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MORPHOPATHOLOGICAL STUDY OF THE INTERACTION BETWEEN THE FRESHWATER PEARL MUSSEL AND THE ATLANTIC SALMON

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[Suena una melodía de fondo]. Cojo el móvil como puedo dentro del saco de dormir. Son las 05:00 h y es el momento en que te das cuenta de la mala noche que has pasado. Siempre igual, tanta prisa por colocar la tienda que luego duermes sobre una zona inclinada y acabas rodando en una esquina de la tienda. Tras vestirme lo más rápidamente, salgo del saco y caliento un café con leche en el *campingaz*. ¡Quién tuviera en ese momento un zumo de naranja recién exprimido, unos churros y unas tostadas con ajo y aceite! Mientras se calienta el café, hago un repaso mental de la ruta y todo lo que habías preparado minuciosamente los días antes. El plan era redondo: hacer cima por una nueva cara de la montaña, con unas vistas espectaculares de todo el macizo y una ruta muy poco concurrida. Además, como no había casi ninguna información de la ruta a seguir, había consultado a guías de la zona que me advirtieron de los pasos complicados, que es así como nos referimos en la montaña a las zonas claves donde tienes que prestar más atención aún si cabe. Tras acabarme el café, y mientras me meto unos pocos frutos secos en la boca, acabo de empaquetar todo el material en la mochila, cierro la tienda y salgo pitando. Son ya las 05:38 h y no puedo perder un minuto más. Ya voy tarde.

Con la luz del frontal voy buscando los hitos, esos montículos de piedras amontonadas por otros montañeros para no perderse en casos de mala visibilidad. Al no haber un camino claramente marcado en el terreno, sin ellos no sería capaz de encontrar la ruta en la oscuridad. Además, es curioso cómo esos hitos son mucho más frecuentes y grandes en las zonas en las que es más fácil desorientarse o desubicarse. Tiene sentido, alguien ya tuvo problemas aquí para encontrar la dirección correcta. A medida que voy ganando altura, empieza a amanecer y ya puedo vislumbrar el espectacular entorno de mi alrededor: por un lado, una pared que se alza sobre mi cabeza y por el otro un profundo valle bajo mis pies que se pierde entre las nubes. Soy positivo, ¡parece que hoy voy a tener suerte y el día me va a acompañar! También es el momento en el que me doy cuenta de que es necesario desviarse del camino principal, ya que recuerdo algunas indicaciones que me habían dicho en la taberna del pueblo: “cuando llegues al lado de un bloque enorme, desvíate hacia el noroeste sin perder altura”. Hacia allí me dirijo, siguiendo las indicaciones del mapa de forma ordenada y meticulosa, afianzando sistemáticamente cada paso y con una visión panorámica y completa del entorno. Pero justo en ese momento cuando ya había avanzado durante una hora, presiento que me estoy metiendo en lío. Qué raro, yo complicándome la vida. La canal por la que estoy subiendo no es la adecuada, ¡era la de mi izquierda! La opción rápida sería atravesar directamente de una canal a otra; seguro que lo conseguiría. Pero hay un duendecito dentro de mi cabeza, que me aconseja: “no pasa nada por perder dos horas, rehaces tus pasos y vuelves a empezar”. Dicho y hecho. Tras haber acometido esta embarcada, desciendo por las huellas que había dejado y me dispongo a continuar, ahora sí, por la canal correcta. Esta vez tengo que fijarme más en los colores del mapa y en aquellas referencias clave que me puedan servir para no volverme a confundir. Así, fui cogiendo poco a poco más altura, descubriendo y disfrutando de las vistas, y también de la compañía de un pequeño y colorido treparriscos, una pareja de alimoches y de una hembra de rebeco con su

cría. Con un último esfuerzo, acabé en la cumbre a las 15:38 h, agotado pero satisfecho con la experiencia de haber hecho cima y ver todo el resultado bajo tus pies.

No obstante, no puedo relajarme pues se aproximan unas nubes intimidantes y el objetivo final aún no está conseguido, pues este ha de ser —como siempre en montaña— llegar sano y salvo al punto de partida. Además, estás cansado, deshidratado y no paras de pensar en tomarte una cerveza o comerte un kebab en buena compañía; y eso te puede hacer cometer errores. Pero también se te vienen a la cabeza un montón de buenos recuerdos que hacen que pongas todo tu empeño y concentración en la vuelta, sano y salvo, al punto de partida. Finalmente, son las 18:20 h y llegas jodido pero contento a la tienda, te descalzas y avisas en casa de que todo ha ido bien.

