intraspecific variation

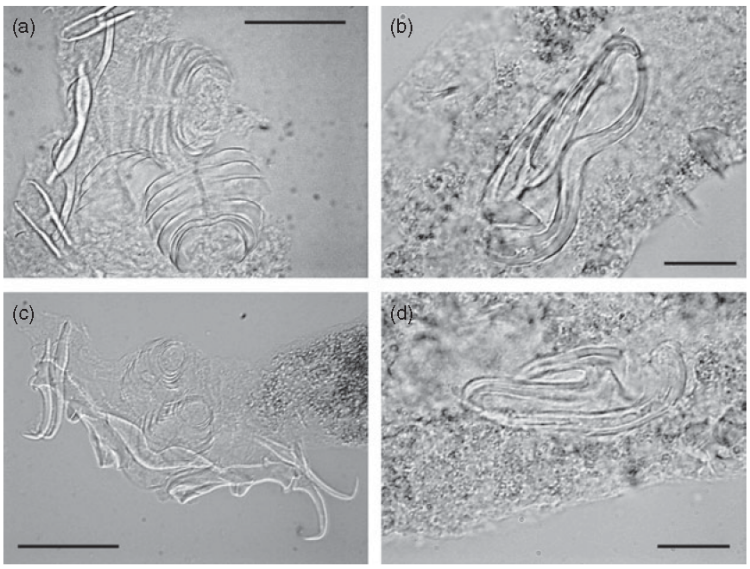

Fig. 1. The haptors of (a) *Lamellodiscus falcus s.l.* and (b) *L. theroni s.l.* and copulatory organs of (c) *L. falcus s.l.* and (d) *L. theroni s.l.* from *Diplodus puntazzo* from the Spanish Mediterranean. Scale bars: a, b = 500 μm; c, d = 100 μm.

provided by Kaci-Chaouch *et al.* (2008) for both *L. ergensi* and *L. ignoratus*. We agree with Poisot & Desdevises (2010) and Poisot *et al.* (2011) who suggested that this degree of molecular divergence in specimens of *Lamellodiscus* does not reinforce the separation of species despite their morphometric variations (*L. falcus* versus *L. ignoratus* and *L. theroni* versus *L. ergensi*). Recent studies emphasized that generalist *Lamellodiscus* species (i.e. members of *L. ergensi* and *L. ignoratus* groups) show higher morphometric variability on their different hosts than specialist species (e.g. *L. bidens* and *L. hilii*) (Poisot & Desdevises, 2010). This intraspecific variability induced by the host, combined with the possibility of morphometric differentiations caused by geographical variation, could explain many morphological variations. Studies incorporating morphological, morphometrical and genetic characterization are thus essential to understand the *Lamellodiscus* species classification.

With reference to digenean identification, specimens of *Peracreadium* sp. collected from both localities apparently belong to *P. characis* (see Bartoli *et al.*, 1989), since the general morphology mostly corresponded to this species but our individuals were somewhat smaller. The results obtained from the molecular analyses confirm that all

Table 5. Comparative morphometrics (μm) of the digenean species *Peracreadium characis* infecting *Diplodus puntazzo*; *n* = number of specimens examined and range in size given in parentheses.

Morphometrics	Present study, Mar Menor, *n* = 8	Present study, Santa Pola, *n* = 5	Bartoli *et al.* (1989), *n* = 10
Body length	1682 ± 276 (1470–2140)	1789 ± 150 (1615–2056)	2940 (2080–4250)
Body max. width	575 ± 189 (390–830)	532 ± 156 (364–765)	920 (785–1190)
Hind body max. length	901 ± 214 (976–1230)	895 ± 250 (715–1132)	1610 (910–2470)
Fore body length	518 ± 60 (410–590)	505 ± 55 (478–575)	900 (640–1230)
Oral sucker length	226 ± 26 (190–270)	232 ± 19 (215–255)	270 (210–350)
Oral sucker width	191 ± 33 (160–260)	189 ± 45 (143–248)	300 (230–390)
Pharynx length	174 ± 21 (150–220)	182 ± 34 (156–225)	210 (160–295)
Pharynx width	133 ± 34 (110–190)	112 ± 42 (88–172)	205 (140–300)
Ventral sucker length	263 ± 43 (230–340)	248 ± 39 (223–318)	400 (300–570)
Ventral sucker width	277 ± 26 (250–330)	289 ± 36 (238–314)	400 (310–520)
Anterior testis length	166 ± 42 (120–220)	175 ± 65 (112–258)	275 (220–370)
Anterior testis width	139 ± 54 (85–220)	102 ± 49 (73–192)	270 (210–340)
Posterior testis length	158 ± 50 (100–220)	150 ± 62 (92–235)	260 (190–375)
Posterior testis width	158 ± 65 (110–260)	139 ± 70 (95–247)	260 (210–320)
Ovary length	90 ± 14 (70–100)	86 ± 20 (68–110)	190 (150–290)
Ovary width	91 ± 21 (70 – 100)	94 ± 23 (69–108)	170 (100–250)
Egg length (mean)	71 ± 5 (63–78)	87 ± 7 (69–80)	66 (61–72)
Egg width (mean)	47 ± 5 (43–50)	42 ± 6 (37–50)	33 (30–39)

Table 6. The range of nucleotides (above the diagonal) and genetic distances calculated as percentages (below the diagonal) in ITS1 sequences of *Lamellodiscus ignoratus* and *L. falcus s.l.* analysed in the present study (*L. ignoratus* complex).

	KC470292	KC470293	KC470294	EU259029	EU259027	EU259026	EU259031
L. falcus s.l. KC470292	–	0	0	35	5	3	105
L. falcus s.l. KC470293	0.0	–	0	35	5	3	105
L. falcus s.l. KC470294	0.0	0.0	–	35	5	3	105
L. ignoratus EU259029	8.4	8.4	8.4	–	38	35	103
L. ignoratus EU259027	1.2	1.2	1.2	9.1	–	8	109
L. ignoratus EU259026	7.0	7.0	7.0	8.4	1.9	–	106
L. ignoratus EU259031	25.1	25.1	25.1	24.6	26.0	25.3	–

isolates examined belonged to the same species, because the ITS sequences for isolates from Santa Pola and Mar Menor were identical. The low divergence (0.3%) between these sequences and the sequence for *P. characis* in GenBank fall well below the range for intraspecific sequence divergence given by Jousson *et al.* (1999) for the Opecoelidae, thus supporting the suggestion that specimens from the Mediterranean sharpsnout seabream also belong to *P. characis*. The present study therefore extends the range of the morphometric variation in this species.

Diversity of parasite fauna

The only myxozoan species found in the present study was *Ceratomyxa* sp. from the gall bladder. To date, two species of this genus have been reported in sharpsnout seabream: *C. diplodae* Lubat, Radujkovic, Marques et Bouix, 1989 and *C. puntazzi* Alama-Bermejo, Raga et Holzer, 2011 (Lubat *et al.*, 1989; Alama-Bermejo *et al.*, 2011). Both species parasitize the gall bladder and are very similar morphologically. Some myxozoan species are known to be highly damaging for aquaculture. In fact, one of the most pathogenic parasites for the culture of sharpsnout seabream has been the intestinal myxozoan, *Enteromyxum leei* Diamant, Lom et Dyková, 1994 (Montero *et al.*, 2007; Muñoz *et al.*, 2007; Álvarez-Pellitero *et al.*, 2008). Species of *Ceratomyxa* such as *C. sparusaurati* Sitjà-Bobadilla et Álvarez-Pellitero, 1995 in the gilthead seabream *Sparus aurata* L. (Palenzuela *et al.*, 1997) and *C. diplodae* in the sharpsnout seabream (Lubat *et al.*, 1989; Katharios *et al.*, 2007) have also been related to severe pathologies in Mediterranean sparids.

The most abundant group was the Monogenea, including eight species; all of them from the orobranchial cavity, usually on the gills (see table 2). Five of these species were monopisthocotyleans, four belonging to the genus *Lamellodiscus*. This genus is specific for the family Sparidae which can be parasitized by one or more species of *Lamellodiscus* (see Desdevises *et al.*, 2002). The fifth monopisthocotylean found in this study was the capsalid *Encotyllabe vallei* Monticelli, 1907, previously reported from the gills and pharynx of sharpsnout seabream and other sparids, including *S. aurata* (Radujkovic & Euzet, 1989). The remaining three monogenean species were microcotylid polyopisthocotyleans. Of these, *Atrispinum seminalis* has been recorded on the gills of five different species of *Diplodus* (see Gibson *et al.*, 2005), including the farmed sharpsnout seabream (Di Cave *et al.*, 2002; Athanassopoulou *et al.*, 2005). *Sparicotyle chrysophrii* is often recorded in wild and farmed *S. aurata* (e.g. Euzet & Audouin, 1959; Radujkovic & Euzet, 1989; Antonelli *et al.*, 2010a). Although this parasite has been found occasionally in farmed fish (Di Cave *et al.*, 2002), this study provides the first record in wild populations of sharpsnout seabream. The third microcotylid reported here, Microcotylidae gen. sp., has not been classified beyond family level since only one specimen was found, and its morphology does not correspond to any of the three genera previously reported in sharpsnout seabream, or to any other genus within the family (Mamaev & Parukhin, 1976; Maillard & Noisy, 1979; Mamaev, 1986; Radujkovic & Raibaut, 1989). Among the microcotylid species reported to date in sharpsnout seabream, *S. chrysophrii* appears most similar due to the presence of genital armature with three types of spines and a single vagina, and the lack of sclerotized plate. However, the number of the peripheral spines and the number of the haptoral clamps of *S. chrysophrii* are distinctly higher (Antonelli *et al.*, 2010a). More specimens of this morphotype should be examined in order to describe this possible new species.

Monopisthocotylean monogeneans represent a minor problem for the sparid cultures (Antonelli *et al.*, 2010b; Sánchez-García *et al.*, 2011). There are exceptional cases where some *Lamellodiscus* species have been related to

Table 7. The range of nucleotides (above the diagonal) and genetic distances calculated as percentages (below the diagonal) in ITS1 sequences of *Lamellodiscus ergensi* and *L. theroni s.l.* analysed in the present study (*L. ergensi* complex).

	KC470295	KC470296	KC470297	EU259056	EU259057	EU259058	EU259059
L. theroni s.l. KC470295	–	1	1	1	1	57	61
L. theroni s.l. KC470296	0.2	–	0	0	0	56	60
L. theroni s.l. KC470297	0.2	0.0	–	0	0	56	60
L. ergensi EU259056	0.2	0.0	0.0	–	0	56	60
L. ergensi EU259057	0.2	0.0	0.0	0.0	–	56	60
L. ergensi EU259058	13.3	13.3	13.3	13.1	13.1	–	8
L. ergensi EU259059	14.2	14.0	14.0	14.0	14.0	1.9	–

severe lesions and problems in some cultured sparids (Roubal, 1994), including the sharpsnout seabream (Katharios *et al.*, 2006). However, Sánchez-García *et al.* (2011) indicated that although the attachment mechanism of *Lamellodiscus* spp. seems quite traumatic (mostly due to the hooks piercing the gill epithelium) the damage provoked by these tiny parasites is mild and localized. In fact, despite the fact that some wild or captive sharpsnout seabream harboured more than 500 individuals of *Lamellodiscus* spp., fish were apparently healthy. In contrast, microcotylids, and *S. chrysophrii* in particular, represent a major parasitological problem in sparid cultures. This species is a well-known parasite of *S. aurata*, very often related to lethal epizootics in Mediterranean cultures (Faisal & Imam, 1990; Sanz, 1992; Sitjà-Bobadilla & Álvarez-Pellitero, 2009). *Sparicotyle chrysophrii* has also been reported to cause severe mortalities in sharpsnout seabream (Di Cave *et al.*, 2002). The finding of *S. chrysophrii* in wild sharpsnout seabream not only extends our knowledge on the occurrence of this emerging parasite, but also confirms that infected wild sharpsnout seabream can act as reservoirs of this monogenean for cultured gilthead seabream (Athanassopoulou *et al.*, 2005). No data exist on epizootics related to *A. seminalis*, but this parasite also seems potentially pathogenic, as its attachment and feeding strategies are similar to those of *S. chrysophrii*. Moreover, being monoxenous, it could also reach high loads in cultures.

With reference to digeneans, the derogenid *Magnibursatus bartolii* was found on gills or the oesophagus. As gills are not a usual infection site for digeneans, these parasites could have been regurgitated by fish; however, other hemiuroids, such as *Aponurus* spp. are frequently found on gills (Carreras-Aubets *et al.*, 2011). *Magnibursatus bartolii* has not been reported previously in sharpsnout seabream, but this parasite has been found in other sparids, such as *Boops boops* L. (the type-host) from the Spanish Mediterranean (Pérez-del-Olmo *et al.*, 2007) or *S. aurata* from Tunisia (Gargouri Ben Abdallah & Maamouri, 2008). More recently, a new species of *Magnibursatus*, *M. diplodii* Bayoumy & Abu-Taweel, 2012 was described in *Diplodus sargus* L. (Bayoumy & Abu-Taweel, 2012). The intestinal fellodistomids *Steringotrema pagelli* and *Proctoeces maculatus* were only found in fish from Mar Menor. This is the first record of *S. pagelli* in the sharpsnout seabream. *Proctoeces maculatus* has been cited previously in a survey of this fish off the Tunisian coast (Gargouri Ben Abdallah & Maamouri, 2008). This parasite has been recorded previously in other sparids (and interestingly also in one gobiid and pleuronectiforms, see Gibson *et al.*, 2005). The monorchid *Monorchis monorchis* and the opecoelid *Peracreadium characis* were previously reported in sharpsnout seabream, although only *P. characis* is strictly specific to this host (Bartoli *et al.*, 1989, 2005).

Metacercariae of *Cardiocephaloides longicollis* and *Galactosomum* sp. are reported for the first time from the brain of sharpsnout seabream. Although this is the first record of both parasites in sharpsnout seabream, it is not surprising since both exhibit low specificity towards second intermediate hosts (Naidenova & Mordvinova, 1997; Gibson *et al.*, 2005; Osset *et al.*, 2005, Culurgioni *et al.*, 2007). In heavy infections, parasites of the brain (including that of *C. longicollis*) have significant pathological effects as they can influence host behaviour in favour of parasite transmission to the final host (Chappel *et al.*, 1994; Lafferty, 2008; Fredensborg & Longoria, 2012). These species must be monitored, as they infect a vital organ, and could display synergic effects in heavy mixed infections.

The low number of crustacean parasites may be related to the loss of the individuals attached to skin during fish collection and processing (including handling or storage at −20°C). Although this is the first report of *Caligus ligusticus* in sharpsnout seabream, this species has been reported previously in sparid fish, including other *Diplodus* species, such as *D. sargus* (see Raibaut *et al.*, 1998 and references therein). Other caligids are known to parasitize sparid cultures (Ragias *et al.*, 2004; Mladineo, 2006) and they should be considered as potential pathogens for fish cultures as they have often been associated with important economic losses in farmed fish (Dawson, 1998; Lester & Hayward, 2006; Costello, 2009). *Lernanthropus vorax* has been often cited in wild sharpsnout seabream (Radujkovic & Raibaut, 1989; Raibaut *et al.*, 1998), and no pathological effects were reported. Nevertheless the presence of *L. vorax* should not be undervalued since other species of the same genus, such as *L. kroyeri* Van Beneden, 1851, have been frequently related to outbreaks and mortalities in sea bass culture (Yardimci & Pekmezci, 2012). The isopod *Gnathia vorax* was found for the first time on sharpsnout seabream. However, its presence is not surprising since gnathiid praniza larvae are non-specific, being found previously in other Mediterranean sparids (González *et al.*, 2004). The species of this family are considered as potential pathogens in culture conditions, due the anaemia provoked by haematophagus feeding (Marino *et al.*, 2004).

Parasite fauna and fish captivity

The current study provides useful information about the sharpsnout seabream parasites that can be transferred from the wild and survive in culture conditions. We observed that most parasites living in the external environment (i.e. ectoparasites and parasites of the digestive tract) disappeared under conditions of captivity, with the exception of *Lamellodiscus* spp. It is well known that most external parasites are highly susceptible to artificial culture conditions (Woo, 2006). Parasites on the skin and gills are affected by water quality and fish health, and parasites in the digestive tract are also affected by the food supplied in cultures. In contrast, parasites living in the blood and tissues can survive in such internal environments. The prevalence and abundance of metacercariae of *C. longicollis* did not vary significantly in captivity. Moreover, this was the only digenean found in fish captive for 20 days, probably because the metacercariae were protected within the brain tissues and are normally resistant encysted stages with little metabolic activity (Paperna & Dzikowski, 2006). A similar situation was observed for the myxozoan *Ceratomyxa* sp. protected within the gall bladder.

The most problematic parasites are usually the monoxenous species such as *Lamellodiscus* spp., which survive in cultures and are able to re-infect fish, reaching abnormally high parasite burdens. However, the importance of heteroxenous parasites should not be

underestimated, because in the surroundings of the sea cages or in the nets a substantial number of crustaceans and polychaetes that could act as intermediate/alternate hosts may be present.

The information provided in this study allows us to conclude which parasites could be a risk to sharpsnout seabream culture. There are three main groups (myxozoans, monogenean microcotylids and crustaceans) that should be taken into consideration for the culture of this fish. The myxozoan *Enteromyxum leei* was not found in the present study, although it has been reported in numerous cases, both in the wild and in culture (Le Breton & Marques, 1995; Merella *et al.*, 2005; Álvarez-Pellitero *et al.*, 2008), even in cultures in the same location (San Pedro del Pinatar) in Mar Menor (Montero *et al.*, 2007). The microcotylid *S. chrysophrii* is an important finding. Its presence could cause respiratory dysfunction due to the epithelial damage and anaemia (Sitjà-Bobadilla & Álvarez-Pellitero, 2009). More crucial is the confirmation that wild sharpsnout seabream harbour one of the most damaging parasites in cultured *S. aurata* and therefore can act as reservoirs. The same effect could be expected in infections with other microcotylids such as *A. seminalis*. In fact, Merella *et al.* (2005) reported an epizootic induced by the high prevalence and intensity of the microcotylid *Atrispinum salpae* (Parona & Perugia, 1890) on cultured sharpsnout seabream. The third important group to take into consideration are the crustaceans. Although their numbers were quite low and they disappeared after 20 days of captivity, species of families such as caligids can provoke severe pathological problems. In fact, the main problem for salmon mariculture is the parasitosis caused by the caligid *Lepeophteirus salmonis* (see Johnson & Fast, 2004). Moreover, in Mediterranean cultures crustaceans have been reported to provoke severe damage of different fish species (Costello, 2009; Yardimci & Pekmezci, 2012).

In view of the significant economic value of this fish, a detailed risk assessment study of sharpsnout seabream would be necessary to relaunch the culture of this species, minimizing possible future parasite problems. It should also be noted that most parasites of sharpsnout seabream recorded in present study are shared by the main fish species in Mediterranean aquaculture, such as the gilthead seabream. The proximity of cages containing this fish species could clearly result in cross-infections.

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Conflict of interest

None.

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CHAPTER 5

Risk assessment for parasites in cultures of the sharpsnout seabream *Diplodus puntazzo* (Sparidae) in the Spanish Mediterranean. Prospects of cross infections with *Sparus aurata*

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Risk assessment

ABSTRACT

The sharpsnout seabream *Diplodus puntazzo* is of interest in Mediterranean fish farming. Disease is an important problem because parasites can spread quickly in culture conditions and fish often develop high parasite burdens. Here we assess the risk that documented parasites pose to the sustainability of *D. puntazzo* farming. This study specifically considers metazoan and protist parasites recorded from wild and farmed *D. puntazzo* in scientific literature. Risk assessment studies involve the identification, characterization and qualitative quantification of the risk in question (parasitoses in this case) and the probability of establishment. We considered the parasite species which may be difficult to manage as a priority for research into potential management strategies. Those parasites which could be transmitted from cultures of gilthead seabream (*Sparus aurata*) were also included in this study. Four groups of parasites represented a risk to *D. puntazzo* farming, ranging from moderate to high: Ciliophora, Myxozoa, Monogenea and Copepoda. Three parasite species were considered high risk to *D. puntazzo* cultures: *Amyloodinium* sp., *Cryptocaryon* sp. and *Enteromyxum leei*. These species were responsible for high mortalities in cultures of these and other fish species. In addition *Sparicotyle chrysophrii*, *Caligus ligusticus* and *Gnathia vorax* entail a moderate risk to *D. puntazzo* Mediterranean farms. No important episodes have been related to caligids and isopods in Mediterranean sparids, nevertheless they should be properly managed to prevent future problems.

1. Introduction

The main fish species bred in Mediterranean aquaculture are the European seabass *Dicentrarchus labrax* L. (Moronidae) and even more so the gilthead seabream *Sparus aurata* L. (Sparidae) (APROMAR, 2010). In this scenario, market diversification is encouraged by researchers, producers and related institutions. The sharpsnout seabream *Diplodus puntazzo* Cetti, 1777 is one of the best options for diversification given its growth rate and market value. Moreover, its physiological requirements are similar to those of *S. aurata*, the main species farmed in the Mediterranean and a very well-known culture model (Abellán and Basurco, 1999). Diseases are important constraints to developing new cultures as cultured fish often develop higher parasitic burdens than wild ones, as fish-farming conditions promote infection by some parasite species (McVicar, 1997). During the first years of experimental culture of *D. puntazzo* at least three parasite species have caused substantial mortality rates: the ciliate *Cryptocaryon irritans* Brown, 1951, the myxozoan *Enteromyxum leei* (Diamant, Lom et Dyková,

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1994) and the monogenean *Sparicotyle chrysophrii* (Van Beneden et Hesse, 1863) (Le Breton and Marques, 1995; Athanassopoulou et al., 1999; Golomazou et al., 2004; Merella et al., 2005; Montero et al., 2007). Although these species belong to very different parasite groups, they all have fish-to-fish transmission mechanisms, which increase parasite burdens exponentially in high density cultures (Woo, 2006). Around 50 parasite species have been reported to date in *D. puntazzo* (see references in Table 1), and a recent study found 19 parasite species in wild *D. puntazzo* from the Spanish Mediterranean, nine of which were recorded for the first time (Sánchez-García et al., 2013). Although some of these species are known pathogens of *D. puntazzo* and other fish cultures, the potential risk of each parasite must be analysed to identify sources and determine which species could hamper these cultures.

The European Food Safety Authority (EFSA) is an independent agency commissioned by the European Union (EU) for food and feed safety risk assessment. According to EFSA "risk assessment is a scientifically-based process consisting in the evaluation, either qualitatively or quantitatively, of the probability and the potential impact of some hazard" (EFSA, 2008). Risk assessment studies should also be well-documented, flexible, consistent and open to review (Miller et al., 1993). These types of studies have been performed for protist and metazoan parasites of aquaculture fishes from different countries (e.g., Akoll et al., 2012; Hutson et al., 2007; Nowak, 2004). We would highlight the case of Australia, where several guidelines for risk analysis have been published (AQIS, 1999; Fletcher et al., 2004; DAFF, 2011). One of the EFSA (2009a) guidelines states the need for risk assessment research in the context of fish aquaculture welfare, providing methodological guidance to assess risk for animal welfare and health in European countries (EFSA, 2008, 2009a,b). However, risk assessment studies are lacking for fish species of Mediterranean aquaculture.

The present study aims to assess parasitological risks in *D. puntazzo* in the Mediterranean. This fish is not only interesting due to its suitability to aquaculture but also because of the many potential cross infections between *D. puntazzo* and *S. aurata*, as both hosts share many parasite species (Gibson et al., 2005; Sánchez-García et al., 2013). For this reason we qualitatively assessed the risk to sustainability of Mediterranean *D. puntazzo* farming based on its documented parasites and pathologies, as well as those affecting *S. aurata*. This is the first risk assessment for pathogens in Mediterranean fish farming.

2. Material and methods

Qualitative risk assessment has been ascertained from the bibliography (EFSA, 2008), which establishes risk categories. Following EFSA guidelines, first we identified the most likely parasites to proliferate in aquaculture (*Hazard identification*). Then we estimated the risk posed by those parasite species which may be difficult to control and have immediate priority for management strategies (*Hazard characterization and exposure assessment*). Finally, we considered treatment options for each parasite, according to scientific and specialized bibliography (Schmahl and Mehlhorn, 1985, 1988; Schmahl and Taraschewsh, 1987; Schmahl et al., 1988; Woo, 2006; Noga, 2009).

2.1. Hazard identification

All parasites found in both in wild and farmed Mediterranean *D. puntazzo* were categorized. Those parasites present in farmed *S. aurata* were also included considering the probability of cross infections between wild and farmed fish, or between farmed fish reared in neighbouring cages (Athanassopoulou et al., 2005; Golomazou et al., 2006; Mladineo and Maršić-Lučić, 2007; Montero et al., 2007). Parasite records were taken from the literature and the Host-Parasite Database of the Natural History Museum, London (Gibson et al., 2005).

2.2. Hazard characterization and exposure assessment

We qualitatively estimated the risk of the parasites becoming potential pathogens. Five categories (from negligible to extreme) were established. Risk was identified for each parasite species by combining the categories obtained for: (1) the *consequence* and (2) the *probability* of becoming established. As the EFSA does not provide specific guidelines to calculate these categories, we have used the matrix provided by the Australian Government Department of Agriculture (DAFF) (see Table 1 in AFFA, 2001).

2.2.1. Consequence of entry and establishment

The *consequence* of parasite establishment was determined using four descriptive scales to categorize the degree of potential damage they could cause to *D. puntazzo* (and *S. aurata*). Four risk factors were considered: (1) marketability; (2) sublethal effects on fish; (3) mass mortalities; and (4) consumer health. Parasites presenting all four factors entail *extreme consequence*; three factors imply *high consequence*; two factors *moderate consequence*; one factor *low consequence*; no factors *negligible consequence*. The data were obtained from previous studies on *D. puntazzo* and *S. aurata* parasites (see references in Tables 1 and 2).

2.2.2. Probability of establishment

We estimated the *probability* of a pathogen transfer to farmed fish considering the *pathway* (route of infection), and exposure assessment. We established the final probability of parasite establishment and proliferation in farmed *D. puntazzo* by combining exposure and infection route assessments using a matrix for combining descriptive probabilities (Table 5 in AFFA, 2001). We again used the matrix suggested by DAFF as no guidelines are provided by EFSA. *Probability* categories ranged from negligible to extreme.

2.2.2.1. Exposure assessment. To estimate exposure, we considered the probability of a parasite occurring in *D. puntazzo* farming areas. The five qualitative levels of farmed-fish exposure to parasites from wild and farmed *D. puntazzo* (and farmed *S. aurata*) were ranked from negligible to extreme. *D. puntazzo* is a sedentary and gregarious species (like *S. aurata*), and parasite exchanges from long distance migrations are unexpected (Abellán and Basurco,

Table 1
Parasites of wild and farmed *Diplodus puntazzo* in the Mediterranean Sea.