En la montaña, el reto es hacer sencillo lo difícil, explorando un nuevo camino que nunca se había hecho. Del mismo modo, esta larga y sinuosa aventura habría sido imposible realizarla solo, sin cada una de las personas habéis estado presentes en cada uno de los pequeños detalles de esta etapa. En especial quiero agradecer a las directoras Maribel, Paz y el director Rober; al equipo de COPEMOL y GapaVet, en especial a Adolfo, Ana, Catuxa, Eva, Jaime, Manuela, Meli, Moncho, Nerea, Paolo, Rafi, Rocío, Sandra, Sonia, Uxía, Xan y Xoel; a las compañeras y compañeros de la Facultad de Veterinaria Mónica Nuria, Luciano y Manolo; a las investigadoras e investigadores Alexandra Elbakyan, Amílcar Teixeira, Antonio Villalba, Antonio Rodríguez, Elena Alonso, Irene Salinas, Jouni Taskinen y Rafael Araujo. Por último, pero no por ello menos importante, agradecer a la familia, Antonio, Carmen, María y Jandra; amigas y amigos de aventuras, del caseto, de la facultad, de conversaciones de taberna y de mucho más.

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SUMMARY [RESUMO]

SUMMARY

Freshwater mussels of the order Unionida are known as naiads, according to the mythological female nature deities which preside bodies of fresh water and protect the water quality and the freshwater ecosystem. Hence, naiads are considered keystone species and ecosystem engineers, providing a direct contribution to human and animal health. Freshwater mussels are characterized by a complex life cycle where the embryo is brooded within the marsupium, a cavity located in the demibranchs of the adult mussels. After completing the embryogenesis, the recently formed larvae are released into the water column and attach mostly to a fish host to metamorphose into juvenile mussels. This interaction with fish is regarded as a parasitic mechanism for dispersal along the rivers, nutrition from the fish and protection. There are two parasitic larval types, the lasidium and the glochidium. The glochidium bears two pairs of valves that are closed by a larval adductor muscle in response to the contact with a fish epithelium, e.g., the epidermis of the skin or the gill epithelium. Thus, the glochidium clasps and encysts to a fish host, to develop and metamorphose after a very variable period, ranging from days to several months, depending on the unionid species. Afterward, the young mussels detach from the fish to continue their infaunal mode of life, buried into the river substratum. Once their reach maturity, adults locate semi-buried into the riverbed, being capable of uptaking a large amount of water into the pallial cavity through the inhalant opening. Their ciliated ctenidia filter and separate the suspended material, select the desirable food by the labial palps, and then, the filtered water is expelled through the exhalant opening. Besides their critical role in the ecosystems, unionids are worldwide imperiled by a synergy of extrinsic factors, all under the influence of global change. Therefore, the conservation of the most endangered populations of freshwater mussels has been converted into a priority, focusing on the selection of species and populations, habitat restoration and captive propagation.

The Freshwater Pearl Mussel (FPM), *Margaritifera margaritifera* (L.), is a naiad with one of the most extreme longevities and reproductive strategies among unionids. As a drawback, the *M. margaritifera* glochidia have a very small size, needing to encyst into the gills of specific populations of only two suitable fish hosts, the Atlantic salmon (*Salmo salar* L.) and the brown trout (*Salmo trutta* L.). In addition, larvae develop for 6–11 months in the fish gills to grow five times in size, and finally, metamorphose into juvenile naiads.

Regarding its conservation status, *M. margaritifera* is one of the most endangered bivalves, considered *Critically Endangered* in Europe due to the highly reduced and fragmented populations, especially at the meridional limit of its European distribution. Because the FPM is a keystone, an umbrella, an indicator, and a flagship species, it can be considered a target animal for the conservation of aquatic ecosystems, and the protection of human and animal health. To ensure the survival of the FPM and reinforce the most affected populations, one of the extreme actions relies on the breeding programmes of juvenile naiads which needs to replicate part of the life cycle of the FPM in captivity. Thus, fish are artificially exposed to *M. margaritifera* glochidia, allowing the encysted larvae to develop and metamorphose into juveniles. However, the high host specificity of the FPMs towards certain Atlantic salmon or brown trout

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populations frequently leads to low larval survivals and growths during the parasitic stage. Despite these constraints during the culturing procedures, there is very little information regarding its larval development in the fish gills and the metamorphosis into juveniles. Once the metamorphosis is complete, the juveniles detached from the fish gills and are collected to continue the breeding during their free-living stages. These first juvenile stages are considered one of the most critical stages during the FPM captive breeding, but there is no information regarding the optimal time to collect the juveniles or the outcome that the parasitism might cause for the acquisition of resources for the next post-parasitic phase.

Due to this compulsory parasitism, healthy and suitable fish are needed to complete the parasitic stage. One of the two suitable hosts is the Atlantic salmon (*S. salar*), an anadromous salmonid fish characterized by a remarkable life cycle: the young salmons, located at the river headwaters, smoltificate, migrate to the open ocean to grow up, and return to their natal rivers to spawn. This homing behaviour confers to salmon extremely important functions in the ecosystems, providing nutrients from marine to inland habitats. In relation to humans, wild salmon exhibits a great symbolism and provides relevant economic, cultural and social values to the local communities where these fish migrate through. Moreover, before travelling downstream to the sea, the young salmon serves as one of the exclusive hosts for FPM, where the salmon fry and parr closely cohabit with the mussel populations.

In Europe, the Atlantic salmon is considered *Vulnerable*, despite its extinction in most of the rivers located at the meridional area of its distribution. Precisely in these southern regions, the poor regional support for the conservation of Atlantic salmon resembles that occurring with the FPM, where both species are at the meridional limit of their geographical distribution. On the contrary, the Atlantic salmon aquaculture has grown exponentially and supposes nowadays the first fish production industry worldwide, led by Norway and Chile. However, this successful salmon farming must face new challenges and diseases which threaten fish health.

The fish gills suppose the exclusive target organ for the glochidium of *M. margaritifera*; thus, understanding the normal anatomy and functionality of the gills is the first step to study the parasitic interaction between the FPM and the Atlantic salmon. Gills conform a highly vascularized and multifunctional organ specialized in the exchange of gases, salts, water and nitrogenous wastes; essential to maintain fish homeostasis. To do so, gills exhibit an intricate macro- and microscopical anatomy, with highly vascularized holobranches, filaments and lamellae, creating a highly efficient counter-current flow between the water and the blood. Lamellae could be regarded as envelope-like structures lined by a thin epithelium which reduced the diffusion distance between the blood and the outer environment. Among lamellae and all over the filamental edges, other specialized cells are involved with the secretion of mucus, osmoregulation and immune defense. Despite this normal structure and functionality, gills exhibit tremendous plasticity, nevertheless, the relation between the morphopathological gill changes their pathophysiological effects on fish remains poorly understood.

The disease status caused by the parasitic glochidia into fish is known as glochidiosis. Despite the impact of glochidiosis on fish health has become a recent concern in the conservation of naiads, there are very few descriptions of the morphopathological changes associated with this disease, where proliferative gill lesions are frequently reported. In the same

way, only a few studies described a detrimental impact on the gill functionality, as a respiratory and osmoregulatory impairment, accompanied by the activation of systemic compensatory mechanisms, e.g., increasing the haematocrit levels. In *M. margaritifera* glochidiosis, it is needed to comprehend the physiopathological effects and how fish gills recover after the parasitism to find an optimal balance between the FPM culture and the fish health. Moreover, an early detachment of larvae is frequently described during the first 40 days of the parasitism, where the subjacent mechanism is completely unknown, only related to the high host specificity of *M. margaritifera*. Hence, a complex host-parasite seems to occur between the FPM and salmonid fish, where the defensive, compensatory and reparative mechanisms of the gills should be elucidated.

Since the recession and culturing of the FPM, there is a need to understand the pathogenesis of *M. margaritifera* glochidiosis. However, there are many knowledge gaps regarding the larval development and the evolution of lesions during glochidiosis. Thus, the main aim of this Ph.D. thesis is to characterize both the morphopathological changes during the most critical changes of *M. margaritifera* glochidiosis, together with the larval morphogenesis and metamorphosis, utilizing stereomicroscopy, light microscopy and scanning electron microscopy. This approach would serve to deepen into the pathogenesis of glochidiosis, and to find tools and strategies to preserve the fish health and to optimize the larval development during the parasitic stage within the FPM culture.