Group	Taxon	Microhabitat	Origin	Identified in Spain	Zone (North/South)	References
APICOMPLEXA	*Eimeria* sp.	Gut/Spleen	Farm	No	N	Álvárez-Pellitero et al. (1995), Athanassopoulou et al. (1999)
DINOFLAGELLATA	*Amyloodinium ocellatum*	Gills/Skin	Farm	No	N	Mladineo (2006)
CILIOPHORA	*Cryptocaryon irritans*	Gills/Skin	Farm	Yes	N	Montero et al. (2007)
	Trichodinia sp.	Gills	Farm	No	N	Rodgers and Furones (1998), Toksen (2006)
KINETOPLASTIDA	*Cryptobia* sp.	Gills	Farm	No	N	Feng et al. (1997), Toksen (2006), Guo et al. (2009)
CNIDARIA, MYXOZOA	*Ceratomyxa* sp.	Gall bladder	Wild/Farm	Yes	N	Angelucci et al. (2008), Sánchez-García et al. (2013)
	Ceratomyxa diplodae	Gall bladder	Farm	Yes	N	Merella et al. (2005), Mladineo (2006), Rigos and Katharios (2010)
	Ceratomyxa puntazzi	Gall bladder	Wild	Yes	N	Alama-Bermejo et al. (2011)
	Ceratomyxa sparusaurati	Gall bladder	Farm	No	N	Mladineo (2006)
	Enteromyxum leei	Intestine	Farm	Yes	N	Montero et al. (2007), Álvárez-Pellitero et al. (2008)
	Myxobolus sp.	Intestine	Farm	No	N	Golomazou et al. (2009)
	Polysporoplasma sparis	Kidney	Farm	No	N	Sitja-Bobadilla and Álvárez-Pellitero (2001), Mladineo (2006)
MONOGENEA						
Monopisthocotylea	*Encotyllabe vallei*	Gills	Wild	Yes	N	Sánchez-García et al. (2013)
	Furnestinia echeneis	Gills	Farm	No	N	Quaglio et al. (2007), Rigos and Katharios (2010)
	Lamellodiscus bidens	Gills	Wild/Farm	Yes	N	Katharios (2006), Sánchez-García et al. (2013)
	L. elegans	Gills	Wild	No	N	Mladineo (2007)
	L. ergensi	Gills	Wild/Farm	No	N/S	Radujkovic and Euzet (1989), Katharios (2006)
	L. falcus	Gills	Wild	Yes	N	Sánchez-García et al. (2013)
	L. hilii	Gills	Wild/Farm	Yes	N/S	Di Cave (1998), Sánchez-García et al. (2013)
	L. ignoratus	Gills	Wild/Farm	No	N	Radujkovic and Euzet (1989), Merella et al. (2005)
	L. impervious	Gills	Wild	No	S	Euzet (1984)
	L. theroni	Gills	Wild	Yes	N	Sánchez-García et al. (2013)
Polyopisthocotylea	*Atriaster heterodus*	Gills	Wild	Yes	N	Sánchez-García et al. (2013)
	Atrispinum acarne	Gills	Wild	Yes	N	Sánchez-García et al. (2013)
	A. salpae	Gills	Wild/Farm	No	N	Radujkovic and Euzet (1989), Merella et al. (2005)
	A. seminalis	Gills	Farm	No	N	Di Cave et al. (2003), Athanassopoulou et al. (2005)
	Polylabris sp.	Gills	Farm	No	N	Quaglio et al. (2007)
	P. tubicirrus	Gills	Farm	No	N	Di Cave (2005)
	Sparicotyle chrysophrii	Gills	Wild/Farm	Yes	N	Di Cave (2003), De Vico (2008), Mladineo (2007), Sánchez-García et al. (2013)

Table 1 (*Continued*)

Group	Taxon	Microhabitat	Origin	Identified in Spain	Zone (North/South)	References
TREMATODA	*Allopodocotyle pedicellata*	Intestine	Wild	No	N	Papoutsoglou (1976)
	Cardiocephaloides longicollis	Brain	Wild	Yes	N	Chappel (1994), Sánchez-García et al. (2013)
	Galactosomum sp.	Brain	Wild	Yes	N	Sánchez-García et al. (2013)
	Lepocreadium album	Intestine	Wild	No	N	Papoutsoglou (1976)
	Macvicaria crassigula	Intestine	Wild	No	N	Papoutsoglou (1976)
	Magnibursatus bartolii	Gills	Wild	Yes	N	Sánchez-García et al. (2013)
	Monorchis monorchis	Pyloric caeca	Wild	Yes	N	Sánchez-García et al. (2013)
	Peracreadium characis	Intestine	Wild	Yes	N	Sánchez-García et al. (2013)
	Proctoeces maculatus	Intestine	Wild	Yes	N	Sánchez-García et al. (2013)
	Pseudopycnadena fischthali	Intestine	Wild	No	N	Gargouri Ben Abdallah and Maamouri (2008)
	Steringotrema pagelli	Intestine	Wild	Yes	N	Sánchez-García et al. (2013)
COPEPODA	*Caligus ligusticus*	Gills	Wild	Yes	N	Sánchez-García et al. (2013)
	C. minimus	Gills	Farm	No	N	Mladineo (2006)
	Clavellotis characis		Wild	No	N	Radujkovic and Raibaut (1989)
	Colobomatus agasizzi	Frontal sinus	Wild	No	N	Raibaut et al. (1998)
	Lernaelophus sultanus	Skin	Wild	No	N	Varvarigos (2007)
	Lernanthropus brevis	Gills	Wild/Farm	Yes	N	Merella et al. (2005), Merella et al. (2005), Sánchez-García et al. (2013)
	Pseudoencanhus kerkennensis		Wild	No	N	Raibaut et al. (1998)
ISOPODA	*Gnathia vorax*	Gills	Wild	Yes	N	Marino et al. (2004), Sánchez-García et al. (2013)

1999). The level assigned depended on the geographical location of the parasites, taking into account the trade relations between northern and southern Mediterranean countries. Low parasite exposure levels have been assigned to parasites of wild or farmed *D. puntazzo* from southern Mediterranean countries, due to the low levels of importation of aquaculture fish from African countries. We also considered that *D. puntazzo* fry are currently produced in a few Mediterranean farms, mainly in Greece and Turkey, and distributed to fish farms in other northern Mediterranean countries (FEAP, 2008). Parasites from both wild and farmed *D. puntazzo* from the northern Mediterranean (excluding Spain) present a moderate probability of exposure, whereas parasites from wild and farmed *D. puntazzo* reported specifically in Spain are considered to pose an extreme exposure level. Similarly, farmed *S. aurata* parasites from any Mediterranean country outside Spain are considered to pose a low probability of exposure, while exposure of parasites present in *S. aurata* from Spanish Mediterranean farms is considered high, as farming areas of this species and *D. puntazzo* overlap.

2.2.2.2. Pathway assessment. The five qualitative levels to evaluate pathway assessment were also ranked from negligible to extreme, considering the route of infection (Hutson et al., 2007). Parasites known to infect *S. aurata* alone (and not *D. puntazzo*) would present a minimal risk to *D. puntazzo* and were considered to possess a negligible pathway risk. Low pathway risk corresponded to heteroxenous parasites with complex indirect life cycles requiring two or more specific intermediate host species. These parasites would have a limited ability to establish and proliferate in farmed fish. The probability of infection would increase for heteroxenous parasites with only one intermediate host species to complete their life cycle, particularly when the other hosts are close to sea-cages. Those parasite species were considered to present a moderate pathway risk for farmed fish. High level risk would correspond to parasites with direct life cycles and common pathogens in aquaculture able to reproduce rapidly. Parasites already established in farmed *D. puntazzo* populations in Western Mediterranean farms were considered to present an extreme pathway risk.

3. Results

Parasites in wild and farmed *Diplodus puntazzo* and farmed *Sparus aurata* previously reported in the Mediterranean are shown in Tables 1 and 2, respectively. Monogenea was the most diverse group in both fish species: 17 species in *D. puntazzo* and nine in *S. aurata*. The second most diverse group was Digenea, with 11 species

Table 2
Parasites of farmed *Sparus aurata* in the Mediterranean Sea.

Group	Taxon	Microhabitat	Identified in Spain	Zone (North/South)	References
APICOMPLEXA	*Eimeria* sp.*	Intestine	Yes	N	Álvárez-Pellitero et al. (1995)
	Eimeria sparis	Intestine	Yes	N	Álvárez-Pellitero et al. (1995), Sitjà-Bobadilla et al. (1996), Álvárez-Pellitero et al. (1997)
	Goussia sparis	Intestine	Yes	N	Sitjà-Bobadilla et al. (1996), Álvárez-Pellitero et al. (1997)
DINOFLAGELLATA	*Amyloodinium* sp.	Gills	Yes	N	Paperna et al. (1980), Álvárez-Pellitero et al. (1995)
	*Amyloodinium ocellatum**	Gills	No	N	Paperna et al. (1980), Fioravanti et al. (2006)
CILIOPHORA	Unidentified ciliates	Gills	Yes	N	Álvárez-Pellitero et al. (1995)
	*Cryptocaryon irritans**	Gills/Skin	No	N	Fioravanti (2006)
	Trichodinia sp.*	Gills	Yes	N	Álvárez-Pellitero (1995)
CNIDARIA, MYXOZOA	*Ceratomyxa* sp.*	Gall bladder	Yes	N	Álvárez-Pellitero et al. (1995)
	*Ceratomyxa sparusaurati**	Gall bladder	No	N	Palenzuela et al. (1997)
	*Enteromyxum leei**	Intestine	Yes	N	Fioravanti et al. (2006)
	Kudoa sp.	Mesenteries	No	N	Paperna (1982), Golomazou et al. (2006)
	Leptotheca sp.	Kidney	Yes	N	Álvárez-Pellitero et al. (1995)
	L. sparidarium	Kidney	Yes	N	Sitjà-Bobadilla and Álvárez-Pellitero (2001)
	*Polysporoplasma sparis**	Kidney	Yes	N	Palenzuela et al. (1999)
MONOGENEA					
Monopisthocotylea	*Encotyllabe vallei**	Gills	Yes	N	Fernández-Jover et al. (2010)
	Dactylogyrus sp.	Gills	No	N	Rodgers and Furones (1998), Abdelmonem et al. (2010)
	*Furnestinia echeneis**	Gills	Yes	N	Sánchez-García et al. (2011)
	Gyrodactylus sp.	Gills	No	N	Colorni (1989), De Liberato et al. (2000)
	G. orecchiae	Gills	No	N	Paladini et al. (2009)
	*L. elegans**	Gills	No	N	Mladineo (2006)
Polyopisthocotylea	*Atrispinum salpae**	Gills	No	N	De Liberato et al. (2000)
	*Polylabris tubicirrus**	Gills	No	N	Athanassopoulou et al. (2005)
	*Sparicotyle chrysophrii**	Gills	Yes	N	Sitjà-Bobadilla and Álvárez-Pellitero (2009)
NEMATODA	*Contracaecum* sp.	Intestine	No	N	Salati et al. (2013)
	Hysterothylacium aduncum	Digestive tract	No	S	Kalay et al. (2009)
TREMATODA	*Allopodocotyle pedicellata**	Intestine	No	N	Mariniello et al. (2000)
	Aporocotilidae sp.	Gills	Yes	N	Padros et al. (2001), Fioravanti (2006)
	Cardicola aurata	Gills	Yes	N	Padrós et al. (2001); Holzer et al. (2008)
	Lepocreadium pegorchis	Intestine	No	N	Mariniello et al. (2000)
	Macvicaria maillardi	Intestine	No	N	Mariniello et al. (2000)
	M. obovata	Intestine	No	N	Mariniello et al. (2000)
	*Monorchis monorchis**	Pyloric caeca	No	N	Mariniello et al. (2000)
	Pycaenoide senegalensis	Intestine	No	N	Mariniello et al. (2000)
COPEPODA	*Caligus* sp.	Gills	No	N	Fioravanti et al. (2006)
	Ergasilus sp.	–	No	N	Fioravanti et al. (2006)
ISOPODA	*Anilocra physodes*	Gills/Mouth	No	N	Smit et al. (2004), Kayis et al. (2009)
	Ceratothoa oestroides	Gills/Mouth	No	N	Mladineo (2006)
	C. parallela	Gills/Mouth	No	N	Papanagiotou and Trilles (2001)

* Species of parasites also found in *Diplodus puntazzo*.

identified in *D. puntazzo* and eight in *S. aurata*. Sixteen parasite species reported in farmed *S. aurata* have also been reported in *D. puntazzo*.

Rank of consequence and probability of establishment for each parasite species are shown in Tables 3 and 4, respectively. No species of parasites were considered to entail an extreme *consequence*, whereas high *consequence* was assigned to *Amyloodinium* spp. (Dinoflagellata), *Cryptocaryon irritans* (including the unidentified *Cryptocaryon*-like parasites) (Ciliophora) and *Enteromyxum leei* (Myxozoa). Several parasites posed an extreme *probability* of establishment and proliferation: one ciliate species (*Cryptocaryon irritans*), three myxozoan species (*Ceratomyxa* sp., *Ceratomyxa puntazzi* Alama-Bermejo, Raga et Holzer, 2011 and *E. leei*), three monogenean species (*Lamellodiscus bidens* Euzet, 1984, *L. hilii* Euzet, 1984 and *Sparicotyle chrysophrii*), and one copepod species (*Lernanthropus brevis*).

Table 3
Consequence of parasite establishment for *Diplodus puntazzo* Mediterranean cultures.

Parasite taxa	Marketability	Pathology	Potential mass mortality	Consumer health	Consequence
APICOMPLEXA					
Eimeria sp.	–	X	X	–	Moderate
E. sparis	–	X	–	–	Low
Goussia sparis	–	–	–	–	Negligible
DINOFLAGELLATA					
Amyloodinium sp.	X	X	X	–	High
Amyloodinium ocellatum	X	X	X	–	High
CILIOPHORA					
Unidentified ciliates	X	X	X	–	High
Cryptocaryon irritans	X	X	X	–	High
Trichodina sp.	–	X	–	–	Low
KINETOPLASTIDA					
Cryptobia sp.	–	X	–	–	Low
CNIDARIA, MYXOZOA					
Ceratomyxa sp.	–	–	–	–	Negligible
Ceratomyxa diplodae	–	–	–	–	Negligible
Ceratomyxa sparusaurati	X	X	–	–	Moderate
Ceratomyxa puntazzi	–	X	–	–	Low
Enteromyxum leei	X	X	X	–	High
Kudoa sp.	X	X	–	–	Moderate
Leptotheca sp.	–	–	–	–	Negligible
L. sparidarum	–	X	–	–	Low
Myxobolus sp.	–	X	–	–	Low
Polysporoplasma sparis	–	X	–	–	Low
MONOGENEA					
Monopisthocotylea					
Dactylogyrus sp.	–	X	–	–	Low
Encotyllabe vallei,	–	–	–	–	Negligible
Furnestinia echeneis	–	X	–	–	Low
Gyrodactylus sp.	–	X	–	–	Low
G. orecchidae	–	X	–	–	Low
Lamellodiscus bidens	–	X	–	–	Low
L. elegans	–	–	–	–	Negligible
L. ergensi	–	X	–	–	Low
L. falcus	–	X	–	–	Low
L. hilii	–	–	–	–	Negligible
L. ignoratus	–	–	–	–	Negligible
L. impervius	–	–	–	–	Negligible
L. theroni	–	X	–	–	Low
Polyopisthocotylea					
Atriaster heterodus	–	–	–	–	Negligible
Atrispinum acarne	–	–	–	–	Negligible
A. salpae	–	X	X	–	Moderate
A. seminalis	–	X	X	–	Moderate
Polylabris sp.	–	X	–	–	Low
Polylabris tubicirrus	–	X	–	–	Low
Sparicotyle chrysophrii	–	X	X	–	Moderate
TREMATODES					
Allopodocotyle pedicellata	–	–	–	–	Negligible
Aporocotylidae sp.	–	X	–	–	Low
Cardicola aurata	–	X	–	–	Low
Cardiocephaloides longicollis	–	X	–	–	Low
Galactosomum sp.	–	–	–	–	Negligible
Lepocreadium album	–	–	–	–	Negligible
Macvicaria crassigula	–	–	–	–	Negligible
M. maillardi	–	–	–	–	Negligible
M. obovata	–	–	–	–	Negligible
Magnibursatus bartolii	–	–	–	–	Negligible
Monorchis monorchis	–	–	–	–	Negligible
Peracreadium characis	–	–	–	–	Negligible
Proctoeces maculatus	–	–	–	–	Negligible
Pseudopycnadena fischthali	–	–	–	–	Negligible
Steringotrema pagelli	–	–	–	–	Negligible
NEMATODA					
Contracaecum sp.	–	–	–	X	Low
Hysterothylacium aduncum	–	–	–	–	Negligible
COPEPODA					
Caligus sp.	X	X	–	–	Moderate
C. ligusticus	X	X	–	–	Moderate
C. minimus	X	X	–	–	Moderate

Table 3 (*Continued*)

Parasite taxa	Marketability	Pathology	Potential mass mortality	Consumer health	Consequence
Clavellotis characis	X	–	–	–	Low
Colobomatus agassizi	–	–	–	–	Negligible
Ergasilus sp.	–	–	–	–	Negligible
Lernaeolophus sultanus	X	X	–	–	Moderate
*Lernanthropus brevis*Syn. *Lernanthropus vorax*	–	X	–	–	Low
Pseudoencanthus kerkennensis	–	–	–	–	Negligible
ISOPODA					
Anilocra physodes	X	X	–	–	Moderate
Ceratothoa oestroides	X	X	–	–	Moderate
C. parallela	X	X	–	–	Moderate
Ganthia vorax	X	X	–	–	Moderate

Results of the risk study (Table 4) indicated that *Amyloodinium* spp., *C. irritans* and *E. leei* entail high risk to sharpsnout seabream cultures in Spain. The dinoflagellata *Amyloodinium ocellatum* (Brown, 1931), the myxozoa *C. sparusaurati* Sitjà-Bobadilla, Palenzuela and Álvarez-Pellitero, 2007, the monogenean *S. chrysophrii*, the isopod *Gnathia vorax* (Lucas, 1849) and the copepod *Caligus ligusticus* Brian, 1906 would represent a moderate risk. Almost all these parasitic species, with the exception of myxozoans, can be treated with antiparasitic drugs with different efficiency rates.

4. Discussion

This is the first risk analysis made in European aquaculture, implementing the EFSA protocols for marine cultures. The methodology used is directly applicable to any farmed aquatic species. Risk assessment studies of one particular species have been exclusively based on data reported for this species, but we recommend considering the information about pathogens of neighbouring fish species, especially for closely phylogenetically related species such as *D. puntazzo* and *S. aurata*. It is important to consider that risk assessment was based on currently available information on parasite presence and distribution; however, some parasite species may not have been detected in wild and farmed *D. puntazzo* populations before and, therefore, further revisions including latest records are to be recommended in the future.

The potential risk of each parasite group will be discussed separately.

4.1. Protists

The protist parasites referred herein, correspond to four different phyla, and only two of them pose a risk to *D. puntazzo* cultures: Ciliophora (*Cryptocaryon* sp. and unidentified *Cryptocaryon*-like ciliates) and Dinoflagellata (*Amyloodinium* sp. and *A. ocellatum*). *C. irritans* is a harmful pathogen, emergent in the Mediterranean, extremely virulent, highly unspecific and with direct life cycle (Noga, 2009). There is extreme to high *probability* of exposure to *C. irritans* and a high *consequence* for Spanish Mediterranean farmed *D. puntazzo*, as it has previously caused mass mortality and important economic losses in farming of this fish (Montero et al., 2007). This parasite is difficult to eradicate, as topical treatments are barely effective because many stages are encapsulated or protected within fish skin and can survive and reinfect fish (Noga, 2009). The second type of protist parasites which entail a high risk to *D. puntazzo* cultures are *Amyloodinium* spp., which are considered important due to their high pathogenicity and difficult control (Alvárez-Pellitero et al., 1995; Rodgers and Furones, 1998; Noga, 2009). Although pathologies related to *Amyloodinium* spp. have not been reported in *D. puntazzo* cultures to date, these unspecific parasites should be monitored to prevent future diseases in fish farms. Similarly to ciliate parasites, useful treatments against dinoflagellates mostly affect free-living stages. Prophylactic measures are the most effective to prevent parasites from entering cultures.

4.2. Myxozoa

Myxozoans are normally heteroxenous parasites, whose development in cultures depends on alternative host proximity (Yokoyama et al., 2012). Of the 10 myxozoan species described in *D. puntazzo* and *S. aurata*, only *Ceratomyxa sparusaurati* Sitjà-Bobadilla, Palenzuela et Alvárez-Pellitero, 2007 and *Enteromyxum leei* entail moderate and high risks, respectively to *D. puntazzo* cultures (Table 4). These two parasites have often been recorded and identified in cultures of both species, which indicates that their life cycles can be completed in culture conditions. *Ceratomyxa* spp. have been recovered from the *D. puntazzo* gall bladder, mostly in farmed fish (Table 1), and they have also been reported from different cultivable fishes of the Serranidae, Sparidae, Mugilidae, etc. (Lubat et al., 1989; Paperna, 1991; Alvárez-Pellitero and Sitjà-Bobadilla, 1993; Sitjà-Bobadilla and Alvárez-Pellitero, 1993). Although external signs of *Ceratomyxa* spp. infections are seldom found, damage to the gall bladder has only been described in *D. labrax* infection by *C. labracis* Sitjà-Bobadilla and Alvárez-Pellitero, 1993 and *C. diplodae* Lubat, Radujkovic, Marques et Bouix, 1989 (Alvárez-Pellitero and Sitjà-Bobadilla, 1993). Moreover, *C. sparusaurati* can cause massive infection of *S. aurata*, associated with trickling mortalities (Palenzuela et al., 1997). *E. leei* is an extremely virulent intestinal parasite, with low host specificity and, unlike the other myxozoans, has a direct life cycle (Golomazou et al., 2004). This parasite is an emergent pathogen, responsible for serious outbreaks in Mediterranean *D. puntazzo* (Alvárez-Pellitero et al., 2008). The *probability* of establishment of other

Table 4
Parasite risk analysis for *Diplodus puntazzo* sea-cage aquaculture in Spanish Mediterranean.

Parasite taxa	Exposure	Pathway	Probability	Consequence	Risk	Ability to treat
APICOMPLEXA						
Eimeria sp.	Moderate	Extreme	Moderate	Moderate	Low	No
E. sparis	High	Negligible	Negligible	Low	Negligible	No
Goussia sparis	High	Negligible	Negligible	Negligible	Negligible	No
DINOFLAGELLATA						
Amyloodinium sp.	High	Extreme	High	High	High	Yes
Amyloodinium ocellatum	Moderate	Extreme	Moderate	High	Moderate	Yes
CILIOPHORA						
Unidentified ciliates	High	Extreme	High	High	High	Yes
Cryptocaryon irritans	Extreme	Extreme	Extreme	High	High	Yes
Trichodina sp.	High	Extreme	High	Low	Low	Yes
KINETOPLASTIDA						
Cryptobia sp.	Moderate	Extreme	Moderate	Low	Negligible	Yes
CNIDARIA, MYXOZOA						
Ceratomyxa sp.	Extreme	Extreme	Extreme	Negligible	Negligible	No
Ceratomyxa diplodae	Moderate	Extreme	Moderate	Negligible	Negligible	No
Ceratomyxa sparusaurati	High	Extreme	High	Moderate	Moderate	No
Ceratomyxa puntazzi	Extreme	Extreme	Extreme	Low	Low	No
Enteromyxum leei	Extreme	Extreme	Extreme	High	High	No
Kudoa sp.	Low	Negligible	Negligible	Moderate	Negligible	No
Leptotheca sp.	High	Negligible	Negligible	Negligible	Negligible	No
L. sparidarum	High	Negligible	Negligible	Low	Negligible	No
Myxobolus sp.	High	Extreme	High	Low	Low	No
Polysporoplasma sparis	High	Extreme	High	Low	Low	No
MONOGENEA						
Monopisthocotylea						
Dactylogyrus sp.	Low	Negligible	Negligible	Low	Negligible	Yes
Encotyllabe vallei	Extreme	High	High	Negligible	Negligible	Yes
Furnestinia echeneis	High	Extreme	High	Low	Low	Yes
Gyrodactylus sp.	Low	Negligible	Negligible	Low	Negligible	Yes
G. orecchidae	Low	Negligible	Negligible	Low	Negligible	Yes
Lamellodiscus bidens	Extreme	Extreme	Extreme	Low	Low	Yes
L. elegans	Moderate	Extreme	Moderate	Negligible	Negligible	Yes
L. ergensi	Moderate	Extreme	Moderate	Low	Negligible	Yes
L. falcus	Extreme	High	High	Low	Low	Yes
L. hilii	Extreme	Extreme	Extreme	Negligible	Negligible	Yes
L. ignoratus	Moderate	Extreme	Moderate	Negligible	Negligible	Yes
L. impervius	Low	High	Low	Negligible	Negligible	Yes
L. theroni	Extreme	High	High	Low	Low	Yes
Polyopisthocotylea						
Atriaster heterodus	Extreme	High	High	Negligible	Negligible	Yes
Atrispinum acarne	Extreme	High	High	Negligible	Negligible	Yes
A. salpae	Moderate	Extreme	Moderate	Moderate	Low	Yes
A. seminalis	Moderate	Extreme	Moderate	Moderate	Low	Yes
Polylabris sp.	Moderate	Extreme	Moderate	Low	Negligible	Yes
Polylabris tubicirrus	Moderate	Extreme	Moderate	Low	Negligible	Yes
Sparicotyle chrysophrii	Extreme	Extreme	Extreme	Moderate	Moderate	Yes
TREMATODA						
Allopodocotyle pedicellata	Moderate	Low	Low	Negligible	Negligible	No
Aporocotylidae sp.	High	Moderate	Moderate	Low	Negligible	No
Cardicola aurata	High	Moderate	Moderate	Low	Negligible	No
Cardiocephaloides longicollis	Extreme	Moderate	Moderate	Low	Negligible	No
Galactosomum sp.	Extreme	Low	Low	Negligible	Negligible	No
Lepocreadium album	Moderate	Low	Low	Negligible	Negligible	No
Macvicaria crassigula	Moderate	Low	Low	Negligible	Negligible	No
M. maillardi	Low	Negligible	Negligible	Negligible	Negligible	No
M. obovata	Low	Negligible	Negligible	Negligible	Negligible	No
Magnibursatus bartolii	Extreme	Low	Low	Negligible	Negligible	No
Monorchis monorchis	Extreme	Low	Low	Negligible	Negligible	No
Peracreadium characis	Extreme	Low	Low	Negligible	Negligible	No
Proctoeces maculatus	Extreme	Low	Low	Negligible	Negligible	No
Pseudopycnadena fischthali	Low	Low	Negligible	Negligible	Negligible	No
Steringotrema pagelli	Extreme	Low	Low	Negligible	Negligible	No
NEMATODA						
Contracaecum sp.	Low	Negligible	Negligible	Low	Negligible	Yes
Hysterothylacium aduncum	Low	Negligible	Negligible	Negligible	Negligible	Yes
COPEPODA						
Caligus sp.	Low	High	Low	Moderate	Negligible	Yes
C. ligusticus	Extreme	High	High	Moderate	Moderate	Yes

Table 4 (*Continued*)

Parasite taxa	Exposure	Pathway	Probability	Consequence	Risk	Ability to treat
C. minimus	Moderate	Extreme	Moderate	Moderate	Low	Yes
Clavellotis characis	Moderate	High	Moderate	Low	Negligible	No
Colobomatus agassizi	Moderate	High	Moderate	Negligible	Negligible	No
Ergasilus sp.	Low	High	Low	Negligible	Negligible	No
Lernaeolophus sultanus	Moderate	Extreme	Moderate	Moderate	Low	No
Lernanthropus brevis Syn. *Lernanthropus vorax*	Extreme	Extreme	Extreme	Low	Low	Yes
Pseudoencanthus kerkennensis	Low	High	Low	Negligible	Negligible	No
ISOPODA						
Anilocra physodes	Low	Negligible	Negligible	Moderate	Negligible	Yes
Ceratothoa oestroides	Low	Negligible	Negligible	Moderate	Negligible	Yes
C. parallela	Low	Negligible	Negligible	Moderate	Negligible	Yes
Ganthia vorax	Extreme	High	High	Moderate	Moderate	Yes

myxozoans such as *Kudoa* sp. is negligible in *D. puntazzo* cultures, as these myxozoans need alternative hosts and only two references report its presence in *S. aurata* but not in *D. puntazzo* (Rodgers and Furones, 1998; Paperna, 1991). However, in the unlikely case that *Kudoa* spp. were to become established in Spanish aquaculture in the future, the *consequence* would be moderate for *D. puntazzo*, as this parasite has been found in the mesenteries and muscle, not related to fish death, although it sometimes causes mio-liquefaction, with consequent economic losses (Golomazou et al., 2004, 2006). Nowadays, the only effective way to control myxozoans is prophylaxis as effective eradication treatment is unavailable (Angelucci et al., 2008). Removal of alternative hosts, if known, prevents completion of the myxozoan's life cycle. Adequate veterinary controls and proper quarantines are indispensable to avoid the widespread contamination of Mediterranean areas.