To morphologically and morphopathologically evaluate this host-parasite interaction, a set of experimental glochidiosis was designed and performed within the FPM culturing programme. Hence, Atlantic salmon—exposed and non-exposed to glochidia—were reared under controlled conditions to induce and maintain an artificial infestation of fish and allow the larva to develop for several months post-exposure (PE). After metamorphosis, the juvenile detachment from the salmon gills was synchronized by increasing the water temperature to facilitate the juvenile collection from the fish tanks. Periodically, 6–10 fish were sampled, anesthetized, euthanized and necropsied, and left gills were observed by stereomicroscopy and light microscopy, and also squashing a few gill filaments. Meanwhile, right gills and most organs were sampled and processed following the routinary techniques employed in histology and scanning electron microscopy. Thus, samples were thoroughly evaluated to qualitatively describe both the morphopathological changes suffered by the fish and the morphogenesis of the parasitic larva.

The results obtained by this approach were divided into three studies. The Study 1 and 2 focused on the gill lesions of Atlantic salmon and the pathogenic mechanisms, during the early and late stages of *M. margaritifera* glochidiosis, respectively. The Study 3 centred on the morphogenesis of *M. margaritifera* larva during its parasitism in the salmon gills, from glochidium to post-larva. The simultaneous evaluation of both gill lesion and larval development allowed to integrate the main highlights of each study, establishing four main events: the encystment of the glochidium into the gill tissue (day 0–3 PE), the premature rejection of immature larva (day 3–14 PE), the development of the mushroom larva (day 14–150 PE), and the metamorphosis and droplet detachment of post-larva (day 150–225 PE).

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M. margaritifera glochidia were attached immediately to the fish gills by pinching the lamellar epithelium, causing an acute proliferative branchitis essential to the cyst formation in a few hours. Afterward, the encysted glochidia lost their typical morphology due to the disappearance of the larval adductor muscle and the granular appearance of the mantle. Despite larva remained closed within the gill tissue, both the internal antigenic structures located within its pallial cavity and/or at the outer surface of the periostracum may trigger this hyperplastic response of the gills.

In spite of the initial glochidial encystment, an early rejection occurred in most fish during the first 14 days, related to an erosive branchitis with a pleomorphic inflammatory infiltrate and epithelial degeneration, described for the first time in suitable species as the Atlantic salmon. Meanwhile, immature larvae detached by an erosive mechanism of the surrounding tissue, suffering occasionally a periostracal damage, and failing to develop and metamorphose into post-larva. As a result of the rupture of the outer barrier, the externalization of internal structures of the larvae may contribute to elicit a more intense inflammatory response at this level. These sequential events during early glochidiosis point towards an idiosyncratic individual response where the immune response of fish is involved but remains completely unraveled in glochidiosis.

Despite all larvae were rejected in some fish, other larvae remained encysted in the gills, continuing their parasitic development. Thus, between the intermediate period between day 14 and 150 PE, the persistent larva acquired an intermediate stage, characterized by the presence of the mushroom body and the zip membrane, two transitory and distinctive structures of this long-lasting parasitism. The zip membrane, located at the valve cleft, may play a unique role in the acquisition of non-particulate nutrients from the fish. Thus, the exchange of non-particulate material through the zip membrane or the thin valves stands out as the most plausible hypothesis to explain the exchange of substances during the *M. margaritifera* parasitism. On the other hand, the mushroom body was the most prominent structure of the larval mantle constituted by highly globose and vacuolized cells. Due to the prominence of the mushroom body and its potential multifunctional role during the parasitic stage of *M. margaritifera*, this distinctive and intermediate parasitic stage was proposed to be termed as mushroom larva. As the larva developed and the zip membrane was created, the gill responses chronified. These simultaneous events suggest a potential role of this membranous structure in the isolation of the putative antigenic material within the pallial cavity to the fish immune system, reducing the activation of the fish immune system and modulating the inflammatory response.

After 200 days of parasitism, *M. margaritifera* metamorphosed into post-larva, allowing to describe the morphogenesis and the acquisition of a complete set of post-larval organs. Following the artificial increase of the water temperature, post-larva detached from the gills by the formation of pedunculated structures and the progressive reduction of the contact area between the parasites and the gills. Due to the similarities with the protruding, pinch-off and detachment of a droplet fluid, this mechanism, described here for the first time, was proposed to be termed as droplet detachment. This non-erosive mechanism of post-larvae contrasts with the rejection of immature larvae. Thus, the post-larval morphology might be involved in the active involvement of the post-larva in response to the increase of the water temperature.

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Meanwhile, the valves remained closed by the zip membrane until it disappeared once detached. Finally, the post-larval organs will be essential for the settlement and deposit-feeding in the riverbed. Among them, the byssal complex of the post-larval foot was described for the first time in unionids at the end of the parasitic stage

The morphopathological evaluation of the gill lesions helps to understand the impact of *M. margaritifera* glochidiosis to fish health and how it may impair the branchial multifunctionality. Besides the reduction of the respiratory capacity due to the proliferative gill lesions, a complex host-parasite interaction is inferred due to the different gill responses between early and late glochidiosis, related to different larval outcomes. The early rejection of immature larvae was associated to an intercellular edema, epithelial degeneration and erosions, which may cause an additional compromise on respiration and also osmoregulation. On the other hand, the persistence of mushroom larvae and the detachment of post-larvae during the late stages maintained the epithelial continuity and minimized the inflammatory and degenerative gill changes. These different morphopathological patterns have important implications for the repair and the return to the normal tissue morphology and functionality after the parasitism. Thus, after the early erosive rejection, the gill healing process consisted of a re-epithelization, inflammation and epithelial remodeling. However, the preservation of the epithelial continuity during the droplet detachment of post-larvae points towards an anticipated epithelial remodeling, achieved by the mutual interplay of apoptosis, polarization and cell shedding. Despite these different reparative mechanisms, Atlantic salmon recovered completely one month after the end of the parasitism. As a result, fish employed as hosts of *M. margaritifera* could be set free into a suitable habitat or even reused in other experimental procedures.

The comparison of the two gill responses and recovery mechanisms during the complex *M. margaritifera* glochidiosis provides the first evidence to suggest that fish that remained parasitized until the post-larval detachment suffer less severe gill damage than fish rejecting prematurely the immature larvae. This relationship between the parasite outcome and the host health suggests an overlap between tolerance and resistance strategies during *M. margaritifera* glochidiosis. However, to support this hypothesis and to better understand the causal relationship between the fish responses and the larval outcomes, further characterization of the role of the immune system is needed.

The complex parasitism—with low survivals and impaired development of the parasitic larva—evidence the need to develop strategies to solve the main constraints during the parasitic stage of the FPM culture. Thus, new tools and methodologies are proposed here to be integrated within the common procedures to monitor and provide a better overview of the larval and post-larval fitness during the FPM culture. In this sense, the semi-transparent valves of the mushroom larva allow to determine and select properly oriented larvae, improving the measurement accuracy during the evaluation of the larval growth. Despite their transparency, the careful stereomicroscopical visualization of the immersed gill tissue reveals as a potential diagnostic tool to *in vivo* determine the larval loads of fish during the FPM parasitism. However, regardless of the technique employed to estimate the number of encysted larvae on the gills, further studies should homogenize the terminology to express the parasitic loads and, ideally, provide detailed information about the methodology employed. Besides larval growth and

survival, the lipid accumulation may constitute a more specific biomarker of the energy storage during the parasitism in the fish gills, and even to predict the subsequent post-larval fitness. To do so, fresh frozen tissue stands out as a complementary histological technique that not only serves to stain lipids but also to reduce the common artefactual shrinkage related to the use of routinary fixative fluids employed in histopathology. Towards the end of the parasitic stage, an important concern is to identify the metamorphosis and to determine the optimal moment to collect the juvenile naiads. Among the morphological changes, the indirect visualization of the adductor muscle by wet mount of the gills is proposed as a potential morphological flagship to easily distinguish the larval metamorphosis and initiate the synchronization of the juvenile collection at the hatchery.