4.3. Monogenea

Most parasites recorded in *D. puntazzo* and *S. aurata* belong to Monogenea, monoxenous ectoparasites of gills and skin. Four different genera entail low to moderate *consequences* for *D. puntazzo* farming. Six species have low, and one moderate risk (Table 4). *Furnestinia echeneis* (Wagener, 1857), *Lamellodiscus* spp. and *Atrispinum* sp. represents a low risk to *D. puntazzo* farming. *F. echeneis* has been described in farmed *D. puntazzo* (not in the wild), this fact together with its direct life cycle and its presence in the farmed *S. aurata* confer this species a high *probability* of establishment; however their possible *consequence* for farmed fish is low, because related pathologies are normally slight and not usually related to fish mortalities (Antonelli et al., 2010; Sánchez-García et al., 2011). A total of eight species of the monopistocothylean genus *Lamellodiscus* have been recorded from wild *D. puntazzo*, however only four of these (*L. biddens* Euzet, 1984, *L. ergensi* Euzet et Oliver, 1966, *L. hilii* Euzet, 1984 and *L. ignoratus* (Palombi, 1943)) parasitized farmed fish. Katharios et al. (2006) and Sánchez-García et al. (2011) studied the pathologies caused by different species of *Lamellodiscus*, finding that some species can cause mild local damage in the gill epithelium, therefore, their *consequences* are ranked from negligible to low (Table 4). *Gyrodactylus* sp. has only been reported in *S. aurata* (De Liberato et al., 2000; Paladini et al., 2009). Although no pathology associated with this parasite has been described, the damage caused by *Gyrodactylus* spp. in fresh water aquaculture is well-known (McVicar, 1997; Arafa et al., 2009). For these reason, although infection of *D. puntazzo* is very unlikely according to current information, these parasites should be considered in the aquaculture industry. Regarding polyopisthocotylean monogeneans, three species pose risk to *D. puntazzo*. The genus *Atrispinum* is herein represented by three species, two of which are low risk. Daily mortalities of *S. aurata* due to high prevalence and intensity of *A. salpae* have been documented in western Mediterranean fish farming facilities (Parona et Perugia, 1890) (Merella et al., 2005; De Liberato et al., 2000). Di Cave et al. (2003) also reported mass mortality of *D. puntazzo* caused by *A. seminalis* (Euzet et Maillard, 1973) in Italian fish farms. Another polyopisthocotylean, *S. chrysophrii*, is one of the most threatening ectoparasites for *S. aurata* cultures, producing mortalities in farmed fish (Faisal and Imam, 1990; Sanz, 1992), and secondary infections are frequently detected in parasitized fish (Padrós and Crespo, 1995; Cruz e Silva et al., 1997; Caffara et al., 2005). Furthermore, *S. chrysophrii* is commonly found in cultured *D. puntazzo* (Mladineo, 2006) with mortalities reported in Greek farms (Di Cave et al., 2003) and recently this parasite was also reported in wild *D. puntazzo* (Sánchez-García et al., 2013). Considering the high pathogenicity of this monogenean species in *D. puntazzo* and *S. aurata*, we determined they pose a risk to *D. puntazzo* aquaculture (moderate).

Many species of monogeneans have been reported to infect *S. aurata* (Gibson et al., 2005), but they have not been considered in this study as they were not reported in the Mediterranean. However we must make a special reference to some monogeneans such as the benedenids, skin monogeneans which are linked to both damage and mortality (Ogawa, 2004; Moreira et al., 2013). In particular, *Neobenedenia melleni* (MacCallum, 1927) has been reported in *S. aurata* in the Red Sea, as well as in other fish species cultured in warm to temperate waters (Colorni, 1994; Whittington and Horton, 1996). We must consider potential infections of these unspecific parasites especially in terms of the quarantine of imported fish.

Monogenean parasite infections are difficult to control in sea cages but reinfection can be prevented with strategically sequenced treatments to break their life cycle in vulnerable stages (larvae and adults) (Ernst et al.,

2005). However, the scarcity of authorized anthelmintic treatments and their limited efficacy makes the situation even more difficult to handle (Sitjà-Bobadilla et al., 2006). Further research should be done to find eco-friendly and efficient measures and treatments against monogeneans.

4.4. Trematoda

To our knowledge, there are no references of trematodes parasitizing farmed *D. puntazzo* and only eight species have been recorded to infect farmed *S. aurata* (Tables 1 and 2). Trematodes have complex heteroxenous life cycles. The proliferation of these parasites in farmed fish is unlikely because a diet based on extruded pellets avoids trophic transmission and hampers the survival of adult trematodes, mostly living within the digestive system (Paperna and Dzikowski, 2006). However some trematodes manage to infect farmed fish, especially when fish are fed with fresh meat or eat infected wild fauna entering the cages. Literature concerning to trematode pathogenicity in the digestive system indicates that they usually do not survive culture conditions and when they do their effect is usually minimal (Woo, 2006; Sánchez-García et al., 2013); therefore, we considered the possible *consequence* of these trematodes negligible for *D. puntazzo* (Table 3). In contrast, the trematodes living within tissues or vascular system can survive culture conditions and are often related to economic losses in aquaculture. Some vascular system flukes (Aporocotylidae) cause important mortalities in fish farms (Ogawa et al., 1989, 2007; Ogawa and Fukudome, 1994; Hayward et al., 2010). In particular, the aporocotylid *Cardicola aurata* Holzer, Montero, Repullés, Sitjà-Bobadilla, Álvarez-Pellitero, Zarza et Raga, 2008 has been related with trickling mortalities of *S. aurata* in cultures, producing impairment of gill function and anaemia (Padrós et al., 2001). In these cases intermediate hosts are supposed to live on or close to the cages and fish are continuously reinfected by emerging cercariae (Paperna and Dzikowski, 2006). *C. aurata* entails a moderate *probability* of reaching farmed *D. puntazzo* as they are usually reared in the same installations where intermediate hosts live. However we classified the risk of this Aporocotylid as negligible because these parasites are always strictly specific (Smith, 2002), and their ability to invade different species is very low. *D. puntazzo*-specific blood flukes have not been recorded to date; however these parasites must always be monitored as their consequences in cultures are often harmful (Crespo et al., 1992; Ogawa and Fukudome, 1994; Diggles and Hutson, 2005). Chemical treatments for *C. aurata* are not available, but other *Cardicola* species, such as those infecting the Pacific Bluefin tuna (*Thunnus orientalis*), have been successfully controlled with antihelmintics that could be tested in Mediterranean facilities (Shirakashi et al., 2012; Ishimaru et al., 2013). Another trematode that could be problematic in aquaculture is the metacercaria of the strigeid *Cardiocephaloides longicollis* (Rudolphi, 1819), a parasite found encysted in groups within the ventricles of the optical lobes of the brain. Final hosts of *C. longicollis* are sea birds. In heavy infections, these metacercariae can cause a significant pathological effect which can alter host behaviour, facilitating parasite transmission (Chappel et al., 1994; Fredensborg and Longoria, 2012; Lafferty, 2008). This parasite has been reported in both *S. aurata* and *D. puntazzo*, and although *C. longicollis* needs several hosts to complete its life cycle, the species represents a moderate *probability* of establishment, as all the intermediate (gastropods) and definitive hosts (sea birds) are known to live close to fish farms. Treatment for trematodes in blood or tissues are either unavailable or hardly effective; therefore, prophylactic measures such as periodical net changes and cleaning of cage structures would be recommendable to remove the invertebrate species hosting the life cycle stages of these trematodes.

4.5. Nematoda

According to the bibliography, *S. aurata* and *Diplodus* spp. from the Mediterranean can occasionally be parasitized by *Contracaecum* (Anisakidae) and *Hysterothylacium* (Raphidascaridae) (Kalay et al., 2009; Salati et al., 2013). References to *D. puntazzo* are lacking but apparently all marine teleosts are susceptible to these parasites as they infect many paratenic hosts during their life cycle. Recently Marino et al. (2013) experimentally infected *S. aurata* with *Anisakis pegreffi*. Their experiments show that fish can be susceptible to the parasite, but likelihood of infection in the wild is low due to differences between fish and parasite habitats [i.e. sparids are neritic and demersal (Bauchot and Hureau, 1986) while the *Anisakis* spp. life cycle is mostly oceanic and pelagic (Nagasawa, 1990)]. However, like trematodes, these nematodes have complex life cycles, and are trophically transmitted; therefore, the use of extruded pellets impedes infections. Consequently, we consider that the *probability* of establishment and proliferation in *D. puntazzo* farming is negligible (Table 4). Nematode parasites rarely cause problems to fish: migrating larvae of anisakids can cause tissue damage (Hauck and May, 1977; Poynton, 1993; Noga, 2009), but no reports exist related to sparids (see Peñalver et al., 2010). Nevertheless, the potential *consequence* of these nematodes cannot be neglected due to the high risk they pose to human health by consumption. The final hosts of anisakids are marine vertebrates, but they are accidental human pathogens, with *Anisakis* spp., *Pseudoterranova* spp. and (more rarely) *Contracaecum* sp. causing anisakidosis (Adams et al., 1997). Raphidascarids cannot be transmitted to humans, as their definitive hosts are fish, but they have also been related to allergic manifestations such as urticaria and anaphylaxis (Fernández-Caldas et al., 1998; Audicana and Kennedy, 2008).

Nematode control in aquaculture installations is essential, because the possible presence of anisakids can alarm consumers and lead to serious economic losses. Although some anthelmintic treatments are effective against adult nematodes within fish guts, encysted nematodes are difficult to eradicate. Control of these parasites requires preventive methods, mainly a diet based on extruded pellets avoiding trophic transmission.

4.6. Copepoda

Caligidae is the most representative group of copepods infecting *D. puntazzo* and *S. aurata*. These parasites

have direct life cycles (Kabata, 1979), which favours direct contagion and reinfections. Caligids cause high mortalities in aquaculture, causing host osmoregulatory failure, anaemia and ulcerations that facilitate secondary infections. In salmonid aquaculture, lesions cause important economic loses (Costello, 2009). Caligid infections are often reported in farmed marine fishes from the Mediterranean region (Papoutsoglou et al., 1996; Ragias et al., 2004), two of them reported in wild (*Caligus ligusticus*; Sánchez-García et al., 2013) and farmed (*C. minimus*; Mladineo, 2006) *D. puntazzo*. Papoutsoglou et al. (1996) and Ragias et al. (2004) described severe pathologies caused by *C. minimus* Otto, 1821 mainly in cultured *D. labrax* during winter months. In addition Pavoletti et al. (1999) also reported a disease outbreak in *D. labrax* caused by *C. minimus* and *C. ligusticus* Brian, 1906. We therefore classify them as moderate and low risk parasites for *D. puntazzo* aquaculture, respectively, based on their incidence in farmed and wild fish, and their pathogenic potential. The current situation indicates that despite the available treatments, species of this genus should be monitored with caution. The pennellid *Lernaeolophus sultanus* (Milne Edwards, 1840) is an unspecific parasite reported for the first time in cage-reared *D. puntazzo* in Greece (Varvarigos, 2007). The author related it with localized deformities together with a decrease in fish body condition and growth retardation. The presence of mature parasites in farmed fish may pose a threat to marine aquaculture (Varvarigos, 2007). In Spain, most *D. puntazzo* fingerlings have been imported from Greek aquaculture installations; subsequently their *probability* and *consequence* is moderate for Spanish aquaculture facilities (Table 4). The third copepod genus to be considered is *Lernanthropus*, with just one species reported, *L. brevis* Richiardi, 1879. To date no studies have reported pathological signs related to *L. brevis*; however some studies have described infections of the species *L. kroyeri* (Van Beneden et Hesse, 1851) on cage-reared *D. labrax* from Greece associated to erosion, desquamation and necrosis of the secondary lamellae, affecting host condition (Manera and Dezfuli, 2003). The pathogenic potential observed in congeneric species, together with their extreme *probability* of establishment in *D. puntazzo* farms, implies that *L. brevis* should be considered a potential pathogen and the presence of this parasite should be controlled.

It is well known that both wild and cultured fishes are reservoirs of infection for sea lice and other parasitic copepods (Paperna, 1975; Carvajal et al., 1998; Ho and Nagasawa, 2001). In order to find appropriate sites for aquaculture, the presence of neighbouring wild hosts, as well as distance and position (mainly with respect to water movements) of close aquaculture installations should be considered. Parasite species with broad host ranges and/or abundant wild hosts in the vicinity of aquaculture sites are generally considerably more difficult to control (Johnson et al., 2004).

4.7. Isopoda

Infections caused by zupheae and pranizae of *Gnathia* spp. have been reported in many fish species of the Mediterranean basin (Grau et al., 1999; González et al., 2004; Alas et al., 2009). *G. vorax* is the only species reported in wild *D. puntazzo*, with no published data on pathological effects. However, some authors have reported mortalities related to infections in eels and cultured mugilids caused by severe skin lesions and anaemia (Paperna and Overstreet, 1981; Mugridge and Stallybrass, 1983). The unspecificity and potential pathogenicity of gnathiids imply a high *probability* of establishment and a moderate *consequence* for confined marine fish. Young free-living infective larvae can occur in greater numbers in inshore plankton samples than in offshore samples (Paperna and Overstreet, 1981). Thus, cage location must be carefully considered to avoid larval transmission to *D. puntazzo*.

The three other isopod species considered in the present work are the cymothoids *Anilocra physodes* (Linnaeus, 1758), *Ceratothoa oestroides* (Risso, 1826) and *C. parallela* (Otto, 1828). These parasites cause economic losses in cultures of *D. labrax* and *S. aurata* from the Central and Eastern Mediterranean, related to anaemia, emaciation and skin damage (Papanagiotou and Trilles, 2001; Horton and Okamura, 2003; Mladineo, 2006). Over the last few years, diseases related to isopod parasites have become more frequent, especially in *D. labrax* (Papanagiotou and Trilles, 2001; Vagianou et al., 2006, 2009). The fact that *C. oestroides* usually parasitize cultured Mediterranean *D. labrax* must be considered, as these isopods are very unspecific and could be transferred from nearby cages, even between different fish species (Mladineo et al., 2009). However, currently we considered that cymothoid isopods entail a negligible *probability* of establishment, as they have not been reported in farmed sparids (as *S. aurata*) in the Spanish Mediterranean. Control of cymothoids is laborious and only partially effective, mainly requiring modifications to risky farming routines. Further studies are required to improve prophylaxis and therapy against these parasites.

5. Conclusions

In summary, the current study illustrates which parasite species pose a threat to *D. puntazzo* cultures. Three species of parasites entail a high risk to *D. puntazzo* cultures: *Amyloodinium* sp., *Cryptocaryon* sp. and *Enteromyxum leei*. These species had previously been related with high mortalities, except for *Amyloodinium* sp., already known as an unspecific high-risk parasite (Noga, 2009). In the same way, *Sparicotyle chrysophrii*, *Caligus ligusticus and Gnathia vorax* entail a moderate risk to *D. puntazzo* Mediterranean farms, as they are responsible for highly harmful outbreaks in *D. puntazzo* and *S. aurata* (such as *S. chrysophrii*) or in other fish species (such as *C. ligusticus* and *G. vorax*). This study has not considered infections from other fish species cultured in the same area (i.e. the moronid *Dicentrarchus labrax*) as they are not phylogenetically close and have not been reported to date. However, transmissions from the above species are not impossible and cross infections should be controlled, especially in mixed cultures.

Most parasites entailing an important risk to *D. puntazzo* farming have previously been identified on *S. aurata* cultures. Protists, myxozoans and the monogenean *S. chrysophrii* are all well known pathogens of Mediterranean *S. aurata* farms. Therefore, for parasite species found in both

fishes, it is important to consider that cross-infection can increase infection rates (Mladineo and Maršić-Lučić, 2007). Moreover *D. puntazzo* is one of the fish species reported by Dempster et al. (2002) to live associated to sea cages in the Mediterranean and, therefore, wild populations of this species could be a source of pathogen transmission to farmed fishes of the same or other species.

Parasite risk assessment provides important scientific information which can be implemented in aquaculture decision making to improve health and safety. Potentially risky pathogens should be monitored and considered in terms of fish-farm location: parasitic fauna in the surrounding environment, water streams and proximity of neighbouring cages, potential contagion from other cultured species, etc. Moreover, all these aspects should be monitored in order to guide the preparation of contingency plans and general disease management options.

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CHAPTER 6

Comparative study of three attachment mechanisms of diplectanid monogenean

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Comparative study of the three attachment mechanisms of diplectanid monogeneans

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ABSTRACT

One of the main characteristics of the monogenean family Diplectanidae Monticelli, 1903 is their complex haptor formed by 2 pairs of hooks, transversal bars, 14 peripheral marginal hooks, and accessory adhesive organ (lamellodisc or squamodisc) that can be present or absent. Sub-family Lamellodiscinae Oliver, 1969 presents one or two lamellodiscs, formed by several overlapped lamellar esclerites (lamellae) which are piled up. Species like *Furnestina echeneis* only have one large ventral lamellodisc. This organ function has been categorized in different ways (i.e. accessory adhesive organ, supplementary or compensating disc or sucker), although its real mode of operation and function is still unclear. Specimens of *Lamellodiscus* and *F. echeneis* were examined. The lamellodisc of *F. echeneis*, studied both in vivo and fixed, seems to work as a sucker: the separated lamellae revolve around the single smallest lamellodisc lamella like the slats of a hand-held fan and create a suction volume. *Lamellodiscus* spp. lamellae (except the basal one) slide in telescopic movement, exerting a posterior and ventral or dorsal force that tightens it to the secondary gill lamellae. This force is contrary to the pulling force of hooks. Opposite forces together with the attachment to two different secondary gill lamellae gives strong binding and stability. These observations were compared with previous knowledge about *Diplectanum aequans* of subfamily Diplectaninae Monticelli, 1903, whose squamodiscs are formed by numerous spines and presents a different attach strategy. *D. aequans* produces extensive and deep alterations in the gill epithelium surrounding the parasite.
The different attachment mechanisms of the diplectanid species can explain the different degrees of damage that each species provoke, and the information provided in this work can be useful for anthelmintic treatment designs.

1. Introduction

The Diplectanidae Monticelli, 1903 is a monopisthocotylean family of monogeneans with many species worldwide (Domingues and Boeger, 2008). Diplectanids are gill parasites that characteristically have a haptor bearing two pairs of lateral large hooks connected to medial esclerotized esclerites, 14 small marginal hooks and, usually, one or two additional medial structures called squamodiscs (Bychowsky, 1957; Desdevises, 2001; Domingues and Boeger, 2008). Only 4 of the 17 genera comprising the family have no squamodiscs: *Lobotrema* Tripathi, 1957; *Murraytrematoides* Yamaguti, 1958; *Murraytrema* Price, 1937; and *Rhabdosynochus* Mizelle and Blatz, 1941 (Bychowsky, 1957; Desdevises, 2001; Domingues and Boeger, 2008). The squamodiscs are composed by dorsal or ventral groups of sclerites whose morphology, arrangement and number are different between different groups of diplectanids (Domingues and Boeger, 2008; Yamaguti, 1963). For instance the squamodiscs of the species within the subfamily Lamellodiscinae (referred as lamellodiscs in this group) are formed by several overlapped sclerotized laminar plates (lamellae) while the squamodiscs of the species of Diplectaninae are formed by several scale-shaped sclerites (see Amine et al., 2007; Desdevises, 2001; Dezfuli et al., 2007; Domingues and Boeger, 2008; Euzet and Audouin, 1959,). Diplectanids are very commonly found in fish from the wild (Domingues and Boeger, 2008) and also in Mediterranean cultured species: *Furnestinia echeneis* (Wagener, 1857) Euzet and Audouin, 1959, (Diplectanidae: Lamellodiscinae), in gilt-head seabream (*Sparus aurata*), *Lamellodiscus* spp. (Diplectanidae: Lamellodiscinae) in gilt-head and sharpsnout seabreams (*Diplodus puntazzo*), and *Diplectanum aequans* (Wagener, 1857) Diesing, 1858 (Diplectanidae: Diplectaninae), in sea bass (*Dicentrarchus labrax*), are frequently reported in Mediterranean aquaculture (Cruz-e-Silva et al., 1997; Dezfuli et al., 2007; Katharios et al., 2006; Merella et al., 2005; Mladineo, 2007; Toksen, 2006; Vagianou et al., 2004, 2006).

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Differences in haptor structure are associated to diverse attachment strategies (Bychowsky, 1957; Dezfuli et al., 2007; Kearn, 1997). These different attachment mechanisms lead to different detrimental effects and damage on host gill tissues. The attachment and related histopathology of *D. aequans*, the currently most pathogenic diplectanid in Mediterranean aquaculture, has been studied (Dezfuli et al., 2007; González-Lanza et al., 1991). However, the attachment mechanisms of other apparently harmless diplectanids (i.e. *F. echeneis* and *Lamellodiscus* spp.) have not been described to date in detail. Recently, Antonelli et al. (2010) reported morphological features of the attachment structures (mostly from the anterior glands) previously not described, although no observations from attached parasites were included. Despite the high diversity and frequency of *Lamellodiscus* spp. in wild and cultured sparid fishes (Cruz-e-Silva et al., 1997; Mladineo, 2007; Reversat et al., 1992) only one single study focused on the pathology caused by two *Lamellodiscus* spp. in cultured sharpsnout seabream exist (Katharios et al., 2006). This lack of studies about the functionality of the different types of squamodiscs suggested the elaboration of the present study.

The aim of this work is to describe and compare comprehensively the attachment mechanisms of *F. echeneis* and two species of *Lamellodiscus* (*L. falcus*Amine et al., 2006 and *L. theroni*Amine et al., 2007) harboured by sharpsnout seabream and the pathology caused in the gills of their host. The present results are also compared with the attachment of the most pathogenic diplectanid, *D. aequans*, in seabass (*D. labrax*).

2. Materials and methods

Parasites were obtained from infected fish from two different Spanish Mediterranean aquaculture facilities in the East coast of Spain. Fifty fish were examined as the source of parasites for this study: 10 infected gilt-head sea breams reared in cages in Burriana (39°53′22″N, 0°5′33″W) from fry produced in a hatchery and 40 infected sharpsnout seabreams captured in Mar Menor (37°41′14″N–0°44′10″E) and reared in the installations of Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA) at San Pedro de Pinatar, Murcia.

Fish were sacrificed by fast medular section. Upon dissection, right gill arches were observed in sea water under a stereomicroscope (magnification ×10–100, Leica MZ 125). In vivo observations of the movement sequence of lamellodiscs and hooks of the monogeneans, free and attached to gill, were recorded by means of an analogical camera *JAI CV-S3200* coupled to the stereomicroscope. Left gill arches were immediately fixed in buffered 10% buffered formalin for histopathology studies in light microscopy and for morphology examination in scanning electron microscopy (SEM).

For an accurate classification, 20 specimens of each species (*F. echeneis*, *L. falcus* and *L. theroni*) were washed in distilled water and examined on semi-permanent preparations in glycerol gelatin under light microscopy. Some living *F. echeneis* specimens were fixed in hot ethanol 70%, dehydrated, stained with iron acetocarmine (Georgiev et al., 1986) and mounted in Canadian balsam. Parasite identification was carried out using Amine and Euzet (2005); Amine et al. (2006, 2007); Euzet and Audouin (1959) and Yamaguti (1963) criteria.

For the haptor study, 10 specimens of each species were examined and drown with the help of a drawing tube. Haptor morphology and attachment mechanism of the host were studied using various microscope techniques for parasites anchored to the host tissue and for released individuals, dead and fixed or in vivo. The semi-permanent preparations in glycerol gelatine of monogeneans and the primary lamellae were examined with light microscope. One parasite of each species was partially digested with a solution consisting in 750 µl TNES urea [10 mM Tris–HCl (pH 8), 125 mM NaCl, 10 mm ethylenediaminetetraacetic acid, 0.5% sodium dodecyl sulphate and 4 M urea], 1 µl of proteinase K [10 mg/ml], and 650 µl of water distilled 15 min at 55 °C (Shinn et al., 2001). The digestion was stopped with buffered formalin and the parasites partially digested were mounted in glycerine. The sclerotized parts of the haptor were studied with differential interference contrast (DIC) microscopy (Leica DMR HC microscope).

For the light histology study left gill arches were embedded in paraffin, serially sectioned at 4 µm with a rotary microtome, stained with haematoxylin and eosin (H-E) and mounted in Entellan™ (Merck) and observed using light microscopy (Leica DMR HC microscope).

For the SEM study, five parasites of each studied species fixed in 10% buffered formalin, free or anchored to gill filament. Samples were dehydrated in an ethanol series, critical point dried in liquid CO_2, mounted on specimen stubs with conductive carbon paint, sputter coated with gold–palladium to a thickness of 25–30 nm in a Bio Rad–Sc 500 coating unit, and examined in a HITACHI S-4100 scanning electron microscope at 5 kV.

The study was complemented with additional paraffin, H-E stained histological sections of *F. echeneis* and *D. aequans* attached to gills of sea bream and sea bass respectively from paraffin blocks of the sample collection of the Fish Diseases Diagnostic Service of the Autonomous University of Barcelona obtained from different aquaculture facilities.