The findings described in this Ph.D. thesis contribute to a better understanding of the pathogenesis of glochidiosis that may aid to manage the parasitic stage during the culture of freshwater mussels. This basic knowledge will contribute to design of further guidelines for managing the parasitic stage during *M. margaritifera* culture, where the optimal larval development and the protection of fish health are the two major priorities. Moreover, these three studies open new research lines where the characterization of the underlying immunitary responses of fish is in the spotlight for being potentially involved with both the course of the disease and the outcome of the larval development. However, these efforts are worthless without the engagement of the general public in the protection of freshwater mussels, and further efforts are also needed to make society understand why the FPM are real jewels in the aquatic ecosystems.

Keywords: *Margaritifera margaritifera*, *Salmo salar,* glochidiosis, gill histopathology, gill healing, larval development, glochidium, host-parasite interaction, parasite detachment, mushroom body, byssal gland.

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RESUMO

Os mexillóns de río da orde Unionida coñécense coma náiades, facendo referencia ás deusas mitolóxicas da natureza que presiden as augas doces, e protexen a calidade da auga e os ecosistemas fluviais. Deste xeito, as náiades son consideradas especies chave e enxeñeiras dos ecosistemas, proporcionando unha contribución directa á saúde humana e animal. Os mexillóns de río caracterízanse por un ciclo de vida complexo onde o embrión é incubado dentro do marsupio, unha cavidade localizada nas demibranquias dos mexillóns adultos. Despois de completar a súa embrioxénese, as larvas recentemente formadas son liberadas á columna de auga para ancorarse maioritariamente a un peixe hóspede, onde acontece a metamorfose a mexillóns xuvenís. Esta interacción co peixe é considerada como un mecanismo parasitario que permite a dispersión dos mexillóns ao longo dos ríos, a súa nutrición do peixe e a súa protección. Existen dous tipos de larvas, o lasidio e o gloquidio. O gloquidio está composto por dous pares de valvas que son pechadas por un músculo adutor en resposta ao contacto co epitelio dun peixe, coma a epiderme da pel ou o epitelio das branquias. Así, o gloquidio ancórase a un peixe hóspede, para desenvolverse e sufrir unha metamorfose a xuvenís, após un período moi variable de tempo, dende días ata varios meses dependendo da especie de uniónido. Despois, os mexillóns novos despréndense do peixe para continuar o seu modo de vida infaunal, soterrándose no substrato do río. Unha vez logran a súa madurez, os adultos localízanse semi-soterrados no leito do río sendo capaces de adquirir unha gran cantidade de auga na cavidade paleal a través da apertura inhalante. Cos seus ctenidios ciliados, estes bivalvos separan o material suspendido seleccionando a comida axeitada por medio dos palpos labiais, e así a auga filtrada acaba sendo expulsada polo apertura exhalante cara ao exterior. Ademais da súa función crítica nos ecosistemas, os uniónidos atópanse mundialmente ameazados pola sinerxía de factores extrínsecos, todos eles baixo a influencia do cambio global. Polo tanto, a conservación das poboacións de mexillóns de río en maior perigo converteuse nunha prioridade, centrándose na selección de especies e poboacións, na restauración do hábitat e na propagación en catividade.

O mexillón de río *Margaritifera margaritifera* (L.) (en inglés "Freshwater Pearl Mussel" ou FPM) é unha náiade cunha das máis extremas lonxevidades e estratexias reprodutivas entre os uniónidos. Porén, os gloquidios de *M. margaritifera* teñen un tamaño moi pequeno e precisan enquistarse nas branquias de poboacións específicas, soamente de dúas únicas especies de peixes hóspede, o salmón atlántico (*Salmo salar* L.) e a troita común (*Salmo trutt*a L.). Ademais, as larvas desenvólvense durante 6–11 meses nas branquias do peixe para medrar cinco veces o seu tamaño e, finalmente, metamorfosear a xuvenís de mexillón.