3. Results

3.1. Attachment of Furnestinia echeneis

The haptor of *F. echeneis* appears as a single large concave structure, hemiellipsoidal, (1/6 of total body length) ventrally opened (Figs. 1B and C and 2A). Haptoral opening is an ellipse which longitudinal diameter (the largest) is more or less elongated depending on the degree of haptor contraction. Haptor is ventrally surrounded by folded vellum of tegument where the peripheral marginal hooks are embedded (Fig. 1D). In SEM observations of detached parasites, the two pairs of main hooks are placed in lateral outgrowths at both sides of the posteromedial part of the haptor ventral margin, while the hooked distal tips protrude posteriorly. The posterior edges of the lamellae of the lamellodisc in SEM mark angulated ribs in the haptoral ventral tegument radially arranged from the annular mark of the edges of the single lamella at the dome of the haptor (Fig. 1B and C). In in vivo and mounted specimens the lamellodisc (ventral) appears as a frame of 9 pairs of elongated sclerotized lamellae which extend along the whole haptor internally supporting it. Lamellae of the lamellodisc, after enzymatic digestion, appear as pairs of independent elongated lamellae (except for the fused posterior pair) and an anteromedial single circular to triangular toroidal lamella (Fig. 1E). The two posterior lamellae are fused at the sagital plain of the worm, forming a T-shaped structure (see Fig. 1E). Paired lamellae are falciform to angulated, sclerotized plates whose posterior edge is a thick rib and the anterior zone is a flattened, thin, crenulated sharp lamina. Proximal tips of the lamellae are blunt and robust while distal tips are pointed. Lamellae are arranged radially overlapping proximal tips and the single annular lamella is placed anterodorsally (Fig. 1E and F). Main paired hooks are small (1/19 of total body length) connected by very thin medial and lateral bars (Fig. 1E).

Haptor size in vivo varies. The hemiellipsoid compresses or extends, elongating or reducing the longitudinal diameter of the haptor. Lamellae of the lamellodisc slide approaching or moving away, what, respectively, compresses or extends the haptor. At the same time, when haptor elongates, lamellae spin like the slats of a hand fan and distal tips of fold ventrally (Fig. 2A). All haptoral hooks also move constantly: Peripheral marginal hooks describe pendular movements in all directions (Fig. 2B), while the main two pairs of hooks spin mainly in frontal plains.

Living specimens of *F. echeneis* are mainly attached to the afferent side of the first lamellae of the gills (smooth areas without secondary lamellae). On gill filaments parasites move frequently, showing a leech-like movement. In order to change the initial position parasites attached with the haptor to gill epithelium elongate the body to stick

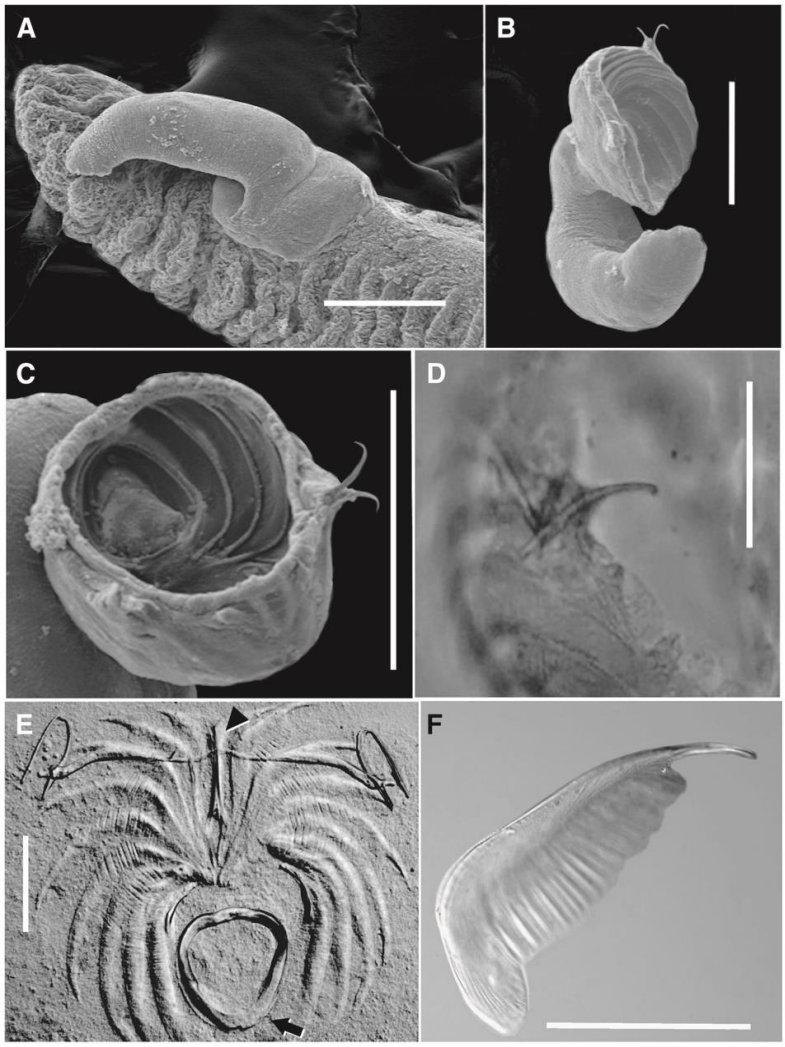

Fig. 1. *Furnestinia echeneis* from *Sparus aurata* L. A–C, SEM images: A, parasite attached to afferent side of first lamella; B, detached specimen with extended haptor; C, detail of compressed haptor. D, in vivo detail of main hooks and peripheral marginal hooks embedded in the vellum. E & F, images of partially digested parasites: E, detail of haptor (arrow head points to the T-shaped fused posterior lamellae and black arrow points the circular lamellae); F, detail of isolated lamellae. Scale bars A–C = 200 μm; D–F = 50 μm.

the anterior end to another area, the haptor expands and detaches, then retracting and bending the body, and finally haptor attaches in the adjacent epithelium. Attachment process is slow, as parasites repetitively compress and extend the haptor while the ventral opening gets deeper in gill epithelium. When the haptor releases the gill, the epithelial area of attachment appears swelled and surrounded by the elliptical depression produced by the compression of the gill epithelium by the haptor margin. Affected epithelia recover the normal smooth aspect within seconds after the haptor detachment.

In the H-E sections, the attached specimens of *F. echeneis* ($n = 7$) were only found on the afferent side of the first lamellae, occupying most of its width. Hooks perforate the gill epithelia, obviously deeper in the case of the main hooks. An apparent suction effect can be observed in the epithelia where the haptor attaches: The epithelium below the concave space of the haptor is somehow dilated while the epithelium contacting with the margins of the haptoral ventral opening is notably compressed. No inflammatory response associated to the attachment site was detected in any case (Fig. 3A and B). In the rest of the gills, no obvious signs of inflammatory response were noticed, and SEM observations of swellings in the afferent side of first lamellae where *F. echeneis* specimens had been attached showed the radial prints of the lamellae ribs and the marked elliptical depression where the haptor margin was placed (Fig. 3C). The insertion points of the main hooks were observed as epithelial depressions (one depression per each lateral pair of hooks) (Fig. 3E). Peripheral marginal hooks attachment is detected as very small epithelial swellings around the elliptical depression (Fig. 3D).

3.2. Attachment of *Lamellodiscus spp*

Haptor of *Lamellodiscus* spp. is dorsoventrally flattened, triangular to T-shaped. The central area of the haptor is ventrally and dorsally bulbous due to the presence of the ventral and dorsal lamellodiscs (Fig. 4C). The 2 lateral outgrowths of the haptor bear the 2 pairs of

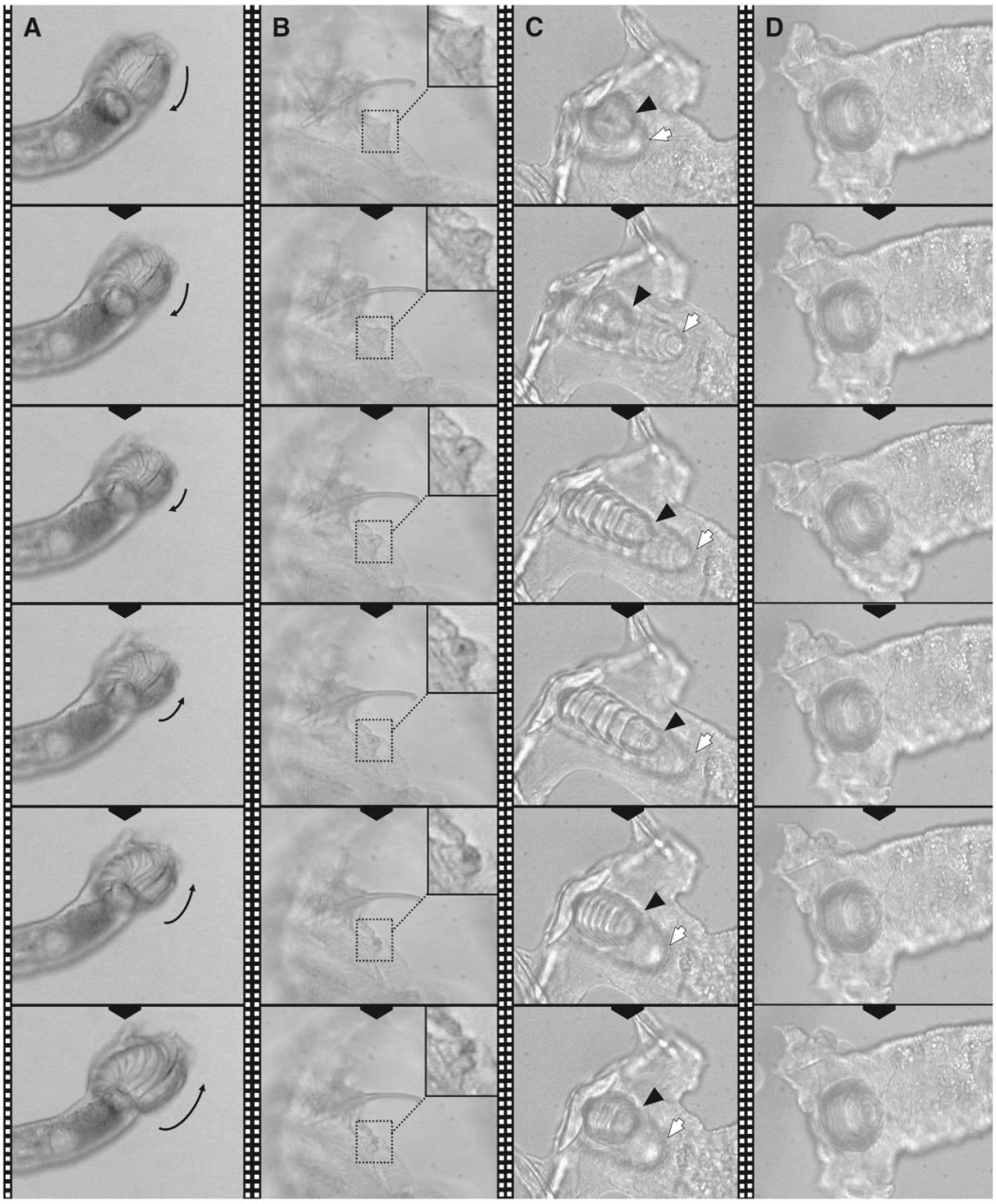

Fig. 2. Video sequences of the haptor movements of two diplectanid species. A and B, movement of the haptor of *Furnestinia echeneis* from *Sparus aurata*: A, haptor expansion, black arrow point to the direction of haptor longitudinal elongation; B, detail of the movement of the *F. echeneis* hooklets. C and D, movement of the haptor of *Lamellodiscus* spp. from *Diplodus puntazzo*: C, lamellodiscs expansion of *L. theroni*, lamellae slide in a telescopic movement, black and white arrows point dorsal and ventral lamellodiscs respectively (note that ventral lamellodisc expands before than dorsal); D, detail of the movement of *L. falcus* hooklets.

main hooks. Peripheral marginal hooks are ventrally and dorsally distributed on the haptor, mainly concentrated in the distal end of the lateral outgrowths. In SEM, relaxed detached specimens exhibit an apical opening on each of the mid-ventral and mid-dorsal swellings where lamellodiscs are (Fig. 4C). The posterior edges of the lamellae of the lamellodiscs are observed within the openings as sharp crenulated borders. Haptor opening is posteriorly covered by a hood-like extension of the tegument (Fig. 4D). Lamellodisc represents 1/12 of total body length in *L. theroni* and 1/10 in *L. falcus* (Fig. 5A and B). Lamellae are independent, wide, sclerotized plates. Different types of lamellae can be distinguished after enzymatic digestion: anteriormost lamella (lamellae 1 in Euzet and Oliver, 1966) of each lamellodisc is toroidal with subcircular shape. The rest of the lamellae are flattened plates with a thicker posterior edge and a wide, thin, sharp anterior zone. More anterior lamellae are elliptical and more posterior ones become gradually wider and more angular, so posteriormost lamellae

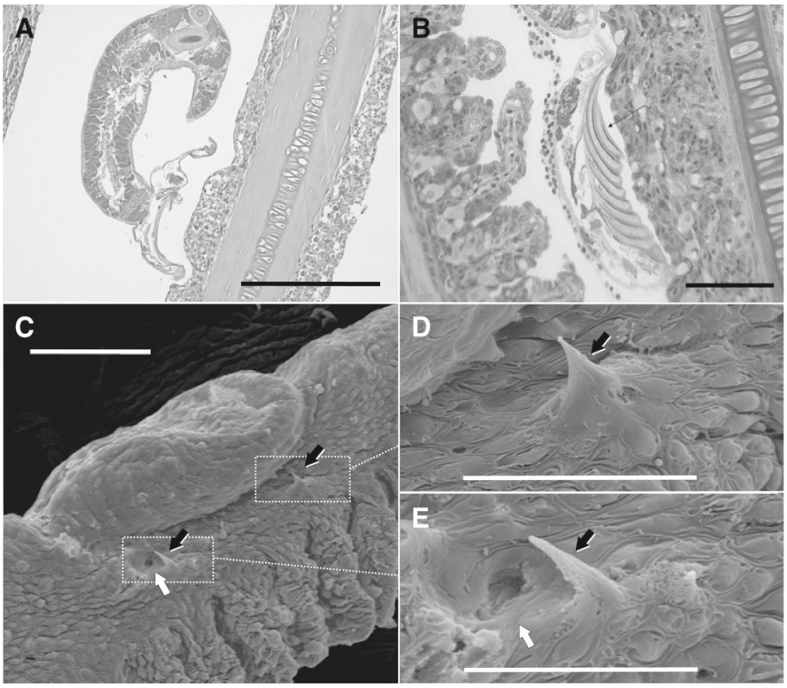

Fig. 3. *Furnestinia echeneis* from *Sparus aurata* II. A and B, H-E sections: A, longitudinal section of *F. echeneis* attached to gill filament; B, detail of the disposition of the haptor lamellae during the attachment. C–E, SEM images: C, *F. echeneis* haptor print on first lamella, with marked epithelial swelling surrounded by an elliptical depression; D and E, details of Fig. 3C, black arrows point to the epithelial swellings produced by the peripheral marginal hooks, white arrows point to the epithelial perforations produced by main hooks. Scale bars: A = 200 μm; B, C = 100 μm; D, F = 50 μm.

are almost rectangular (Fig. 5D). Paired hooks are large (1/10 of total body length, in *L. theroni* and 1/16 in *L. falcus*) connected by thick medial and lateral bars (Fig. 5A and B). In in vivo observations, lamellodiscs are extensible or compressible by the sliding of overlapping sections of the lamellae, in a sort of telescopic movement. In this way when the most posterior lamellae move away from the body longitudinal axis, dorsal and ventral lamellodiscs expand posterodorsally or posteroventrally. Surrounding tegumental swellings also expand and retract with the lamellae displacement. Peripheral marginal hooks describe pendular movements in all directions while the two pairs of hooks move mainly dorsoventrally (Fig. 2C and D). On gills, specimens of *Lamellodiscus* spp. introduce most of the haptor in the interlamellar spaces (between two secondary gill lamellae) to become attached. The rest of the body is free in the branchial chamber, expanding or retracting to reach to different feeding areas. Most of the parasites of both species were allocated close to the efferent side of the first gill lamellae (about 77%, $n = 371$ in 20 first gill lamellae), where secondary lamellae are shorter (Fig. 4A and B). Living parasites usually remain attached, although eventually move to attach into a different interlamellar space, performing a leech-like movement.

In histological sections, interlamellar spaces where the haptors are or were attached appeared expanded, as contiguous secondary gill lamellae separate more than double the normal distance. Frontal sections of attached specimens showed that the lamellae of lamellodiscs displaced from the longitudinal axis, expanding dorsally or ventrally the whole haptor. In attached specimens, the lamellar sliding is reflected in the posterodorsal and posteroventral displacement of the posteriormost lamellae, even overtaking the medial bars. The only apparent contact between the lamellodiscs and the gill epithelium seem to be that of the posterior margins of the most posterior lamellae, where the displacement of the epithelia is also observed (Fig. 6A and B). Within the interlamellar space, left and right dorsal hooks perforate the same secondary lamella while the ventral paired hooks the adjacent one. Hooks deeply penetrate in the epithelia, displacing and often perforating blood vessels of secondary gill lamellae (Fig. 6C and D). Slight epithelial hyperplasia and microhaemorrhages related to hook attachment were sometimes observed. No inflammatory response was detected. Displacement of the epithelium of the secondary gill lamella related to lamellodiscs expansion was also observed in SEM.

3.3. Attachment of *Diplectanum aequans*

The examination of histological sections of gills of *D. labrax* infected with *D. aequans* revealed an inflated haptor, surrounded and trapped by the tissue inflammation. Haptor is in close contact with the epithelia, with the squamodisc spines anteriorly oriented and stuck in the tissues (Fig. 6E and F). The gill structures in contact or close to the attachment area display a clear inflammatory response with extensive epithelial hyperplasia, affecting the gill epithelium of the interlamellar space where the haptor is attached at both right and left sides of the infected first lamellae. The strong inflammatory reaction also involves connective structures of the filament, with granulocytes, lymphocytes, macrophages and usually eosinophylic granular cells are present. Lamellar hyperplasia usually provokes partial or total occlusion of the interlamellar space and sometimes complete lamellar fusion that can be observed even if the parasite cannot be found attached to the lesion.

4. Discussion

The taxonomy of the Diplectanidae is mainly been based on the morphology of the squamodisc and the male genital organ (Desdevises,

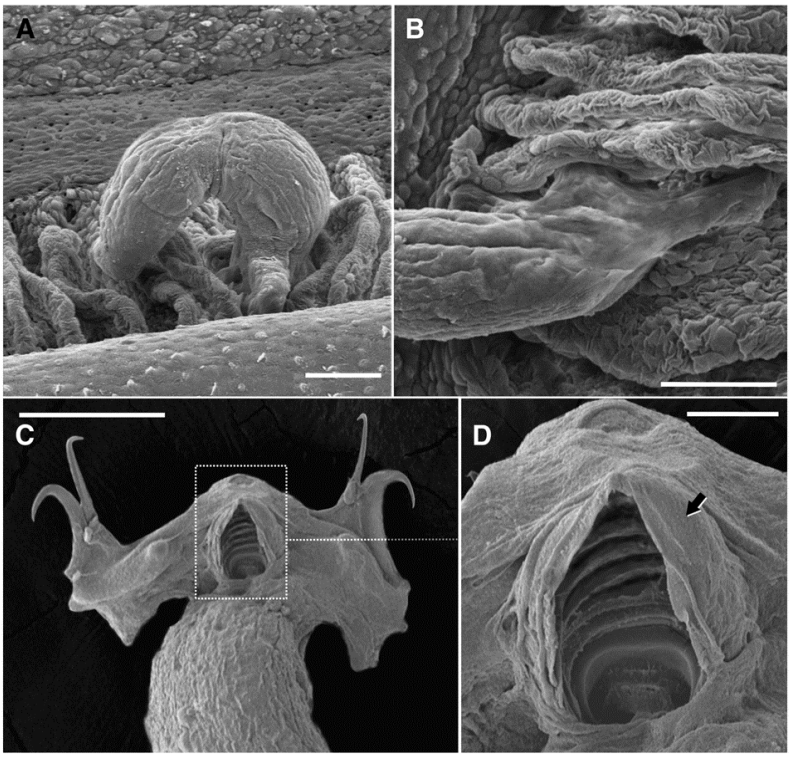

Fig. 4. *Lamellodiscus* spp. from *Diplodus puntazzo*. SEM images of *L. theroni*: A and B, *L. theroni* attached to gill filament, with haptor allocated between two secondary lamellae; C, detached relaxed specimen; D, detail of the squamodisc partially covered by a posterior hood-like extension of the tegument (arrow). Scale bars A–C = 50 μm and D = 10 μm.

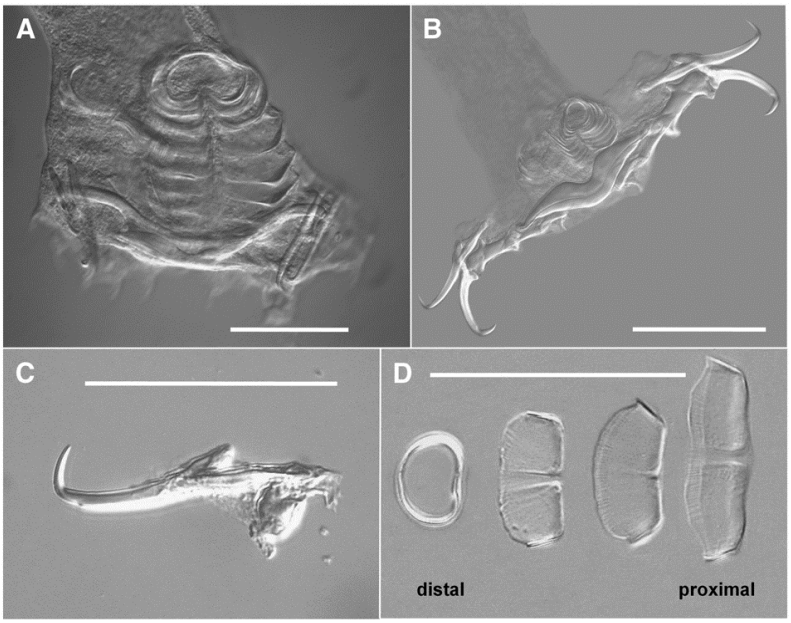

Fig. 5. *Lamellodiscus* spp. from *Diplodus puntazzo*. A and B, DIC images of detached parasites in glycerol gelatin: A, haptor of *L. falcus*; B, haptor of *L. theroni*. C and D, DIC images of partially digested parasites: C, *L. theroni* main hook; D, isolated lamellae of *L. theroni* squamodisc. Scale bars = 50 μm.

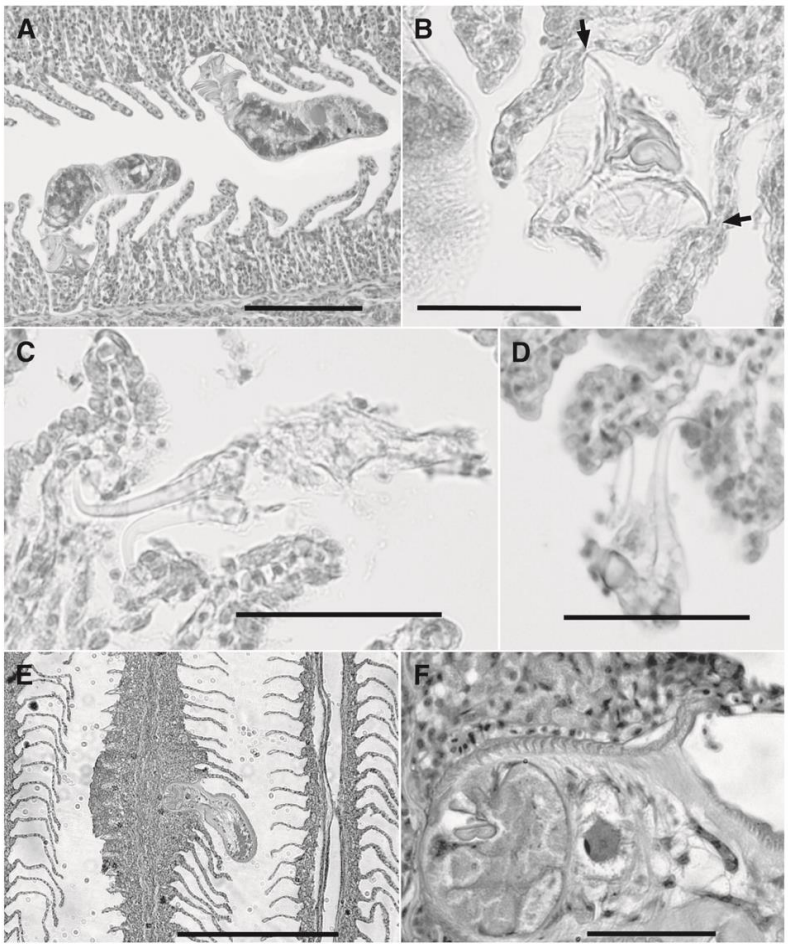

Fig. 6. H-E longitudinal sections of attached diplectanids. A–D, *Lamellodiscus* spp. from *Diplodus puntazzo*: A, two attached parasites with haptor inside interlamellar spaces where distance between the secondary lamellae is increased. B, detail of attached haptor, black arrows point to the contact between parasite and gill epithelium; C and D, detail of main hooks penetrating in gill epithelia, sometimes displacing and perforating blood vessels. E and F, *Diplectanum aequans* from *Dicentrachus labrax*: E, high inflammatory response of the gill epithelium surrounding *D. aequans* attachment; F, detail of an attached *D. aequans* showing close contact and deep penetration of the squamodisc in gill tissue. Scale bars A = 100 μm; B–D and F = 50 μm; E, 500 μm.

2001; Domingues and Boeger, 2008). Drawings in the descriptions of diplectanid species include illustrations of the haptor in ventral or dorsal views which provide little information on the three-dimensional morphology and arrangement of the structures which compose the haptor and, indeed, functional interpretation of this structure is often complicated. Some morphological descriptions include denominations of the squamodisc with terms that implicate a functional interpretation as sucker-like or adhesive organs. Euzet and Oliver (1966), and references therein, referred to these organs in Lamellodiscinae as "une sorte de ventouse," and more recently Domingues and Boeger (2008) reported these structures as "accessory adhesive organs."Bychowsky (1957) reference to this structure is more ambiguous, alluding to them as "supplementary or compensating discs." However there are no studies to explain how the squamodiscs take part in the attachment.

Most of the species of the genera within the Lamellodiscinae possess 2 lamellodiscs (dorsal and ventral) with the exception of *F. echeneis* which has one only ventral lamellodisc relatively larger than the ones in the other two genera (Euzet and Audouin, 1959; Euzet and Oliver, 1966; Desdevises, 2001). Despite this marked morphological difference, molecular studies revealed that *F. echeneis* could even be included within this genus *Lamellodiscus* (Desdevises, 2001). Two groups of species of *Lamellodiscus* have been described depending on the type of morphology of the lamellae (Desdevises, 2001). The "split type" group is characterized for the presence of deeply curved lamellae, divided in two parts by the medial line. The "non-split type" group is characterized for having angular lamellae, joined by the medial line (Desdevises, 2001). Both *L. theroni* and *L. falcus* belong to the "non-split" group, while *F. echeneis* (in view to its close phylogenetical relationship with *Lamellodiscus*) was included within the "split" group (Desdevises, 2001). The present study also reports the separation of the lamellae of the lamellodisc of *F. echeneis* in two lateral groups, although lamellae are very different in shape (narrow and curved) and arrangement (radial) to the ones of *Lamellodiscus*.