Con respecto ao seu estado de conservación, *M. margaritifera* é un dos bivalvos máis ameazados, considerándose en Europa en *Perigo Crítico* debido ás poboacións altamente reducidas e fragmentadas, especialmente no límite meridional da súa distribución europea. Debido a que o FPM é unha especie chave, paraugas, indicadora e bandeira, pode ser considerada un animal obxecto de conservación para a protección dos ecosistemas acuáticos e da saúde humana e animal. Para asegurar a supervivencia de FPM e reforzar as poboacións máis afectadas, unha das accións extremas depende da cría dos mexillóns xuvenís mediante a simulación de parte do ciclo de vida deste mexillón en catividade. Deste xeito, os peixes son

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expostos artificialmente aos gloquidios de *M. margaritifera* para así permitir o enquistamento das larvas e o seu desenvolvemento e metamorfose en xuvenís. Así e todo, a alta especificidade dos FPMs cara certas poboacións de salmón atlántico ou troita común trae consigo unha baixa supervivencia e baixo crecemento das larvas durante o estadio parasitario. A pesar destas restricións durante cultivo, hai moi pouca información con respecto ao seu desenvolvemento larvario e metamorfose nas branquias do peixe. Tras acadar esta metamorfose, os xuvenís caen das branquias do peixe e recóllense para continuar a súa cría durante os estadios de vida libre. Estas primeiras etapas xuvenís son consideradas un dos momentos máis críticos do cultivo de FPM, mais non hai ningunha información con respecto ao tempo óptimo para recoller os xuvenís ou o impacto que supón o parasitismo na adquisición de recursos para a seguinte fase post-parasitaria.

Debido a este parasitismo forzoso, é fundamental a presencia de peixes sans e axeitados para completar a fase parasitaria. Unha destas dúas especies hóspede axeitadas é o salmón atlántico (*S. salar*), un peixe salmónido anádromo caracterizado por un particular ciclo de vida. As crías do salmón, nadas nas cabeceiras dos ríos, sofren unha esmoltificación e migran ao océano para alimentarse e medrar, e despois retornar aos ríos natais para desovar. Este comportamento confire ao salmón funcións extremadamente importantes nos ecosistemas, tansportando nutrientes dende o medio mariño aos hábitats terrestres. En relación aos humanos, o salmón salvaxe posúe un gran simbolismo e proporciona imporantes valores económicos, culturais e sociais ás comunidades locais por onde este peixe migra. Ademais, antes de comezar a súa viaxe augas abaixo, as crías de salmón serven como un dos únicos hóspedes para o FPM, onde os alevíns e pintos de salmón cohabitan estreitamente coas poboacións de náiades.

En Europa, o salmón atlántico é considerado como *Vulnerabe*, a pesar da súa extinción na maioría dos ríos localizados na área meridonal da súa distribución. Precisamente nestas rexións mais ao sur, o escaso apoio rexional para a conservación do salmón atlántico asemella ao que acontece co FPM, onde ambas especies atópanse no límite meridional da súa distribución xeográfica. Con todo, a acuiltura do salmón atlántico medrou exponencialmente e supón hoxe en día a primeira industria produtora de peixe a nivel mundial, liderado por Noruega e Chile. A pesar deste exitoso negocio, a acuicultura ten que afrontar novos desafíos e enfermidades que ameazan a saúde dos peixes.

A branquia dos peixes supón o único órgano diana para a parasitación do gloquidio de *M. margaritifera*. Deste xeito, o primeiro paso para estudar a interacción parasitaria entre o FPM e o salmón atlántico é a comprensión da anatomía e fisioloxía normal das branquias. As branquias consisten nun órgano multifuncional altamente vascularizado e especializado no intercambio de gases, sales, auga e residuos nitroxenados; básico para manter a homeostase do peixe. Para levar a cabo estas funcións, as branquias teñen unha intricada anatomía macro- e microscópica, compostas por holobranquias, filamentos e lamiñas enormemente vascularizados e que crean un eficiente fluxo a contracorrente. Estas lamiñas microscópicas poden compararse con estruturas con forma de sobre recubertas por un epitelio fino que reduce a distancia de difusión entre o sangue e o medio externo. Entre as lamiñas e tamén sobre os bordes dos filamentos, outras células especializadas adoitan estar implicadas na secreción de mucus, na osmorregulación e na defensa inmune. A pesar desta estrutura e funcionalidade

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branquial, este órgano mostra una tremenda plasticidade, pero por contra, a relación entre os cambios morfopatolóxicos da branquia e os efectos fisiopatolóxicos no peixe atópase aínda pouco comprendida.