Combination of the different microscopical techniques, examining living and fixed parasites, attached and non-attached to the host, allow interpreting the role of each part of the haptor in the attachment. Large relative size of the only lamellodisc of *F. echeneis*, especially when it is compared with the small hooks (main and marginal), points out that this is the most important structure for the attachment to the gill tissues. In vivo, *F. echeneis* specimens normally stick the haptor to the smooth

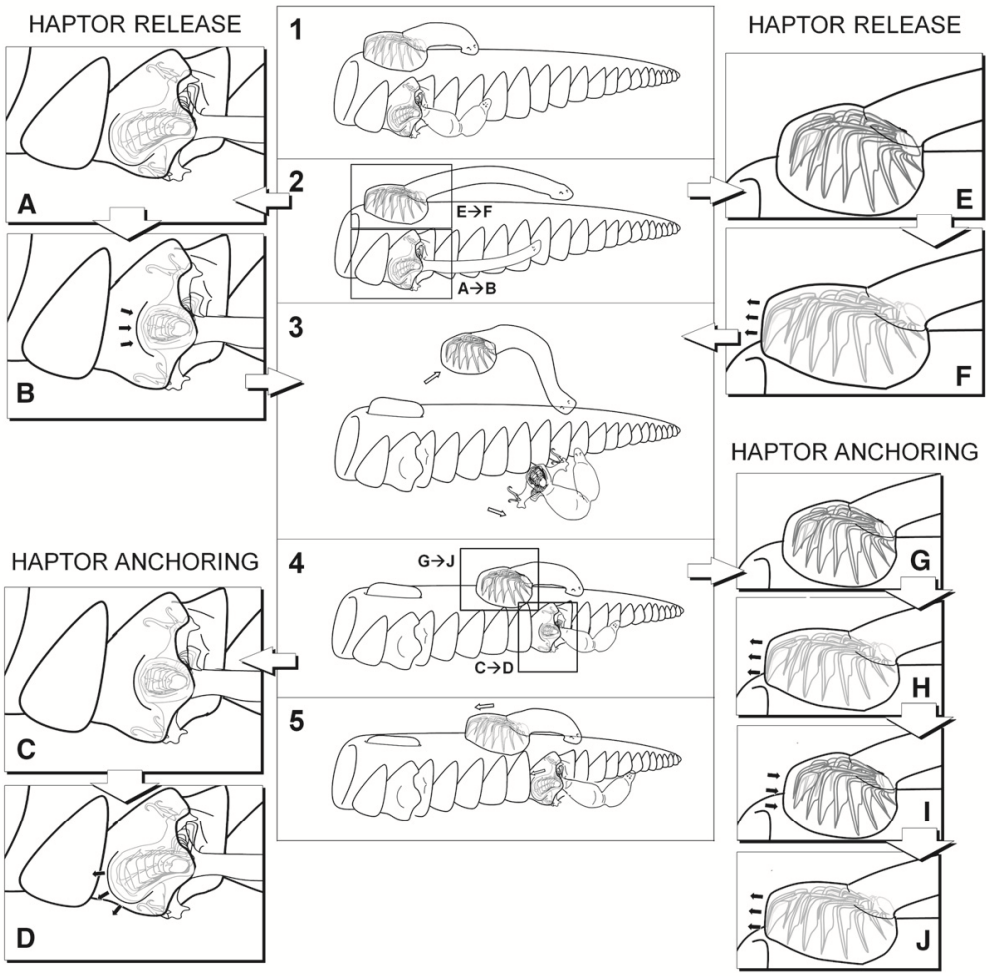

Fig. 7. Diagrammatic representation of the attachment strategy of *Lamellodiscus* spp. and *F. echeneis*. 1–5, pictures corresponding to the movement of both parasite species along the gill filament. A–D, details of *Lamellodiscus* spp. haptor: A, when parasite is attached, the squamodisc is telescopically extended, pushing the contiguous secondary lamellae; B, when parasite detaches the squamodisc becomes contracted; C and D, when *Lamellodiscus* spp. attach to a new interlamellar space performs the inverse sequence. E–J, details of *F. echeneis* haptor: E, when parasite is attached, the haptor is contracted, sucking the epithelium of the afferent side of first lamella; F, when parasite detaches haptor expands stopping suction; G–J, when *F. echeneis* attach to a new gill area sequentially repeats an expansion-contraction of the haptor, what allows to create a vacuum (*note the haptor print in the gill epithelial tissue).

surfaces of the afferent side of first gill filament. The swellings observed in the epithelium where a haptor is or was attached illustrate the suction effect of the *F. echeneis* haptor. Suction is produced when haptor is expanded because the displacement of the sclerotized lamellae of lamellodiscs. Parasites in vivo sequentially repeat an expansion-contraction of the lamellodisc working as a pump creating vacuum and fixing the monogenean to the gill tissues. Antonelli et al., 2010 refer to an internal "sucker" surrounded by the toroidal central lamella. In the histological sections of present study this sucker by the toroidal lamella was not observed but a very thin tegumental layer. Haptor sucks up host epithelium, creating a swelling surrounded by a depression created by the margins of the epithelium. Vellum surrounding the haptor margins can contribute to suction sealing the margins. Hooks would play a secondary role strengthening the attachment and probably helping mostly at the early steps of attachment, when parasite has just moved (Fig. 7, 1–5; E–I).

In a different way to *F. echeneis*, the size of the lamellodiscs of *Lamellodiscus* spp. are similar to those of main hooks, therefore appearing that both kinds of structures are relevant in parasite attachment (Fig. 7, 1–5, A–D). Examination of histological sections showed that the 4 main hooks perforate the epithelium of the two secondary lamellae at both sides the interlamellar space, in 4 anchoring points which impede parasite detachment due their hooked shape. The interpretation of the lamellodiscs function is more complex than the hooks. In living specimens lamellae move telescopically, sliding and pushing the lamellodiscs subperpendicularly from the longitudinal axis. In histological sections of *Lamellodiscus* spp. on the gills, sclerites appear to be superimposed and laterally displaced also suggest the telescopical displacement of the lamellae. Only the distal edge of most proximal *lamellae* (the closest to host when lamellae slide) establishes a line of contact with the host epithelia. The lamellodiscs act like protractile frames performing a pushing force which results in the gill secondary lamellae separation observed in light and SEM microscopy. Lamellodiscs thrust force is opposite to hooks traction force which tightens the epithelia, providing a highly efficient attachment and stability to the worms. Therefore, lamellodiscs appear not to work as suckers. In fact, in SEM, lamellodiscs of non-attached specimens were observed to be only partly (posteriorly) covered by parasite tegument,

indicating that the cup-like structure needed to create a vacuum cannot be displayed. Most of the parasites were found attached to the afferent sides of the gill filament. Interestingly, secondary lamellae are asymmetrical, being shorter at the afferent side, so the attachment surface is smaller for parasites. However, the fact of having most of their body free in the gill chamber will improves movement capability of the anterior region of their bodies for reproduction and feeding.

The lamellodiscs of the species of Lamellodiscinae are arranged in overlapped lamellae. When lamellae slide, the resulting movements of the whole haptor are different depending on their shape, arrangement and size. In general, lamellae display a telescopic movement, more evident in species of other genera as *Calydiscoides* Young, 1969 and *Protolamellodiscus* Oliver, 1969 whose lamellae are packed unpaired cylindrical or conical (Domingues and Boeger, 2008). In the case of *Lamellodiscus* spp., the cylindrical/conical lamellae become unrolled overlapped plates, but they still slide telescopically. *Lamellodiscus* spp. in present study, have non-split lamellae, therefore each one slides as an only piece. Lamellae displacement only contains the posterior component, resulting in the telescopic projection and pushing effect on gill epithelium. In fact, haptor tegument only covers its posterior part ("hooded" but not cup-like), the one that extends. The present study does not include split type species of *Lamellodiscus* spp., but similar attachment behaviour would be predictable as haptors shape and arrangement is the same. Lamellae halves must move together, as lateral components of the force would reduce the efficiency of the attachment due to the buckling effect (Timoshenko and Gere, 1963). Therefore a more or less solid medial junction of the lamellae halves would be expected. When each half lamellae is completely separated, as in *F. echeneis*, each half slides and moves independently. Lamella halves independence allows that, when muscles push them, not only the posterior component but also lateral components of the displacement exist. For this reason lamellae can move as like the slats of a hand-held fan and the whole cup-like haptor expands, resulting in the suction effect. Summarizing, similar structures of common origin result in different movements and attachment strategies. In the case of *F. echeneis* morphological differences are particularly marked as only one large lamellodisc exists. Desdevises (2001) suggested that during the evolution of *F. echeneis*, parasite lose the dorsal lamellodisc hypertrophying the ventral lamellodisc, possibly like an attachment strategy. In fact, *F. echeneis* is larger than the species of *Lamellodiscus*, its haptor is too large to fit inside the interlamellar space, and hooks remain as relatively small structures to be able to fix a big worm; therefore, parasites need to attach by suction to the smooth and larger surfaces of the primary lamellae.

Attachment of *D. aequans* and related pathology was deeply studied by Dezfuli et al. (2007). According to the authors the haptor of *D. aequans* enters inside the interlamellar space, similarly to that of *Lamellodiscus* spp., but *D. aequans* establishes a tight contact with host tissues. The hooks and also the spines present on the squamodisc pierce and lacerate the gill epithelium at numerous points, producing extensive and deep alterations in the gill epithelium surrounding the parasite attachment site. In accordance with present observations, stiff contact of the haptor of *D. aequans* with the epithelium of the interlamellar space is favoured by its inflation, which also erects the squamodisc spines, making parasite detachment more difficult. In addition, the attachment of the parasite seems to be also related to hyperplasia and inflammatory reaction of the epithelia, including tissue oedema what passively traps the haptor of the parasite. This effective passive attachment will nevertheless prevents *D. aequans* from detaching the haptor from the gill and move along the gill filament, as *F. echeneis*, and partially *Lamellodiscus* spp., do.

Main lesions related to monogeneans are associated with their specific attachment modes (Kearn, 1997), although feeding activity of the parasite on the fish gills should also be taken into account. Most of the monopisthocotylean monogeneans attach by hooks that pierce the epithelia of the host (Bychowsky, 1957; Yamaguti, 1963; Kearn, 1997). This traumatic attachment mechanism is one of the reasons why monopisthocotyleans are considered particularly pathogenic (Whittington and Chisholm, 2008). However and amongst Diplectanids, different attachment mechanisms displayed by the different species can explain the different damage intensity that each species provoke. *D. aequans* is the only species with reported serious effects in fish (Dezfuli et al., 2007). In fact, the attachment mechanism of this worm implies extensive friction surfaces of the haptor, with subsequent severe alterations of gills. No significant effects have been reported related to *Lamellodiscus* species, although, according to Katharios et al. (2006), high numbers of *Lamellodiscus* spp. specimens can hamper some fish under culture conditions, as that of the sharpsnout sea bream. *Lamellodiscus* spp. attachment is based on the four attachment points of the hooks and the pressure of the largest lamella edges. Although the large hooks can penetrate deeply the epithelia and, sometimes, perforate a blood vessel causing small haemorrhages, related damage in the gill epithelia can be considered as lower. In reference to the attachment site, *D. aequans* and *Lamellodiscus* spp. are attached to the secondary gill lamellae, causing different damage at this level and therefore impairing the respiratory function (hyperplasia, lamellar fusion, damage of the vascular structures). In contrast *F. echeneis* attachment does not affect respiratory structures and only causes mild damage (epithelial swelling and hooks perforate only superficially the gills tissues).

Several unspecific therapies have been successfully used to control monogenean infestations, but it is important to realize that monogenean species and even populations differ in their sensitivity to treatments. Gill monogeneans are often more resistant to treatments than skin parasites, possibly because the gill chamber provides some protection from drug exposure (Thoney and Hargis, 1991). The information provided in this work can be useful to design specific antihelmintic treatment in terms of efficiency and safety: *D. aequans* attachment has been seem to be tight and passive (the haptor is passively trapped by tissue inflammation), and only aggressive and parasiticide treatments would be effective. Less aggressive treatments would not kill the parasites and parasites will keep alive and continue attached after the treatment. In the case of *Lamellodiscus* spp. and *F. echeneis* their attachment are partially active (lamellodiscs must be protruded and hooklets piercing) or totally active (haptor suction) respectively. These parasites should be more easily eliminated, after a treatment with antihelmintic products as those with spasmodic effect on the parasites (Noga, 2010). Nowadays, formalin is the most common anthelmintic in the Mediterranean fish farms. Formalin is an effective parasiticide in bath treatment, which cross-links proteins resulting in cell death (Van Ham and Hall, 1998). However all uses of formalin have recently been banned in many countries as those in the European Union (Noga, 2010). Other drugs as praziquantel, a spasmodic anthelmintic mainly used for land animals which is also known to impair the neuromuscular system, inhibiting attachment of monogeneans (Noga, 2010). Mebendazole and peroxide are comparatively much less efficient or viable (Noga, 2010; Sitjà-Bobadilla et al., 2006). The actual situation requires from alternative parasiticide treatments to replace effective but banned formalin, and to achieve that goal the kinds of attachment of the monogenean parasites must be considered as a useful guide to find the correct treatments.

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CHAPTER 7

Morphological and attachment changes of *Lamellodiscus theroni* (Monogenea, Diplectanidae) during its post-larval development on fish

Morphological and attachment changes of *Lamellodiscus theroni* (Monogenea, Diplectanidae) during its post-larval development on fish

(Submited to Folia Parasitologica the 3rd of June 2014, accepted with major revision)

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Running header: Sánchez-García N. et al. Post-larval development of *Lamellodiscus theroni*

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Abstract

Species of the genus *Lamellodiscus*, Johnston et Tiegs 1922 (Monogenea, Diplectanidae) are characterized by a complex haptor bearing many different attachment elements: two pairs of main hooks joined by medial bars, 14 peripheral marginal hooks and one or two lamellodiscs, formed by several overlapping sclerotized plates (lamellae). These haptoral structures appear gradually during parasite development and, therefore, attachment strategies vary with developmental stage. The main aim of this work is to study the developmental changes of one species of *Lamellodiscus*, *L. theroni* Amine, Euzet et Kechemir-Issad 2007, under experimental conditions, paying special attention to the gradual variations in attachment strategies and the pathological implications. Throughout the gradual development of the sclerotized structures, six developmental phases have been distinguished in *L. theroni*: phase I, with only 14 peripheral marginal hooks; phase II, with main hooks (ventral and dorsal); phase III, with ventral bar; phase IV, with dorsal bars; phase V, with dorsal and ventral lamellodiscs; and phase VI, adult stage with male copulatory organ. During development, parasites attach to different parts of the first and secondary lamellae: from an unspecific attachment in the early stages, based on piercing any flat gill tissue, until ventral and dorsal main hooks are completely functional and parasites become restricted to the interlamellar space, and finally the definitive adult attachment when lamellodiscs are fully developed. The timing of key events in *L. theroni* development is used to establish adequate intervals for anthelmintic drug administration.

Keywords

chronology, attachment, haptor development, lamellodisc

7.1 Introduction

Monogenean monopisthocotyleans from the family Diplectanidae Monticelli 1903 are characterized by a complex haptor bearing many different attachment elements: two pairs of main hooks joined by medial bars, 14 peripheral marginal hooks and one or two groups of sclerotized rodlets or lamellae called "squamodiscs" or "lamellodiscs" (Bychowsky, 1957; Desdevises, 2001; Domingues and Boeger, 2008; Sánchez-García *et al*,. 2011). Within this family, the species of Lamellodiscus, Johnston et Tiegs 1922 (and Lamellodiscinae Oliver 1969, in general) have one or two lamellodiscs, formed by several overlapping sclerotized plates (lamellae). Sánchez-García *et al*. (2011) studied the functional morphology of the haptor of *Lamellodiscus* spp., indicating the roles of each component of this structure in parasite attachment, as well as the pathological implications. According to the authors, the sclerotized lamellae of the ventral and dorsal lamellodiscs slide, displaying a telescopic movement, pushing and separating the secondary gill lamellae. This thrust force is opposite to the main hooks' traction force which tightens the epithelia, providing highly efficient attachment and stability for the worms. The effects of this type of attachment were studied for *Lamellodiscus theroni* Amine, Euzet et Kechemir-Issad 2007 and *L. falcus* Amine, Euzet et Kechemir-Issad 2006, in sharpsnout seabreams, *Diplodus puntazzo* (Walbaum 1792). In this case, both piercing by hooks and pressure by lamellodiscs were related to some local damage to gills although, in general, the observed effects did not seem severe. In contrast, other authors, such as Katharios *et al*. (2006), have described that high burdens of *Lamellodiscus* spp. can seriously damage cultured sharpsnout seabreams.

The unexpected stronger effect of *Lamellodiscus* spp. in cultures could be explained by the high numbers of parasites, which cause much local epithelial damage and widespread gill malfunctioning. Moreover, we must consider that many different developmental stages live together in natural infections, and each stage often infects different parts of the gill using different attachment mechanisms and, subsequently provoking a combined effect of different types of damages to the gills as a whole (Repullés-Albelda *et al.*, 2011). In the case of *Lamellodiscus* spp.

the attachment strategies have only been described for adults, but not for post-larval stages. Some studies have described details about the development of the haptor of the *Lamellodiscus* spp., providing information about the sequence of development of some of the sclerotized pieces of the haptor (Bychowsky, 1957; Euzet, 1957, Oliver, 1987). The haptor morphology of post-larval specimens differs from that of adults: first having only the 14 peripheral marginal hooks while the rest of the structures appear gradually. Developmental changes in haptor arrangement imply different attachment sites and strategies in time, until all structures become gradually functional. Consequently different pathologies must be caused in host gill tissues. Knowledge about life cycle chronology is often lacking, and it is also important to understand attachment and damage dynamics.

The main aim of this work is to study the developmental changes of L. theroni over time. To do so, we monitored parasite development under experimental conditions, especially studying the morphological changes in the sclerotized structures. We also studied the changes in attachment strategies during haptor development, as well as the pathological implications.

7.2 Materials and Methods

Forty *L. theroni*-infected sharpsnout seabreams (further on referred as donor fish) were collected in May of 2008 from Mar Menor (Spanish Mediterranean: 37º41´N-Oº44´E) and confined in the facilities of the Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA) at San Pedro de Pinatar, Murcia (Spain). Fish were transported alive and maintained in the aquaria of the Central Service for the Support to Experimental Research of the University of Valencia (SCSIE). Once in the SCSIE facilities, ten infected fish (total length 296.1±12.8 cm; weigh 393.3±40.5 g) were killed by cervical decapitation in order to monitor parasite levels (prevalence, 100%; intensity, 985 parasites/infected fish). Gills were checked for parasites under light microscope. Infected fish were used as donors. Naïve fish were acquired from a Greek hatchery, transported to the SCSIE facilities and reared for three years. All fish were maintained in parasite-free aquaria with marine water, salinity 37‰, temperature 20˚C, and photoperiod 8:16.

One hundred and fifty naïve fish (total length 170.8±13.0 mm; weigh 68.5 ±12.3 g) were infected by cohabitation with the donor sharpsnout seabreams for 48h in a 250 l tank. After infection period, fish were transferred to 250 l volume "experimental tanks". "Time 0" of the experiment was assumed as the moment fish were restocked in the "experimental tanks". From this moment, five fish were killed daily by cervical decapitation and analyzed for 22 days. At 22 days post infection (dpi), a second generation of post-larval specimen was found. From each fish, gill arches were isolated in individual Petri dishes in seawater and analyzed immediately after dissection with a Leica MZ AP0 (8X-80X) stereomicroscope using transmitted light. Each first lamella was observed carefully, without removing the parasites from their infection sites. After examination, gill arches with higher parasite abundance were fixed in 4% formalin for histological analysis. Fish skin and fins were also analyzed to look for post-larvae or adults. Specimens were collected and mounted on temporary slides with glycerin jelly after being killed in hot saline solution. The sclerotized parts of the haptor and the male copulatory organ were studied with differential interference contrast (DIC) microscopy (Leica DMR (100X-1000X)). Morphological study of L. theroni development started from the earliest post-larvae, as described by Bychowsky (1957), corresponding to phase I of the present study. The sequence in which each piece appeared was recorded and both shape and growing changes were monitored. The sclerites of L. theroni were drawn with the help of a drawing tube and measured using Image Tool for Windows v 3.1 (see measurements in Fig. 7.1). All measurements are in micrometers unless otherwise indicated. The terminology used to name the different parts of the sclerites of the haptor follows that of the original description (Amine *et al.*, 2007).

For the histological study, the infected gill arches previously fixed in 4% formalin were embedded in paraffin, serially sectioned at 4 µm with a rotary microtome, stained with haematoxylin and eosin (H-E), mounted in Entellan® (Merck, Darmstadt, Germany) and observed using light microscopy (Leica DMR HC microscope).

7.3 Results

7.3.1 Developmental stages

All parasites were found on gills, none on skin or fins. In total 64 parasites were collected, including post-larvae and adults (prevalence 55.71% and intensity 1.64). Oncomiracidia needed at least 15 dpi to develop into adult (phase VI) and 18 dpi to mature and develop eggs. Second generation of post-larval specimens was found at 22 dpi. Morphometric variations during parasite development are shown in Table 1 and graphically represented in Fig. 7.2. Six developmental phases have been distinguished in L. theroni development, according to the first findings of the sclerotized structures (see graphical summary of the developmental events in Fig. 3).

- Phase I (from 1 to 12 dpi; n= 8) (Figs. 7.2, 7.4A): tiny first post-larval stages just after fixation in gills, very similar to oncomiracia but without cilia. Post-larvae characterized by the haptor armed only with marginal hooks (a.k.a. uncinuli). Body elongated and slightly dorso-ventrally flattened. Haptor with 14 sclerotized short hooks (further on referred as peripheral marginal hooks); 12 of them circularly disposed along the haptor edge, and one pair at the haptor center. Hooks with a thick handle and a distal pointed and hooked tip. Although hook shape does not vary along parasite life, they gradually become slightly longer in time. Two pairs of eyespots situated on the anterior part; the two anterior ones smaller and subspherical to piriform and the two posterior ones larger and kidney-shaped. The eyespots are present during the whole parasite life.

- Phase II (from 5 to 15 dpi; n= 14, more than 50% reaching to this stage at day 8) (Figs. 7.2, 7.5A): post-larvae characterized by the first observation of the main hooks (dorsal and ventral; a.k.a. anchors). Total body length very similar to that of the previous phase. The haptoral region becomes notably distinct, wider and longer (approximately half body length). The peripheral marginal hooks maintain the same arrangement, except for the two central ones, which are gradually further apart from the center to the haptor, at the posterior end (Fig. 4A, 5A). From the first

observation, dorsal and ventral pairs of hooks are larger than the peripheral marginal ones, formed by thin straight bars with a hooked posterior end. Ventral hooks are approximately 30% longer than dorsal hooks. During this growth phase, dorsal and ventral hook shapes remain almost invariable, while their length increases slightly.

- Phase III (from 7 to 8 dpi; n= 2) (Figs. 7.2, 7.5A): post-larvae characterized by the first observation of the medial ventral bar. Total body length is still very similar to the previous phases. By contrast, main hooks are distinctly longer and more robust, although, at this phase, ventral hooks are approximately one third longer than the dorsal ones. Moreover ventral hooks start to take on their final appearance and develop an anterior protuberance that will become the hook "guard" (Fig. 7.2). The earliest ventral bar is a small and thin single piece, slightly bent and pointed at both ends, arranged perpendicularly to the main hook; its length is still half that of the adult.

- Phase IV (from 8 to 16 dpi; n= 16, more than 50% reaching to this stage at day 11) (Figs. 7.2, 7.5A): post-larvae characterized by the first observation of the lateral dorsal bars. At this stage, body length increases for first time during development; size of dorsal and ventral hooks continues increasing, and both appear more robust. The early "guards" are perceptible in both pairs of hooks, differing slightly between ventral and dorsal hooks: the guard projection of the ventral hooks is elongated and ventro-anteriorly oriented, while that of the dorsal hooks is a short ventral swelling, becoming more apparent in the later stages. The ventral bar is a thin curved bar, longer but similar in shape to that of the previous stage. The early dorsal bars are only composed of a pair of single small elongated sclerites (left and right), perpendicular to oblique to the ventral bar, which appear to be the axial parts. Dorsal bars noticeably grow during this phase, even developing a distal protuberance, slightly similar to that in the axial part in adults.

- Phase V (from 9 to 16 dpi; n= 20, more than 50% reaching to this stage at day 14) (Figs. 7.2, 7.5A): post-larvae characterized by the first observation of the dorsal and ventral lamellodiscs. At this phase worms' total length increases

noticeably and all haptoral sclerites are present. All peripheral marginal hooks are now at the haptor edge. Dorsal and ventral hooks are similar and have almost the definitive adult length and thickness. Their morphology also becomes very similar to that of adults: the posteroventral hook guards are marked and enable clear differentiation between dorsal and ventral pairs; the guard is an oblique digitiform projection in the ventral hooks and a soft swelling in the dorsal ones. Ventral bar reaches almost the definitive length and, although its definitive morphology is not totally developed, the lateral ends become gradually more robust, leaving the medial bend as a narrow notch, typical of the adult ventral bar. Each dorsal bar develops into two overlapping pieces, which are the axial and lateral parts. Dorsal bars are still thin and start to develop their species-distinctive protuberances, while their arrangement gradually turns from oblique to almost perpendicular to the longitudinal axis (lined up with ventral bar). Early lamellodiscs are located at dorsal and ventral sides, anterior to the other sclerites. Not all lamellae can be observed at this stage (from 4 to 8), appearing as soft narrow primordial plates, except for the already circular anteriormost lamella.

- Phase VI (from 15 dpi; n= 23, more than 50% reaching to this stage at day 15) (Figs. 7.2, 7.6A): adult worms characterized by the first observation of the male copulatory organ. Haptor is totally developed and all sclerotized structures have their definitive size and shape. Body elongates and the haptor grows laterally, giving the final T-shape to the body. Dorsal and ventral pairs of hooks and dorsal and ventral bars elongate and thicken until acquiring their final arrangement, shape and thickness. The lamellodisc area represents 1/12 of the total body length. Lamellae are fully developed. Male copulatory organ is composed of the species-typical two sclerotized, articulated pieces, with the impair piece with a curved tip. The first intrauterine eggs are detectable at 18 dpi.

7.3.2 Attachment development

The analysis of histological sections revealed that parasites modified their attachment mechanism depending on the available functional sclerotized structures present in their haptor. During phase I, the only haptoral sclerites were the

peripheral marginal hooks; at this phase the haptor is a flattened surface with the 14 small marginal hooks more or less uniformly arranged around the haptor (except for the central ventral pair) and mostly oriented to the ventral side (Fig. 7.4). Histological sections showed that parasites used the marginal hooks to attach mainly on the flattened surfaces of the gill filaments or between secondary gill lamellae. When L. theroni were attached on flattened surfaces, all marginal hooks showed the same ventral orientation (Fig. 7.4E). Nevertheless in some slides we observed the marginal hooks ability to orientate dorsally (Fig. 7.4D) attaching the monogenean onto two contiguous lamellae.

During phases II, III and IV, the components of the haptor gradually appeared and took part in the attachment. These stages were usually found between secondary gill lamellae, normally quite external and far from the basal lamina (Fig. 7.5). At phase II the sizes of dorsal hooks appeared to be too small, and tips did not manage to pierce the gill tissue (Fig. 7.5B). Meanwhile ventral hook tips protruded from the haptor tegument, reaching and piercing the tissue of the same secondary gill lamella. Dorsal hooks started to take part in the attachment at phase III; therefore the ventral hooks were attached to secondary gill lamellae while the dorsal hooks, oriented backwards, pierced the contiguous secondary gill lamella, pulling the gill lamellae towards the parasites. This type of attachment is facilitated at phase IV by the longer and more robust main hooks, which penetrate deeper into gill tissues. During these phases, the peripheral marginal hooks were seen to be orientated dorsally and ventrally, hooking the two contiguous lamellae of the interlamellar spaces (Fig. 7.5B).

At phase V, lamellae of the lamellodiscs were thin and narrow, apparently useless (Fig. 7.5C). They became gradually thicker and larger, acquiring the definitive capability to slide performing a telescopic movement at phase VI (Fig. 7.6). During this growth period, the lamellodiscs could gradually push the secondary lamellae further. Finally, the adult worms became attached via the previously described mechanism of pushing the tissues with dorsal and ventral lamellodiscs while pulling with the dorsal and ventral hooks. At this stage,

peripheral marginal hooks are very small in relation to body length, but were observed to contribute to attachment piercing ventral and dorsal tissues.

7.4 Discussion

Most of the studies on monogenean development (including that of *Lamellodiscus elegans* described by Bychowsky, 1957) refer to morphological changes in relation to body growth, rather than considering the timing of these changes (Prost, 1963; Kearn, 1968; Ogawa, 1988; Roubal and Diggles, 1993; Dzika *et al.*, 2009). However, growth of monogeneans seems to follow different developmental timing, depending on the biological needs of each stage (Repullés-Albelda *et al.,* 2011). The present study indicates that in *L. theroni* total and partial growth varies in time, depending on the biological needs of each life period.

7.4.1 Morphological comments

The development of *L. theroni* is, in general, very similar to that of other diplectanid species as well as to other species of closely phylogenetically related monopisthocotylean families as tetraonchids and dactylogyrids (Bychowsky, 1957; Euzet, 1957; Euzet and Audouin, 1959; Kearn, 1968; Oliver, 1987). However, some specific differences were observed in this species. Compared with previous developmental studies of a *Lamellodiscus* species (*L. elegans*) the earliest post-larvae, corresponding to phase I, were longer (60-70 µm long, Bychowsky, 1957). The development of the sclerotized pieces of the haptor followed the same order previously described by the above mentioned author, although differences were observed in the stage at which some structures were fully developed. The size of the main pairs of hooks (ventral and dorsal) of *L. theroni* gradually increased from their earliest detection at phase II, acquiring their definitive size and shape when all haptor structures were fully developed (phase VI). Interestingly, Bychowsky (1957) reported that the main hooks were characterized by rapid growth, acquiring their definitive size and shape in phase IV (previous to lamellodisc detection). Bychowsky (1957) also observed that both pairs of main hooks began to form almost simultaneously; in contrast we found that ventral hooks (the largest in adults)

were bigger than dorsal hooks from the beginning of development. These differences could be related with the variable dimensions of the main hooks in adults of both species, which are proportionally larger in *L. theroni* than in *L. elegans*. Shape and growth of ventral and dorsal bars are very similar to those described by Bychowsky (1957).

As previously described in *L. elegans* (Bychowsky, 1957), dorsal and ventral lamellodiscs were simultaneously noticeable, although not all lamellae appeared at the same time, being incomplete and thin in the earliest observations. No partially developed copulatory organs were found, appearing to be totally formed from their first observation. Neither did Bychowsky (1957) report different growing stages of the copulatory sclerites.

The body of *L. theroni* grew relatively slowly during the first five stages, only increasing 2 fifths of the adult length from phase I to phase V, in 9 dpi (see Table 7.1). By contrast, it grew the remaining 3 fifths to reach the adult length (at phase VI) in just 5 days. Therefore it grew fastest when all the sclerites were present in the haptor. This kind of growth has been described in other monogeneans (see Repullés-Albelda *et al.*, 2011). In this group, the early parasite life is a critical period when main parasite investments are spent on fully developing its haptor components, thus guaranteeing its lasting and stable attachment to the fish surface tissues. Once the attachment structures are effective, parasites can feed, grow and reproduce. Adequate attachments can secure larger parasites to the host tissues. Similarly, Kearn (1968) reported that the diplectanid *Diplectanum aequans* suffered metamorphic alterations during the development to change from being attached to skin at the earliest stages of development to the attachment in their definitive habitat, the interlamellar space. Interestingly, *L. theroni* was always found on gills, and not on skin, as Bychowsky (1957) reported for *L. elegans*. Other organisms such as sessile mollusks with metamorphic free-swimming larvae also show a similar growth pattern, investing first in fixing onto a substrate properly before continuing their development (Porri *et al.*, 2007). According to Repullés-Albelda *et al.* (2011), when parasites invest more resources in reproduction, growth slows down again. Further studies based in soft anatomical parts are needed to explore the processes

of development and maturation of the adult specimens (stage VI), as the size range is very wide at this stage.

7.4.2 Attachment development

The histological study of *L. theroni* showed that during phase I, marginal hooks were the only sclerotized attachment structures of the haptor. As these hooks are short and ventral, the specimens mainly lie over the tissues, piercing them superficially. Despite this simple attachment mechanism, their small size allows parasites to attach to any gill surface: usually being found on flat smooth surfaces but also penetrating deeply into the interlamellar space. In the latter case, most marginal hooks were oriented ventrally, but others were oriented so as to pierce tissues placed posteriorly, or even dorsally (Fig. 7.4). This observation agrees with Sánchez-García *et al.* (2011), describing the movements of the marginal hooks of *Lamellodiscus* spp. as multidirectionally pendular. The non-selective attachment together with the small size of parasites increase the diversity of the gill areas that the earliest post-larvae of *L. theroni* can colonize, favouring their displacement along the gill filaments. This type of attachment is also suitable for skin attachment, as Bychowsky (1957) described, although *L. theroni* were not observed in this habitat in this study. Other strategies, as the secretion of adhesive substances, can also be involved in parasite attachment at this and the following stages (Paling 1966).

As parasite body size increases, new more stable attachment mechanisms must develop, being proportional in size to total parasite length. The appearance of ventral and dorsal hooks means an important change in parasite attachment strategy; these long hooks pierce fish tissues deeply and apparently become the main attachment devices, as peripheral marginal hooks would become accessory attachment structures. These hooks continue working as accessory attachment points to increase contact with the gill tissues during all parasite life. Although ventral and dorsal hooks were detected simultaneously, only ventral hooks seemed to be functional at phase II, as dorsal hooks were short and did not exceed the haptor margins, hampering host tissue piercing. Histological sections showed that

parasites lay on their ventral sides, where the ventral hooks pierce, and in the anterior end, the parasite mouth was orientated to the dorsal side. From phase III, both dorsal and ventral hooks were functional, piercing the contiguous secondary lamellae besides the interlamellar space, as described for adults in Sánchez-García *et al.* (2011) (Fig. 7.2). Later development of ventral and dorsal bars (phases III and IV) did not initially take part in the attachment. However, as the haptor widens, ventral and dorsal bars gradually arrange perpendicularly to the longitudinal axis. When the haptor of L. theroni acquires the transversal elongated T-shape characteristic of adults, these bars are thick and robust, and probably improving the skeletal support and providing fastening points for the hooks musculature (see musculature arrangement in *Diplectanum aequans* in Paling, 1966).

According to Sánchez-García *et al.* (2011) the attachment of the adult *Lamellodiscus* spp. also requires the thrust force of the lamellodiscs, opposite to the traction force of the main hooks. For this purpose these sclerotized lamellae must slide telescopically to project dorsally and ventrally. However, the histological sections of *L. theroni* showed the early lamellodiscs (phase V) were incomplete and thin, not large enough to push gill lamellae apart (Fig. 7.5C). They enlarge during phase VI until becoming functional, contributing to more efficient attachment of the larger worms.

7.4.3 Pathogenic and therapeutic considerations

The monopisthocotylean attachment system based on hooks has been considered particularly pathogenic (Whittington and Chisholm, 2008); however, the different degree of pathologies also depends on the attachment strategies and habits during the distinct post-larval stages of each species. Effects of *Lamellodiscus* spp. on fish are usually considered as mild, although Katharios *et al.* (2006) reported mortalities of sharpsnout seabreams, associated to hyperplasia of the epithelial tissue between the secondary lamellae and, in some cases, cell hypertrophy. In our experiment, the first post-larval monogeneans obtained from gills were already attached by the tiny marginal hooks although, due the small size of the peripheral marginal hooks, piercings were quite superficial and were not

related with noticeable lesions; nevertheless these small piercings can lead to secondary infections. Larger haptoral hooks increase the number and seriousness of intrusive lesions: dorsal and ventral hooks can penetrate deep inside the epithelia and sometimes perforate the blood vessels, causing small hemorrhages (Sánchez-García *et al.*, 2011). Therefore, during the last and longest period of infection, gill damage would become worse, especially when lamellodiscs begin to take part in the attachment, tightening and increasing their contact with gill tissues.

Lamellodiscus spp. are common gill parasites of Mediterranean sparids, including some cultured species, such as the sharpsnout seabream. The confinement of farmed fish facilitates reinfections, leading to high parasite loads (Dezfuli *et al*,. 2007; Katharios *et al*., 2006; Toksen, 2006; Mladineo, 2006; Sánchez-García *et al.*, 2013), and the joint effect of a high number of small intrusive lesions can finally seriously damage the respiratory epithelia of fish. Moreover, we must consider that fish simultaneously harbor different developmental stages, each having different attachment strategies and habitat preferences, therefore gill damage is more widely extended, affecting not only the interlamellar space where adults attach. In farm conditions, gills of *D. puntazzo* could be parasitized by 17 monogenean species, eight of them *Lamellodiscus* spp., two species of copepods and one isopod (Sánchez-García *et al.*, 2014a). The high parasite loads related to farm conditions, together with the possible synergistic effect of other gill parasite species would entail damages and consequently more serious respiratory problems (Katharios *et al.*, 2006; Sánchez-García *et al.*, 2011).

Knowledge about key developmental events can also help to establish adequate intervals during which to administer anthelmintic treatment, as parasites inside eggs can survive such treatments (Repullés-Albelda *et al.*, 2012). Sánchez-García *et al.* (2011) recommended the use of spasmodic or lethal treatments (as praziquantel or oxidants, respectively) against *Lamellodiscus* spp. In this study, at 20ºC, *L. theroni* became gravid from 18dpi, and a second generation was found on gills at 22 dpi. A two-dose treatment, separated by a minimum of 4 days, could be optimal for long-lasting deworming. In this way, post-larvae and adults would be eliminated by the first dose, while the second dose should be administered after the

all new worms have hatched from the surviving eggs. Nevertheless, detailed information about egg development and time of hatching, as well as knowledge of oncomiracidia survival of *L. theroni* in different conditions must be acquired as periods could be altered by environmental conditions (Kearn, 2004; Mo, 1993; Rubio-Godoy and Tinsley 2002), mainly temperature (Cecchini, 1994; Cecchini *et al.*, 1998).

In conclusion, data obtained from this study indicates that parasite metamorphosis during development implies deep changes of the haptor, meaning a sequential variation of attachment mechanisms and related damage. This study must be considered as a starting point to design effective antihelmintic applications and to predict infection progression of each particular case. Other factors must be strongly considered in further studies, as the effect of temperature, parasite intensity, or the presence of synergistic gill parasites. The study of the development of other species of *Lamellodiscus* with different morphologies can also provide comparative information in order to recognize those aspects modelling timing and infection patterns.

Acknowledgments

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7.5 TABLES AND FIGURES

Table 7.1. Measurements of the sclerotized structures during the development of *Lamellodiscus theroni*. Range in size given in micrometers.

	PHASES					
	I	II	III	IV	V	VI
	n=15	n=8	n=2	n=4	n=21	n=14
Body	144.4-182	152.1-169.7	157.5-159.1	162.9-280.9	257.5-508.5	475.1-887.5
Peripheral marginal hooks	7.2-9.2	7.4-10.0	8.6-10.4	8.0-9.8	8.4-11.6	7.9-10.7
Dorsal hook	-	27.9-44.5	34.9-59.1	55.2-88.6	102.9-126.5	110.6-125.2
Ventral hook	-	39.2-65.8	62.9-84.3	80.5-98.5	111.6-136.2	115.0-141.4
Ventral bar	-	-	43.5-55.3	61.3-78.9	102.9-162.9	143.4-178.2
Dorsal lateral bar (axial/lateral)	-	-	-	38.5-56.9	69.5-91.7/ 58.4-80.8	81.8-93.2/ 71.0-89.0
Lamellodisc width (int/ext)	-	-	-	-	22.3-29.5/ 49.1-69.8	22.4-30.4/ 52.7-66.1
Male copulatory organ	-	-	-	-	-	35.9-93.5

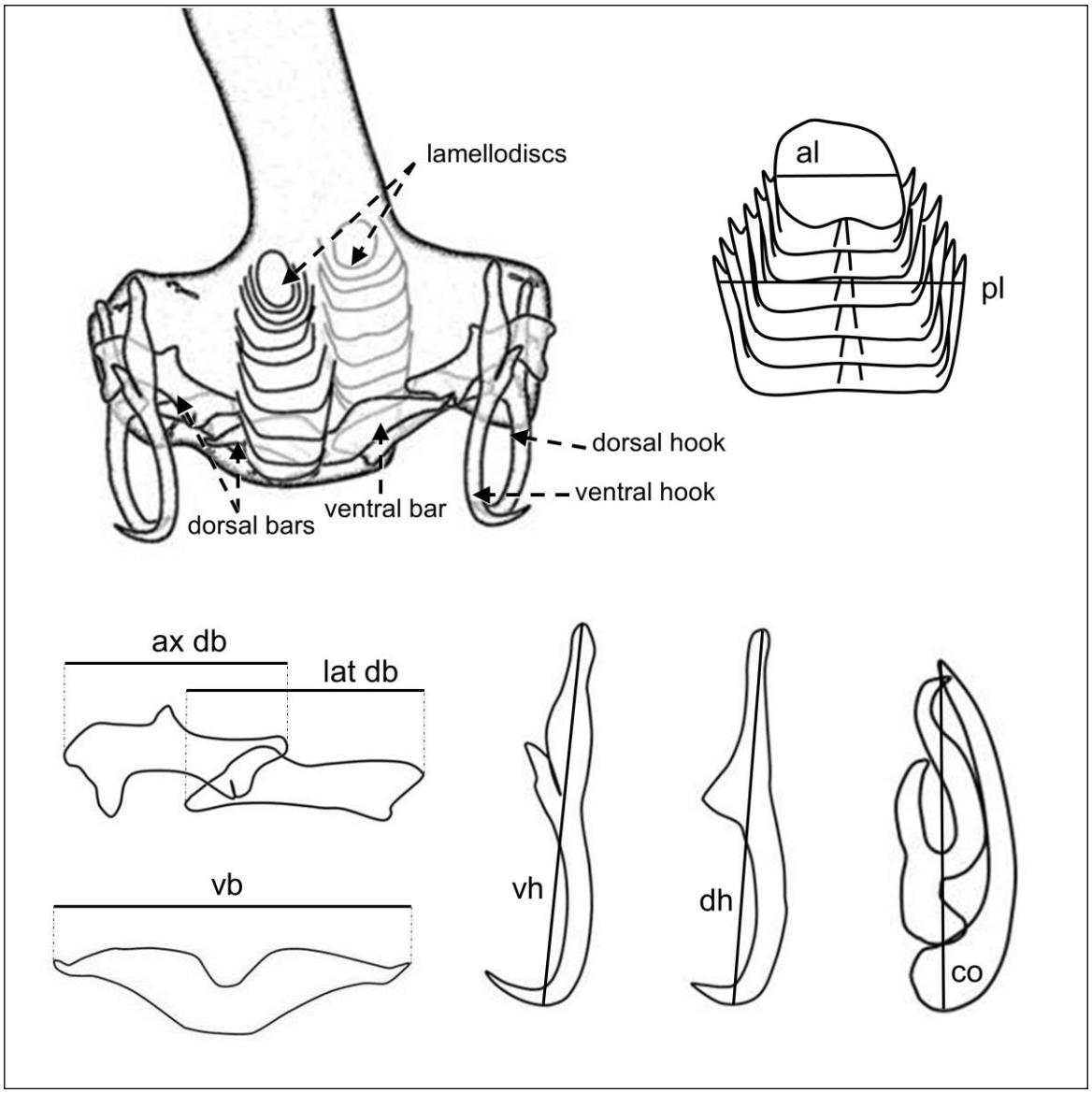

Fig. 7.1. Haptor of *Lamellodiscus theroni*. Measurements taken from the sclerotized pieces. Abbreviations: **al,** length of the anteriormost lamella; **pl,** length of the posteriormost lamella; **axdb,** length of the axial part of the dorsal bar; **latdb,** length of the lateral part of the dorsal bar; **vb,** length of the ventral bar; **vh,** length of the ventral hook; **dh,** length of the dorsal hook; **co,** length of the male copulatory organ

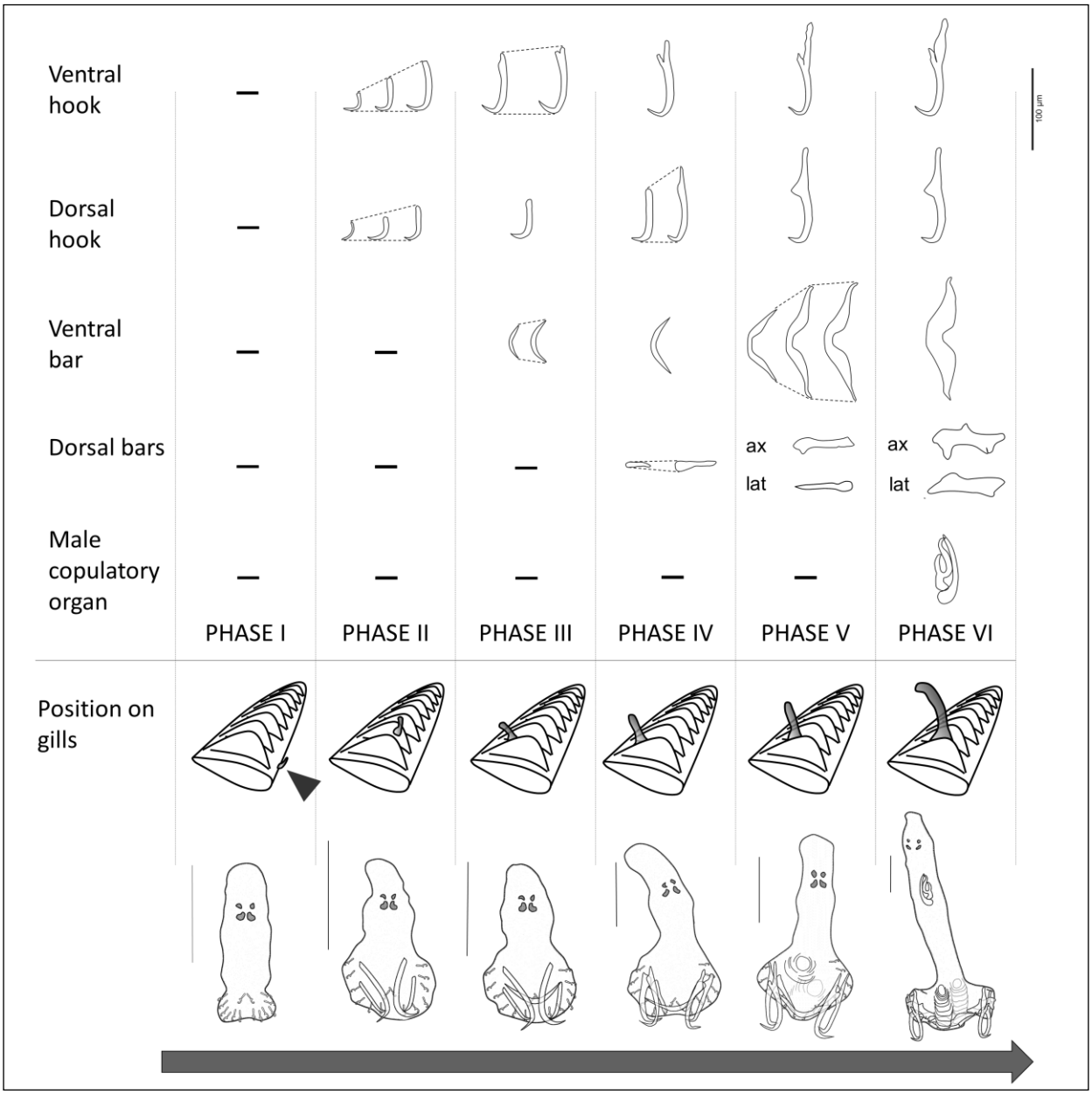

Fig. 7.2. Phases of *Lamellodiscus theroni* development. Morphological changes the sclerotized structures and attachment places of *Lamellodiscus theroni* parasitizing *Diplodus puntazzo* during development. Scale bars, 100µm

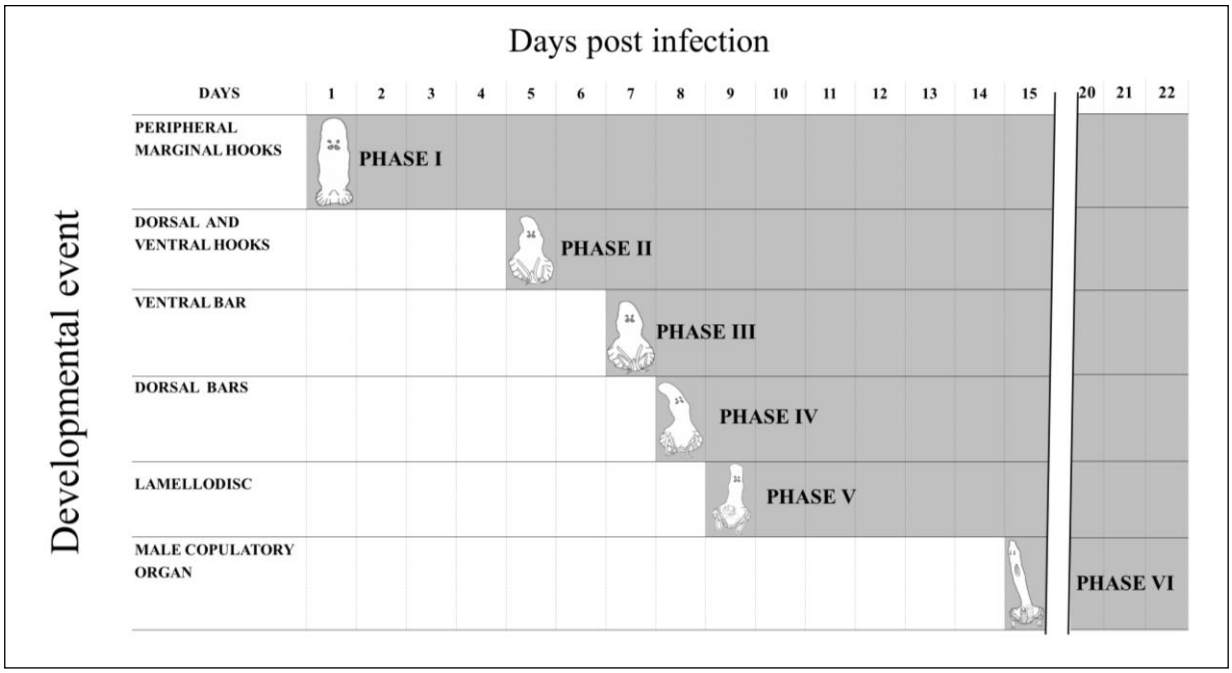

Fig. 7.3. Developmental events of *Lamellodiscus theroni*. Chronology of the developmental events of *Lamellodiscus theroni* parasitizing *Diplodus puntazzo*

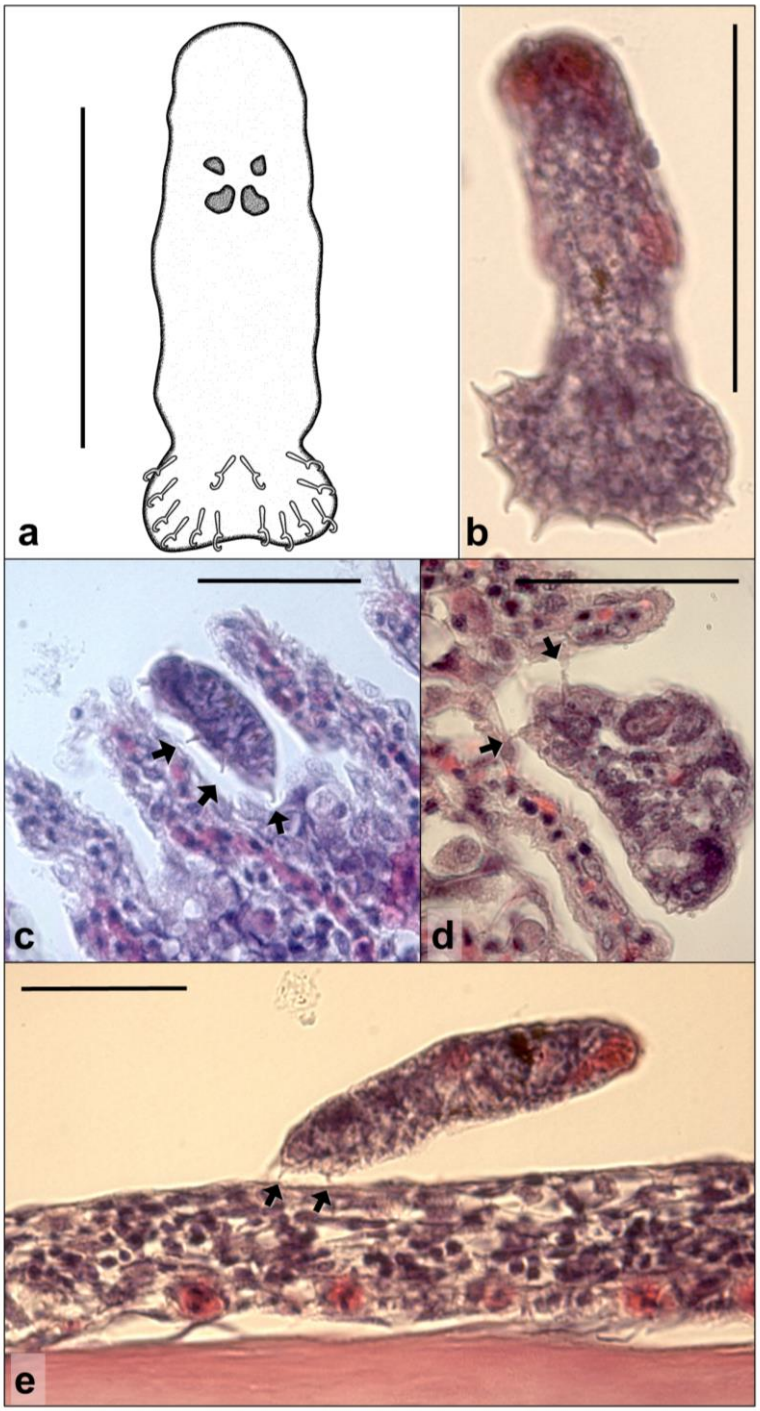

Fig. 7.4. Development of *Lamellodiscus theroni* on the gills of *Diplodus puntazzo*, Phase I. A, drawing of the whole worm; B-E, histological sections obtained from parasitized gills. Parasites in C and D were found within the interlamelar space, while that in E was found on a flat surface of a first lamella. Black arrows point to the peripheral marginal hooks. Scale bars, A=100µm; B-E =50µm

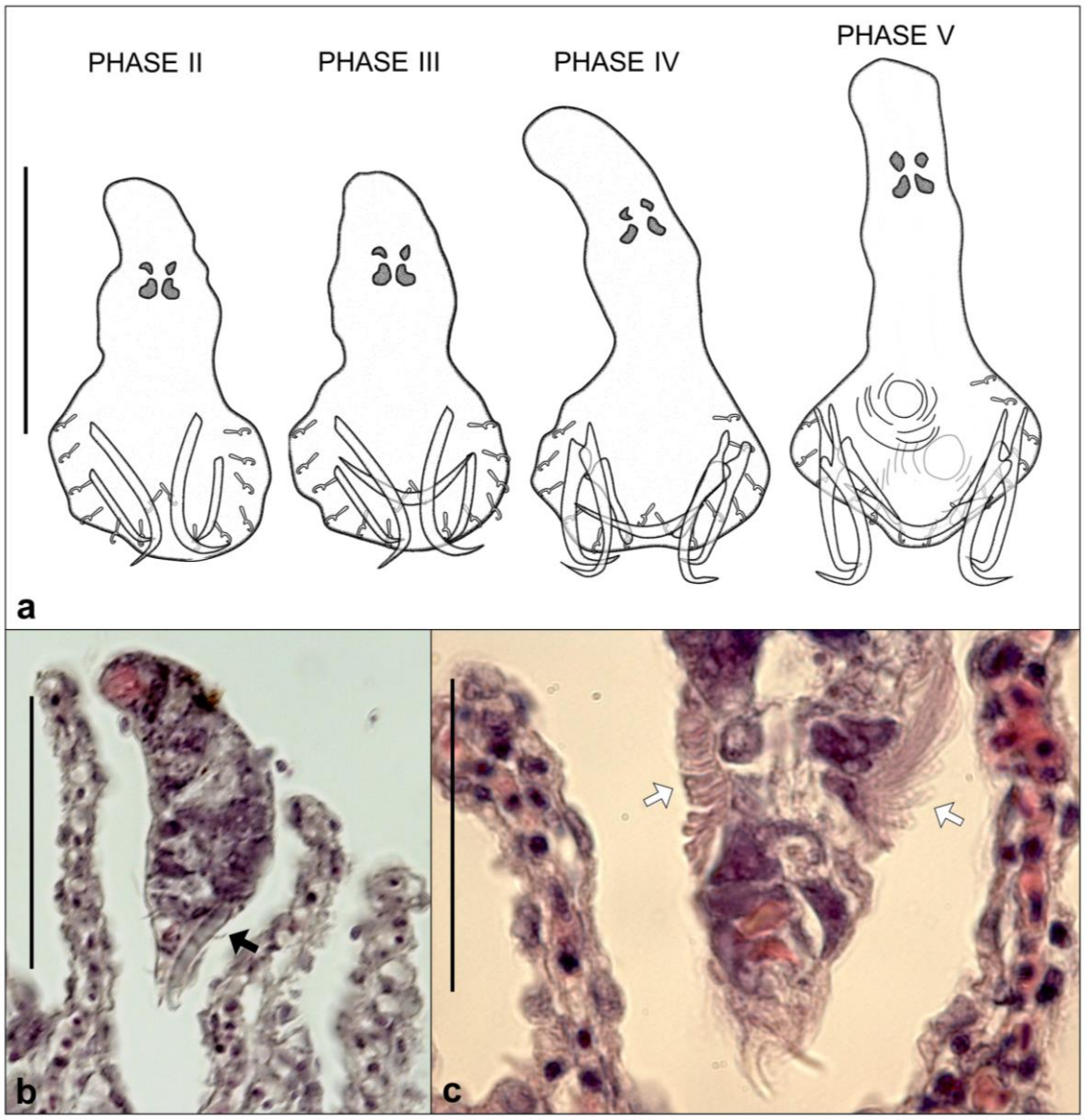

Fig. 7.5. Development of *Lamellodiscus theroni* on the gills of *Diplodus puntazzo*, Phases II to V. A, drawings of whole worms; B and C, histological sections of specimens attached between two secondary lamellas. The black arrow points the ventral hook of a Phase II individual and white arrows point to the rudimentary lamellodiscs of a Phase V individual. Scale bars, A & B=100µm; C=50mm

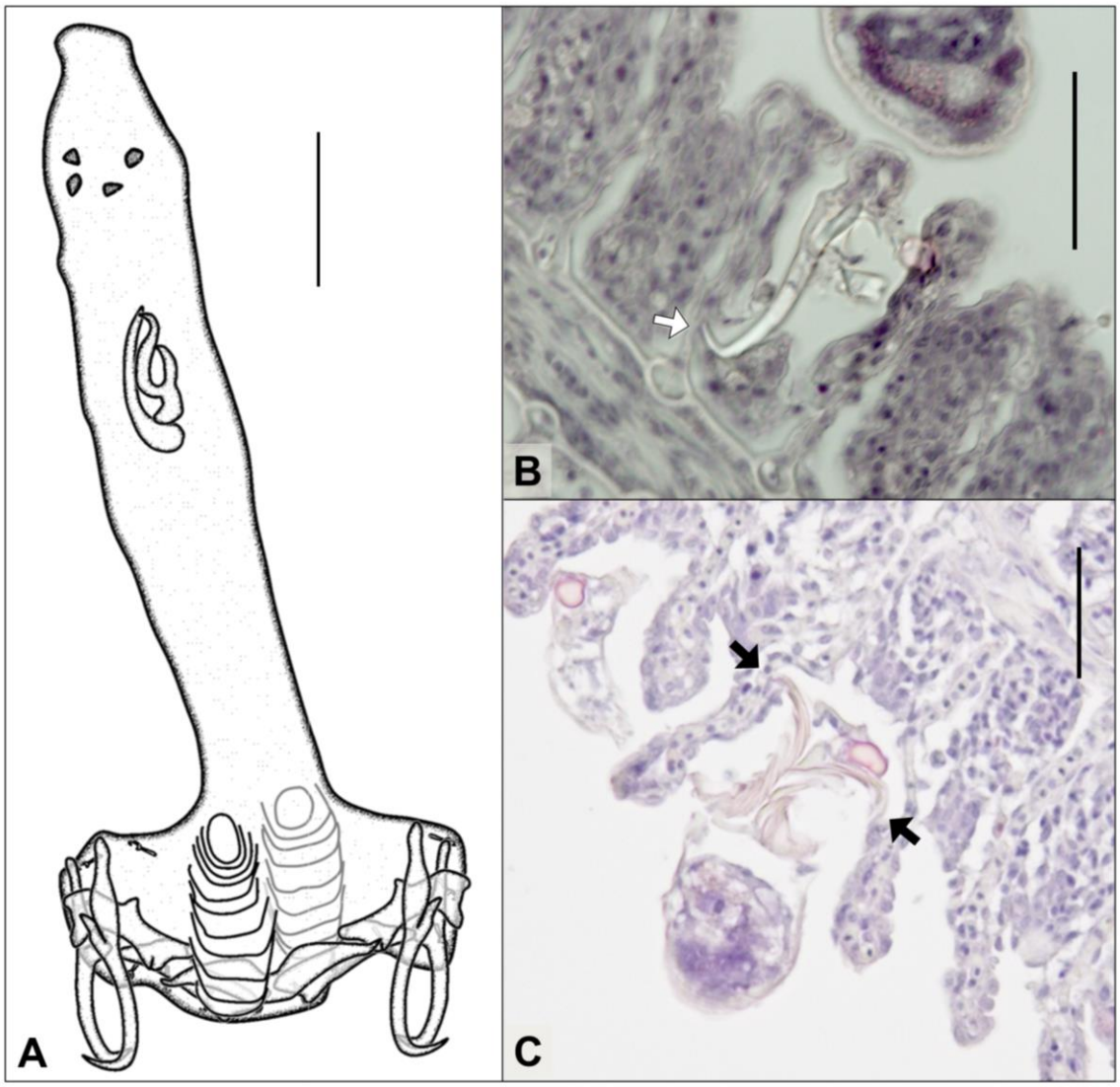

Fig. 7.6. Development of *Lamellodiscus theroni* on the gills of *Diplodus puntazzo,* Phase VI. A, drawing of whole worm; B and C, histological sections of specimens attached deeply inside the interlamellar space. Black arrow points to the dorsal hook piercing the gill epithelium and white arrows point to the gill epithelium pushed by the dorsal and ventral posteriormost lamellae of the lamellodiscs. Scale bars, 100µm

CHAPTER 8

Comparison of the post-larval development of *Lamellodiscus* (Monogenea, Diplectanidae) species. Description of the development of *L. falcus*

Comparison of the post-larval development of *Lamellodiscus* (Monogenea, Diplectanidae) species. Description of the development of *L. falcus*

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8.1 Introduction

Studies on the species of *Lamellodiscus* Johnston & Tiegs 1922 (Monogenea, Diplectanidae) have mainly contributed with new data about taxonomy, host-parasite coevolution or attachment (Amine *et al.*, 2007; Elsayed and Abd El-Monemp, 2011; Poisot and Desdevises, 2010; Poisot *et al.*, 2011; Sánchez-García *et al.*, 2011, 2014a, 2014b); however number of studies regarding to the *Lamellodiscus* development has been limited. Bychowsky (1966) studied for first time the development of *L. elegans* Bychowsky, 1957, providing new knowledge about genus *Lamellodiscus* development. More recently, Sánchez-García *et al.* (2014b) also followed up the development of the sclerotized structures of *L. theroni* Amine, Euzet et Kechemir-Issad 2007 parasitizing *D. puntazzo* in experimental infections, what additionally allowed the study of the developmental chronology. During this study the authors found differences in the moment of development of some haptoral pieces of *L. elegans* and *L. theroni* which were tentatively related with the morphological differences of the adult haptors.

Both species of *Lamellodiscus* have been reported in *Diplodus puntazzo* (Walbaum 1792) (see Sánchez-García *et al.*, 2014a), together with other 6 species: *L. ergensi* Euzet & Oliver 1966, *L. falcus* Amine, Euzet & Kechemir-Issad 2006, *L. hilii* Euzet 1984, *L. ignoratus* Palombi 1943, *L. impervius* Euzet 1984 and *L. bidens* Euzet 1984). Based on previous classifications of the *Lamellodiscus* species depending on the composition of the haptoral sclerotized pieces the 8 species in *D. puntazzo* could be classified in two groups of morphotypes (Amine and Euzet, 2005): in *L. bidens, L. elegans*, *L. falcus*, *L. hilii*, *L. ignoratus* and *L. impervius* each lateral dorsal haptoral bar is formed by only one piece; in *L. ergensi* and *L. theroni* each lateral bar is formed by two pieces. Morphological differences in monogenean haptor imply different attachment strategies and, subsequently, differences in other aspects as ecological, pathological (Sánchez-García *et al.*, 2011), or even developmental (Sánchez-García *et al.*, 2014b).

In this paper we have studied the development of a third *Lamellodiscus* species, *L. falcus*, in experimental infections of *D. puntazzo* in order to record and

compare the morphological changes in time. *L. falcus* is quite similar to *L. elegans*; haptors belong to the same morphological group (single-pieced lateral bars) and, moreover, these two species have relatively short main hooks (slightly overpassing the haptoral margins). Comparing the growth of these 2 similar species with that of *L. theroni*, we also want to investigate how haptor morphology determines the differences on the rhythm of development between species.

8.2 Materials and Methods

Forty *Lamellodiscus falcus*-infected *Diplodus puntazzo* (further on referred as donor fish) were collected in May 2009 from Mar Menor (Murcia, Spain: 37º41´N - Oº44´E) and reared in the installations of Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario (IMIDA) at San Pedro de Pinatar, Murcia. Fish were transported alive and maintained in the facilities of the Central Service for the Support to Experimental Research of the University of Valencia (SCSIE). Once in the SCSIE installations, ten infected fish (total length 305.3±9.2 cm; weigh 399.8±38.7 g) were killed by cervical decapitation in order to monitor parasite levels (prevalence, 100%; intensity, 898 parasites/infected fish). They were used as donor fish. Naïve fish were acquired from a Greek hatchery, transported to the SCSIE aquarium plant and reared along three years. All fish were maintained in parasite-free aquaria with marine water, salinity 37‰, temperature 20˚ and photoperiod 8:16.

One hundred and twenty naïve *D. puntazzo* (total length 168.3±10.8 mm; weigh 66.1 ±14.2 g) were infected by cohabitation with the donor fish for 48h. After infection period, fish were transferred to 250 l volume "experimental tanks". "Time 0" of the experiment was assumed as the moment when the fish were restocked in the "experimental tanks". From this moment, 5 fish were killed and analyzed daily for 22 days, as at 20 dpi a second generation of post-larval specimens was found. Gill arches were isolated from each individual in different Petri dishes with seawater and analyzed fresh immediately after dissection. Each first lamella was observed with a Leica MZ AP0 (8X-80X) stereomicroscope using transmitted light. After examination, gill arches with higher parasites abundance

were fixed in 4% buffered formalin to histological analysis. During the first 5 dpi fish skin and fins were also analyzed to find post-larval parasites. All specimens found were collected and mounted on temporary slides with glycerin jelly after being killed in hot saline solution. The sclerotized parts of the haptor and the male copulatory organ were studied with differential interference contrast (DIC) microscopy (Leica DMR (100X-1000X)). The sclerites of *L. falcus* were drawn with the help of a drawing tube and measured using ImageTool 3.1 (Fig 8.1). The sequence of finding s of each developmental event was recorded and the shape and growing changes monitored.

For the histological study highly infected gill arches previously fixed in 4% buffered formalin were embedded in paraffin, serially sectioned at 4 µm with a rotary microtome, stained with haematoxylin and eosin (H-E), mounted in Entellan TM (Merck) and observed using light microscopy (Leica DMR HC microscope).

To compare the development of the different species of *Lamellodiscus* morphological and morphometrical data were extracted from available previous studies, Sánchez-García *et al.* (2014b) and Bychowsky (1969).

8.3 Results

8.3.1 Morphology

All parasites were found on gills, none of them on skin or fins. Total number of parasites collected was 40 (prevalence 66.6%), corresponding to different developmental stages, 16 of them being adult. Parasites needed at least 14 dpi to develop into adult (with all haptor structures and the copulatory organ) and 16 dpi to become mature worms and develop eggs. Second generation of post-larval specimens was found at 20 dpi. Morphometric variations of the parasite structures during the development are indicated in Table 1 and graphically represented in Fig 8.2. Six developmental phases have been distinguished in *L. falcus* development, according to the first findings of the sclerotized structures (see graphical summary of the developmental events in Fig 8.3).

- Phase I (from 1 to 12 dpi; n= 8) (see table 8.1 and Fig. 8.2): first post-larval phase after oncomiracidia fixation with haptor only armed by marginal hooks (a.k.a. uncinuli). Body elongated and dorso-ventrally flattened. Haptor with 14 sclerotized peripheral marginal hooks, circularly arranged along the haptor edge, except for a pair of them allocated at the center of the haptor. Hooks with a thick handle and a distally curved and pointed tip. Shape remained equal along parasite life, nevertheless length increased slightly between phase I and II.

- Phase II (from 5 to 15 dpi; n= 12, more than 50% reaching to this stage at day 9) (see Fig 8.2, 8.4): post-larvae characterized by the first observation of dorsal and ventral hooks (main hooks). Total body length slightly increased. The haptor region wider and longer than in phase I (approximately half body length). Dorsal and ventral pairs of hooks formed by thin straight bars with hooked distal end. Ventral hooks slightly larger than dorsal ones. Although main hooks were larger than the marginal ones since their first detection, their length increased during this phase, developing the early anterior protuberances that will become the "guard" of the hooks.

- Phase III (from 7 to 8 dpi; n= 1) (see Fig 8.2, 8.4): only one specimen of *L. falcus* belonging to this phase was found. Post-larvae characterized by the first observation of the ventral bar. Body length increased slightly from previous phase. Main hooks distinctly robust; ventral hooks became longer than in previous phase, while dorsal hooks length remained nearly invariable (Table 8.1). Morphology of the main hooks was close to the adult one: early "guards" perceptible in both pairs of hooks but slightly different between ventral and dorsal hooks; the guard projection of the ventral hooks was elongated and perpendicular to main axis, while that of the dorsal ones was a short ventral swelling, more evident in the following phases. At this phase, the ventral bar was a thin and slightly bended bar with both ends pointed, and arranged perpendicularly to the ventral and dorsal pair of hooks. Its length was shorter to those of main hooks, 1/3 of the adult ventral bar.

- Phase IV (from 8 to 16 dpi; n= 11, more than 50% reaching to this stage at day 14) (see Fig 8.2, 8.4): post-larvae characterized by the first observation of the

dorsal bars. Body length experienced a noticeable increase from previous stage. Lengths of dorsal and ventral hooks also increased, appearing more robust. Hooks morphology very similar to that in the adults: the definitive "guard" is a perpendicular digitiform process, slightly oriented to the handle, in the ventral hooks and a soft swelling in the dorsal ones. Ventral medial bar longer and more robust, changing from a bended bar to a more straight bar slightly notched medially. Early dorsal bars, oblique to the ventral bar, were thin and quite sinuous.

- Phase V (from 9 to 16 dpi; n= 3, more than 50% reaching to this stage at day 14) (see Fig 8.2): post-larvae characterized by the first observations of the dorsal and ventral lamellodiscs. Total length almost doubled that from previous phase. All haptoral esclerites were already present at this stage. Dorsal and ventral hooks showed almost their definitive shape and thickness, while length remained almost invariable from the previous phase. Ventral bar increased considerably its length and acquired a more robust appearance, with morphology very similar to that in the adults: fusiform, medially bended or notched, with pointed lateral tips. Dorsal bars acquired almost their definitive shape, although they were slightly shorter and thinner than the adult ones. From first observations of this phase the primordia of the dorsal and anterior lamellodiscs were visible, anterior to the other haptor esclerites. Primordial lamellae appeared as thin narrow curved plates, except for the thin anteriormost lamellae which were elliptical from the first observation.

- Phase VI (from 14 dpi; n= 12, more than 50% reaching to this stage at day 15) (see Fig 8.2, 8.5): adult worms characterized by the first observation of the male copulatory organ. Body length experienced the greatest increase observed during the development. Haptor was totally developed and all sclerotized structures had their definitive size and shape. Main hooks and ventral and dorsal bars experienced a noticeable size increase. Definitive adult lamellae represented 1/10 of total body length. Adult male copulatory organ was composed by the two sclerotized pieces typical of the species.

8.3.2 Attachment development

The attachment strategy changed as the haptor elements develop. At phase I, haptor was only armed by the 14 peripheral marginal hooks; therefore worms used the marginal hooks to attach to the gill tissues, mainly on flat surfaces or between gill secondary lamellae (Fig 8.4). When the early post-larvae were attached on flat surfaces, all peripheral marginal hooks were oriented ventrally, and when they were placed between adjacent secondary lamellae, hooks could be oriented to dorsal and ventral sides. Phase I post-larvae were mainly recovered from the most basal areas of the first gill lamellae.

During the following phases II, III and IV, attachment strategy experienced important changes. These worms were always observed between secondary gill lamellae: dorsal hooks pierced the same secondary lamella while the ventral hooks pierced the contiguous backwards one (Fig 8.4), stretching gill lamellae toward parasites. Main hooks grew gradually, becoming more hooked and piercing more deeply.

During phases V and VI, the development of the lamellodiscs implied the last change in the attachment strategy. Early lamellodiscs were thin and incomplete lamellae, apparently afunctional. When they became thick and large plates acquire the definitive adult capability to slide and project performing a telescopic movement. Worms remained located between the interlamellar spaces, dorsal and ventral lamellodiscs pushing the secondary lamellae, opposite to the stretch action from dorsal and ventral hooks (Fig 8.5). Peripheral marginal hooks worked as accessory attachment points.

8.4 Discussion

The present study indicates that, in *L. falcus*, total and partial growth rhythms vary in time, depending on the biological demands of each life period. All these changes lead to different final haptor morphologies and sizes, what also have to determine the growth patterns. The development of the different sclerotized pieces of the haptor follow the same order than previously described for *L. theroni* and *L.*

elegans (Fig 8.6). However, some aspects of parasite growth are difficult to predict. Interestingly, although *L. facus* is larger than *L. theroni* , it reaches to the definitive size and haptor morphology (phase VI), one day before than *L. theroni* (14 vs. 15 dpi). Perhaps, in this case the growth speed is more determined by the final size of the haptor components, as they are much larger (in absolute and relative numbers) in *L. theroni* (i.e. mean body: ventral hook ratio in *L. theroni*, 1:0.19 and in *L. falcus*, 1:0.10; mean body: ventral bar ratio in *L. theroni* 1:0.25 and in *L. falcus* 1:0.13). More species must be studied to confirm this hypothesis. Sánchez-García *et al.* (2014b) pointed out that differences between *L. theroni* and *L. elegans* could be related to the larger relative size of the main hooks of *L. theroni*. The final composition and arrangement of the haptor sclerites can also determine the development of the *Lamellodiscus* species. For example, according to the classification defined by Amine and Euzet (2005), *L. theroni* belongs to the group of species with lateral bars divided in two pieces while the lateral bars of *L. elegans* and *L. falcus* are composed by a single bar.

No remarkable morphologic differences were found between *L. falcus* and anterior *Lamellodiscus* post-larval descriptions (Bychowsky, 1961; Sánchez-García *et al.* 2014b). The size of the earliest post-larval *L. falcus* specimens was very similar to that of the post-larvae of *L. theroni* (Sánchez-García *et al.* 2014b), however, as Sánchez-García *et al.* (2014) remarked, their length was more than double of that described by Bychowsky (1961) for *L. elegans* (60-70 vs. 161-163 µm). Sánchez-García *et al.* (2014b) also indicated that ventral hooks of *L. theroni* (the largest main hooks in adults) were bigger than dorsal hooks from the beginning of development, in contrast, in *L. falcus*, although ventral hooks are also the largest in adults, both pairs of main hooks showed very similar length from the earliest phase, as described for *L. elegans* (Bychowsky, 1961). Similarly to *L. theroni*, main hooks grew gradually during all developmental stages, only reaching to the definitive size at phase VI (Sánchez-García *et al.*, 2014b). However, in contrast to *L. theroni*, where the definitive shape is also attained at phase VI, the shape of the main hooks of *L. falcus* showed the nearly typical morphological features of the species at phase III. Phases III and IV were characterized by the development of ventral and dorsal bars respectively, similarly to *L. theroni* the time between both

events was short (Sánchez-García *et al.*, 2014b) (Fig 8.6). Ventral and dorsal bars growth along all the development phases, nevertheless the adult morphology was acquired at the last developmental phase. The definitive adult *L. falcus* haptor became completely developed during phase V and VI with the dorsal and ventral lamellodiscs development (Fig 8.6).

Body length of *L. falcus* grew from the phase I, while *L. theroni* maintained the same length from phase I to II, when starts to enlarge its length. These differences in total length growing could be related with the different final body size of both species, being *L. falcus* 10% larger than *L. theroni*. The haptor sclerites of *L. theroni* experienced the large size increases between phase IV and V, when dorsal and ventral main hooks acquired almost their definitive size (Sánchez-García *et al.* 2014b). In contrast during *L. falcus* development, haptor sclerites, except for the ventral bar, experienced the larger increases in two times, between phase III and IV, and between phase V and VI. *L. theroni* main hooks grew faster than those of *L. falcus*, in each phase the length increased range between 20% and 38% of the total size; while in *L. falcus* development the main hooks length increase range was between 0% to 18%. Regarding the shape of the main hooks, differently to *L. theroni*, those of *L. falcus* showed the guards characteristics of the species from phase III, and its morphology is almost that of the adult at phase IV. This type of development of the main hooks is very similar to that of *L. elegans*, which acquire their definitive shape previous to lamellodisc detection (in phase IV) (Bychowsky, 1957). The thicker and longer main hooks of *L. theroni* could need more time of sclerotization to achieve their final profile.

The similar developmental sequence of the haptoral sclerotized of *L. falcus* and *L. theroni* implies their attachment strategies along development are also similar, as the histological study showed confirms. We identified the three periods regarding to the attachment described for *L. theroni* by Sánchez-García *et al.* (2014b) *i)* early post-larvae only attached by the peripheral marginal hooks, mainly on flat surfaces and sometimes in interlamelar spaces; *ii)* phases II to IV post-larvae mainly attached by their main pairs of hooks within the interlamelar space with dorsal and ventral hooks piercing the adjacent secondary lamellae; and *iii)* phases

V to VI, progressively involving the lamellodiscs in the attachment, as they develop and sclerotize, conferring a higher stability and strength to the attachment. Main difference between these species seems to be related with the sizes of the attachment structures. The histological sections of both species show that the main hooks of *L. theroni* penetrate deeper in tissues than *L. falcus*, what can be explained because the main hooks of *L. falcus* are smaller than those of *L. theroni*. According to Sánchez-García *et al.* (2011), hook piercing of *Lamellodiscus* sp. often involved hemorrhages, due to the perforation of vessels. According to the hook size, we can identify now that the species related to these hemorrhages is *L. theroni*, which hooks pierce deep enough to reach to the vessels.

In conclusion, although the attachment strategies of the developmental stages of the species of *Lamellodiscus* are similar, some differences can be found related to the different composition and dimensions of adult sclerites of each species. The timing of these changes seems more variable, as larger pieces take longer times to reach to their final dimensions. These differences can imply shorter or longer periods of more or less harmful damage on gills and, finally, can explain particular pathologies, as hemorrhages, only possible if hooks reach to sizes enough big to penetrate to blood vessels in gills.

8.5 TABLES AND FIGURES

Table 8.1. Measurements of the sclerotized structures during the development of *Lamellodiscus falcus*. All measurements are given in micrometers ± standard deviation.

LENGTH	**PHASES**					
	I	**II**	**III**	**IV**	**V**	**VI**
	n=15	n=5	n=1	n=10	n=3	n=16
Body	161.2±19.2	191.5±15.3	209.5	277.9±44.0	433.1±89.3	753.2±155.9
Peripheral marginal hooks	8.2±1.0	10.0±1.0	9.7	9.8±0.4	9.6±1.0	10.55±1.0
Dorsal hook	-	47.6±5.4	50.4	61.3±5.4	60.5±5.2	69.4±5.0
Ventral hook	-	49.7±8.6	58.1	66.8±5.8	67.4±6.2	77.2±4.4
Ventral bar	-	-	35.3	57.7±15.0	83.7±10.0	97.3±8.2
Dorsal bar	-	-	-	48.4±10.0	51.3±7.3	83.9±8.9
Lamellodisc (int/ext)	-	-	-	-	35.5±16.0/ 66.1±22.8	44.3±2.8/ 85.0±6.9
Mail copulatory organ	-	-	-	-	-	90.1±53.3

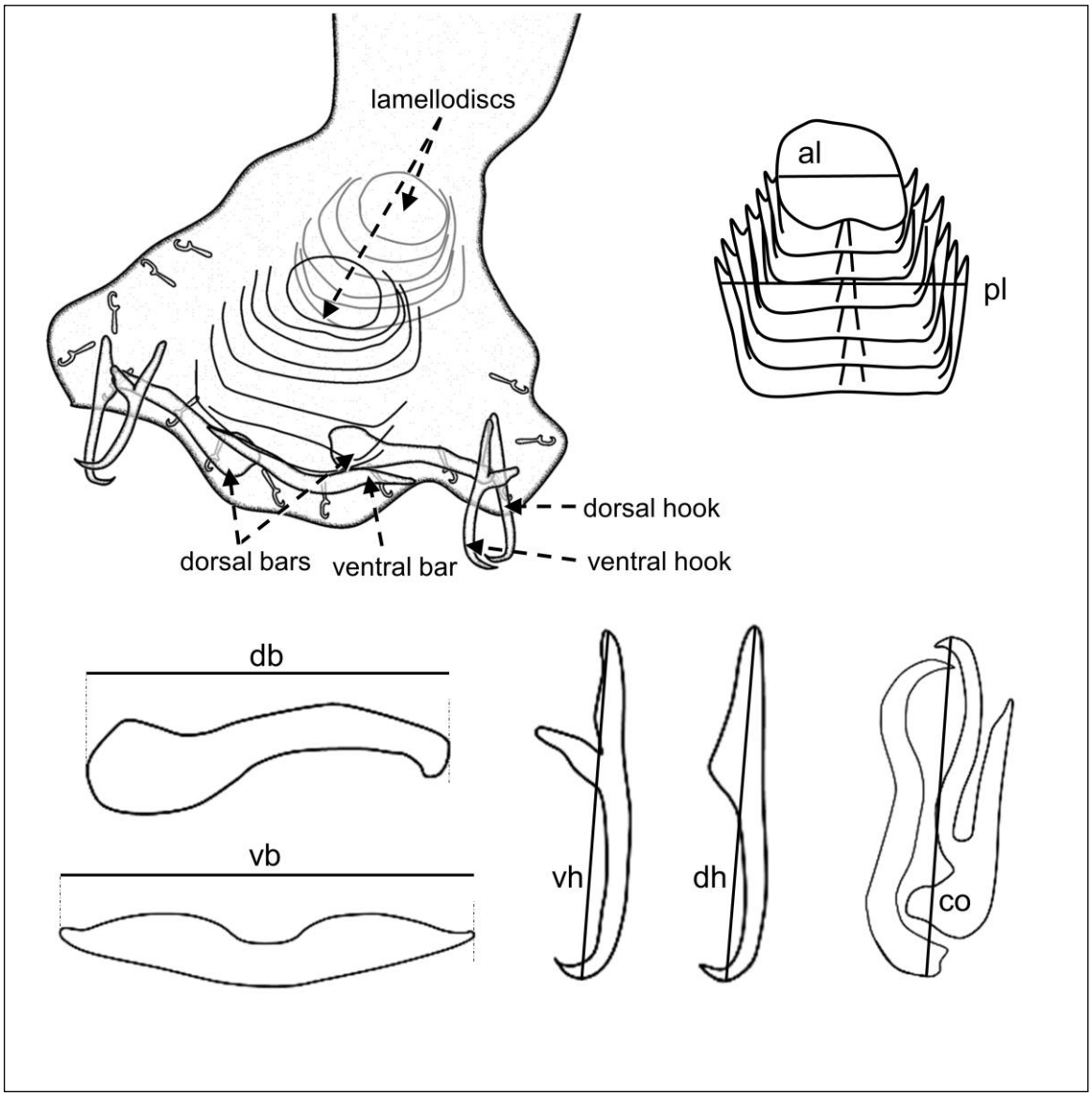

Fig. 8.1. Haptor of *Lamellodiscus falcus*, showing the measurements taken from the sclerotized pieces. Abbreviations: **al,** length of the anteriormost lamella; **pl,** length of the posteriormost lamella; **db,** length of the dorsal bar; **vb,** length of the ventral bar; **vh,** length of the ventral hook; **dh,** length of the dorsal hook; **co,** length of the male copulatory organ

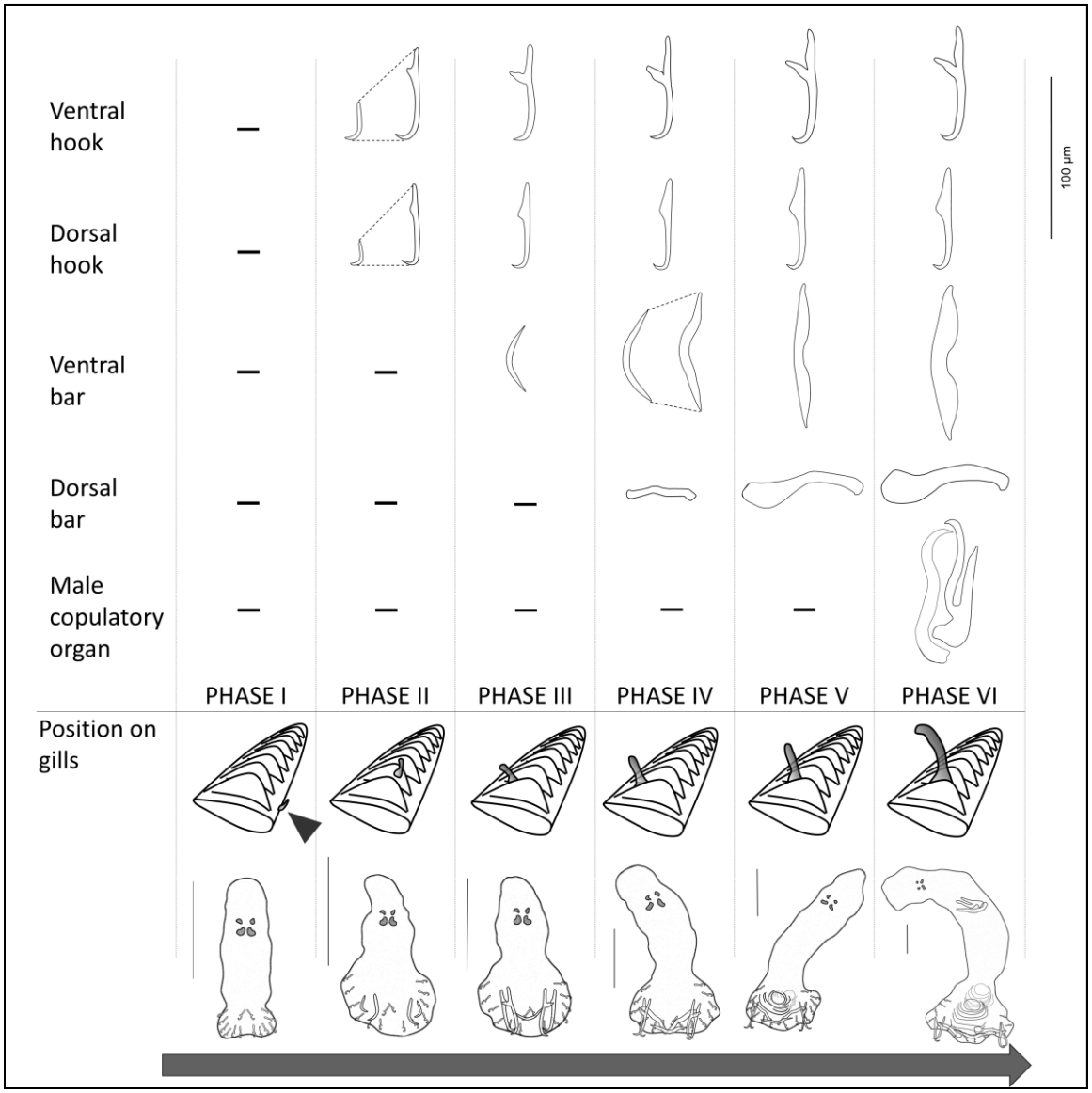

Fig. 8.2. Morphological changes the sclerotized structures and attachment places of *Lamellodiscus falcus* parasitizing *Diplodus puntazzo* during development. Scale bars: 100µm

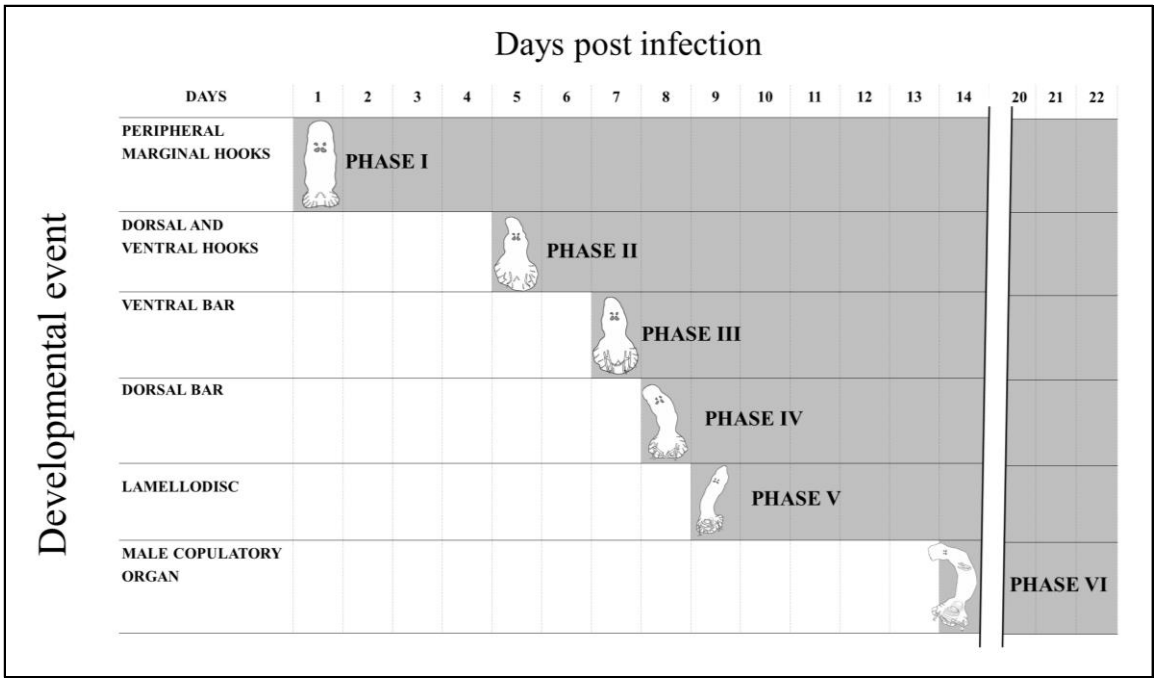

Fig. 8.3. Chronology of the developmental events of *Lamellodiscus falcus* parasitizing *Diplodus puntazzo*

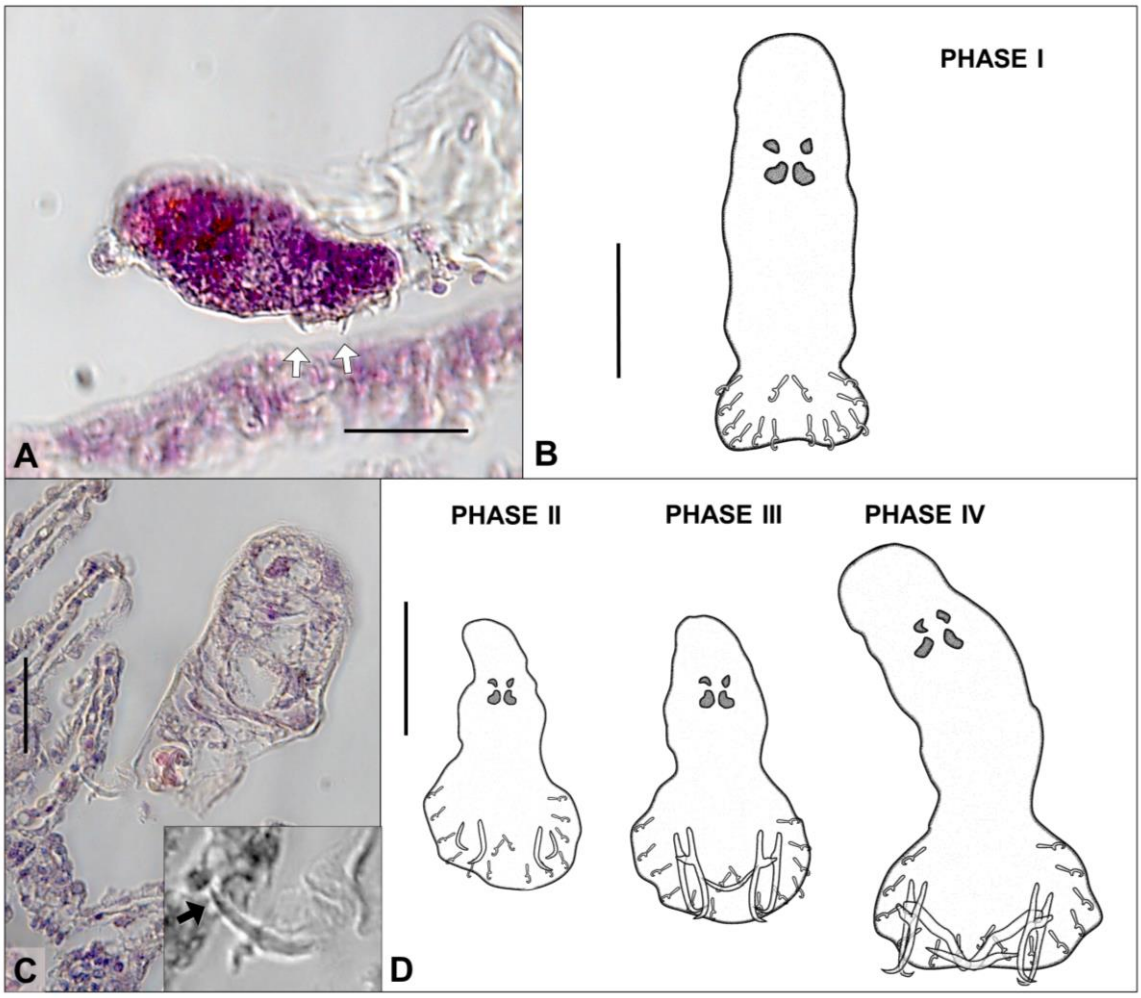

Fig. 8.4. Development of *Lamellodiscus falcus* on the gills of *Diplodus puntazzo*. Phase I to IV. A and C, histological sections obtained from parasitized gills; B and D, drawing of the whole worm. Parasites in C and D were found within the interlamelar space, while that in A was found on a flat surface of a first lamella. White arrows point to the peripheral marginal hooks and the black arrow point to the dorsal hooks. Scale bars, A-B=50µm; C-D=100µm

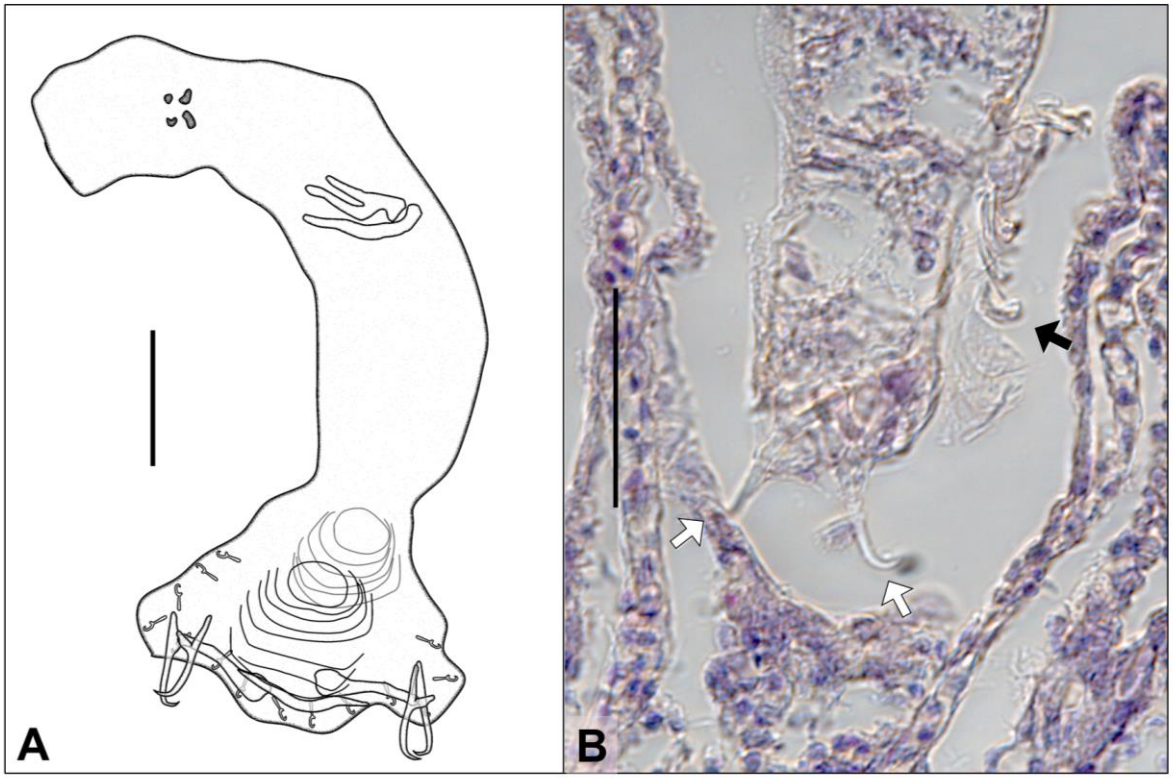

Fig. 8.5. Development of *Lamellodiscus falcus* on the gills of *Diplodus puntazzo*. Phase VI. A, drawing of whole worm; B, histological sections of specimen attached deeply inside the interlamellar space. White arrows points to the main hooks piercing the gill epithelium and black arrow point to the lamellae of the lamellodiscs. Scale bars, 100µm

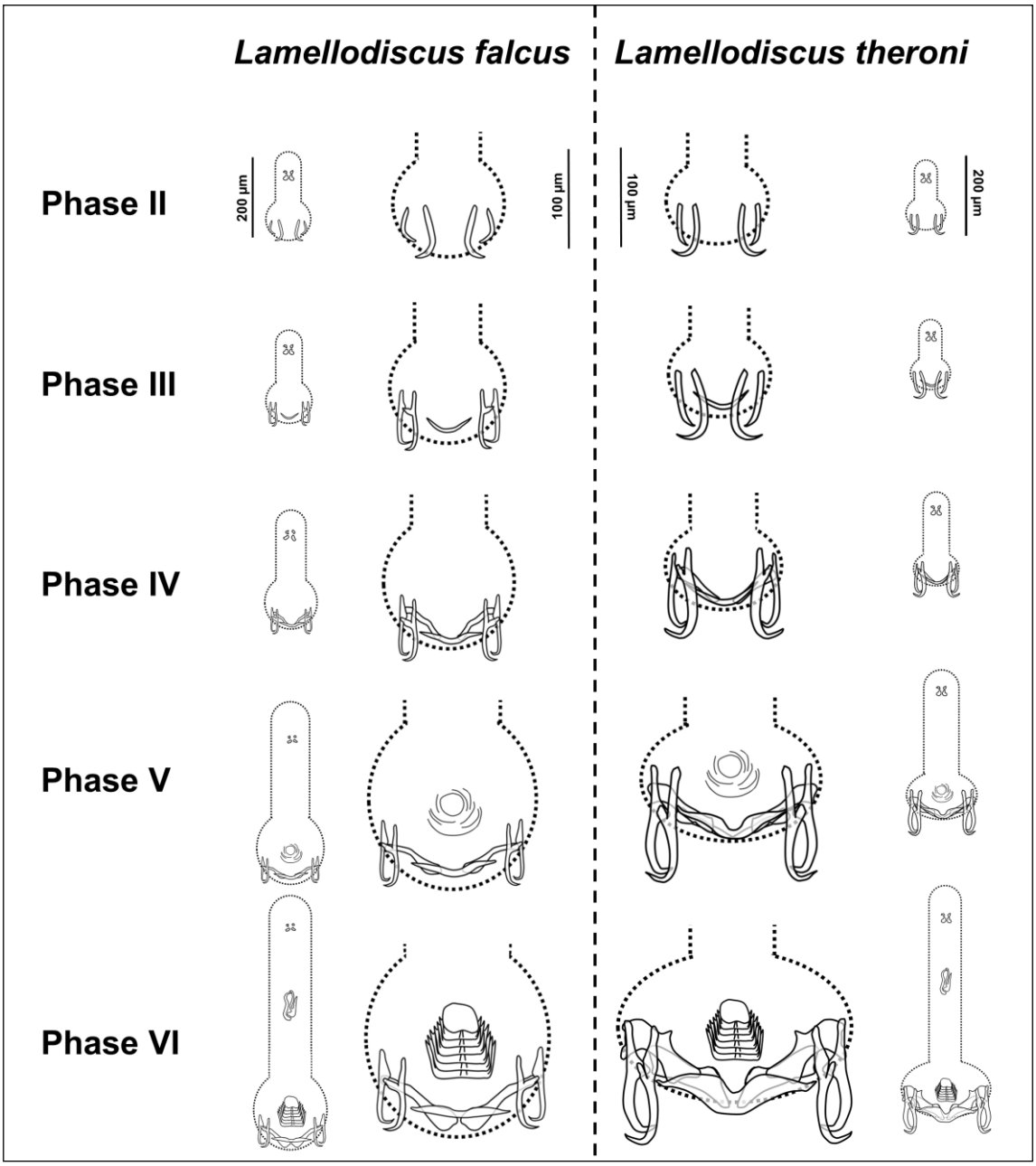

Fig. 8.6. Comparison between development of *Lamellodiscus falcus* and *Lamellodiscus theroni* on the gills of *Diplodus puntazzo*

Conclusions

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

1. The parasite fauna of wild *Diplodus puntazzo* from Santa Pola and Mar Menor in the Spanish Mediterranean is composed by nineteen parasite species, seven of them cited for the first time in *D. puntazzo*: *Microcotyle* sp., *Magnibursatus bartolii, Steringotrema pagelli, Galactosomum sp., Cardiocephaloides longicollis, Caligus ligusticus* and *Gnathia vorax*. We also report the first records in the wild of two parasite species previously found only in farmed *D. puntazzo*: the polyopisthocotylean monogeneans *Atrispinum seminalis* and *Sparicotyle chrysophrii*.

2. Most of the parasites found in the current study are monoxenous (11 species): 8 monogeneans and 3 crustaceans. The heteroxenous species consist of 7 species of trematode and one species of myxozoan.

3. The morphological traits of the species of the monogenean genus *Lamellodiscus* found in present study coincide with those described for *L. falcus* and *L. theroni*. However, their dimensions were mostly similar to those of *L. ignoratus* and *L. ergensi*.

4. The molecular results from *Lamellodiscus falcus sensu lato* and *L. theroni sensu lato* show intraspecific differences in ITS1. However, this variation lies within the range of intraspecific variation provided by previous studies for *L. ergensi* and *L. ignoratus*. Thus, despite the morphometric variation amongst the described species (*L. falcus vs. L. ignoratus* and *L. theroni vs. L. ergensi*), molecular divergences are not enough to support their separation.

5. Spanish Mediterranean specimens of the trematode *Peracreadium characis* analyzed in the present study are somehow smaller than previous descriptions. Therefore, the present study extends the range of the morphometric variation for this species. Molecular analyses confirm that these morphological differences are intraspecific.

6. Most of the ectoparasites living on skin, gills and digestive system disappear in captivity conditions with the exception of the species of the genus *Lamellodiscus*. However some endoparasites remain protected by internal organs, as *Cardiocephaloides longicollis* in the brain and *Ceratomyxa* sp. in the gall-bladder.

7. Three species of parasites entail a "High" risk to *D. puntazzo* in Mediterranean culture conditions: *Amyloodinium* sp., *Cryptocaryon* sp. and *Enteromyxum leei*. Moreover three species of parasites, *Sparicotyle chrisophrii*, *Caligus ligusticus* and *Gnathia vorax*, entail a "Moderate" risk to Spanish Mediterranean farms of *D. puntazzo*. Most of the parasites entailing a "Moderate" to "High" risk to *D. puntazzo* farming have been previously reported in *Sparus aurata* cultures. Cross-infections between both species must be considered.

8. The lamellodiscs of *Lamellodiscus theroni* and *L. falcus* are arranged in overlapped lamellae. Contrary to previous publications, lamellodiscs do not work as suckers. Each independent lamella slides posteriorly resulting in the telescopic projection and creating a pushing effect on the gill epithelium, causing the gill secondary lamellae separation. Lamellodiscs thrust force is opposite to hooks traction force, which tightens the epithelia, providing a highly efficient attachment and stability to the worms.

9. *Furnestinia echeneis* presents a split type lamellodisc, where each half lamellae slides and moves independently. Lamella halves independence allows a postero-lateral displacement (hand fan-like), resulting in a volume increasing and suction effect. The lamellodisc of *F. echeneis* is too large to fit inside the interlamellar space, and works as a large sucker that fixes the monogenean to wide and flat gill tissues. Hooks would play a secondary role strengthening the attachment.

10. *Diplectanum aequans* haptor penetrates inside the interlamellar space, establishing a tight contact with host tissues. The hooks and spines produce extensive and deep alterations in the gill epithelium surrounding the parasite attachment site. The hyperplasia and inflammatory reaction of the epithelia, including tissue oedema, passively trap the haptor of the parasite.

11. Dissimilar attachment mechanisms of diplectanids explain the different damage intensity provoked by each species. *Diplectanum aequans* attachment mechanism implies extensive friction of surfaces contacting the haptor, with subsequent severe alterations of gills. *Diplectanum aequans* and *Lamellodiscus* spp. are attached to the secondary gill lamellae, causing severe damages and therefore impairing the respiratory function (hyperplasia, lamellar fusion and damage of the vascular structures). In contrast, *F. echeneis* attachment does not affect respiratory structures and only causes mild damage (temporary epithelial swelling and superficial perforation of gill tissues by hooks).

12. Information provided about attachment of Diplectanids is useful to design specific antihelmintic treatments: *D. aequans* attachment seems to be tight and passive; therefore parasites are difficult to detach, requiring aggressive and parasiticide treatments. The attachment of *Lamellodiscus* spp. and *F. echeneis* are respectively partially or totally active, and parasites should be more easily eliminated, after a treatment with antihelmintic products as those causing spasmodic effects on parasites.

13. During the *L. theroni* and *L. falcus* post-larval development, six developmental phases have been identified depending on the development of the sclerotized structures of the haptor. I) with 14 peripheral marginal hooks, II) with dorsal and ventral hooks, III and IV) with ventral and dorsal bars, V) with dorsal and ventral lamellodiscs, and finally VI) with developed the copulatory organ.

14. Three developmental phases are crucial for the attachment strategy of the *Lamellodiscus* species during development. In phase I, parasites are only attached by the short 14 peripheral marginal hooks, a slightly stable attachment what allow parasite free displacement on plane surfaces. In phase II, the use of the large main hooks hampers the free movement of the parasite, although parasites are more stably attached to the secondary lamellae within the interlamelar space; and finally, in phase V, the presence of dorsal and ventral lamellodiscs entails a more stable and efficient attachment.

15. Despite the different haptors of *L. falcus, L. theroni* and *L. elegans* their growth patterns were very similar. These similarities imply analogous attachment strategies along development. However some differences can be found related to the different developmental rhythm of sclerites apparently related with their final dimension in each species.

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