A enfermidade causada polos gloquidios coñécese como gloquidiose. A pesar de que o impacto da gloquidiose comezou a ser unha preocupación recente na conservación das náiades, existen moi poucos estudos sobre os cambios morfopatolóxicos asociados a esta enfermidade, nos que foi frecuentemente descrita unha branquite proliferativa. Do mesmo xeito, só uns cantos estudos describiron un impacto prexudicial na funcionalidade branquial, como unha limitación da capacidade respiratoria e osmorregulatoria, acompañado pola activación de mecanismos compensatorios sistémicos, como por exemplo o incremento dos niveis de hematócrito. Na gloquidiose causada por *M. margaritifera* cómpre unha avaliación máis precisa dos efectos fisiopatolóxicos e da capacidade de recuperación branquial trala fin da parasitación, co propósito de atopar un balance óptimo entre o cultivo do FPM e a saúde do peixe. Ademais, a caída temperán das larvas é un problema que adoita ser descrito durante os primeiros 40 días do parasitismo, e onde o mecanismos subxacente permanecen completamente descoñecidos, só relacionados á alta especificidade de *M. margaritifera* polo seu hóspede. Por todo isto, asemella que existe unha complexa interacción hóspede-parasito entre o FPM e peixe, onde os mecanismos de defensa, compensación e reparación branquial precisan ser esclarecidos.

Dende o comezo da redución das poboacións e implantación do cultivo do FPM, existe a necesidade de comprender a patoxenia da gloquidiose *de M. margaritifera*. Con todo, hai moitos baleiros no coñecemento respecto ao desenvolvemento larvario e a evolución das lesións ao longo desta parasitose. Así, o principal obxectivo desta tese de doutoramento é a caracterización dos cambios morfopatolóxicos durante os cambios máis críticos durante a gloquidiose de *M. margaritifera*, xunto a morfoxénese larvaria e metamorfose; mediante o emprego de técnicas de estereomicroscopía, microscopía óptica e microscopía electrónica de barrido. Esta abordaxe poderá servir para afondar na patoxenia da gloquidiose e tamén para atopar ferramentas e estratexias para preservar a saúde de peixe e para optimizar o desenvolvemento larvario durante a fase parasitaria no cultivo do FPM.

Para a avaliación morfolóxica e morfopatolóxica desta interacción hóspede-parasito, deseñouse un conxunto de gloquidioses experimentais que foron levadas a cabo dentro dun programa de cría do FPM. Así, alevíns de salmón atlántico—expostos e non expostos aos gloquidios—foron criados en condicións controladas para inducir e manter unha infestación artificial nos peixes, e así permitir o desenvolvemento das larvas durante varios meses post-exposición (PE). Trala metamorfose, a caída das náiades xuvenís dende as branquias foi sincronizada mediante o aumento da temperatura da auga, e así facilitar a recolección dos xuvenís nos tanques de peixes. Periodicamente, seleccionáronse entre 6 e 10 peixes e de seguido foron anestesiados, eutanasiados e necropsiados. As branquias esquerdas observáronse mediante estereomicroscopía e microscopía óptica, ademais de realizar un "squash" dunha porción dos filamentos branquiais. Namentres, tomáronse mostras das branquias dereitas e demais órganos e procesáronse seguindo as técnicas histolóxicas e de microscopía electrónica de rutina. Así, as mostras foron avaliadas para realizar a descrición cualitativa dos cambios morfopatolóxicos sufridos polos peixes e tamén a morfoxénese das larvas parasitarias.

Os resultados obtidos por medio desta aproximación foron divididos en tres estudos. O Estudo 1 e 2 centráronse nas lesións branquiais do salmón atlántico e nos mecanismos patoxénicos, durante os estadios temperás e tardíos da gloquidiose de *M. margaritifera*, respectivamente. O Estudo 3 centrouse na morfoxénese das larvas de *M. margaritifera* durante o seu parasitismo nas branquias do salmón, dende gloquidio ata post-larva. A avaliación simultánea das lesións branquiais e do desenvolvemento larvario permitiu integrar os principais achados de cada estudo, establecendo catro eventos principais: o enquistamento do gloquidio no tecido branquial (día 0–3 PE), o rexeitamento prematuro da larva inmatura (día 3–14 PE), o desenvolvemento da *larva cogomelo* (día 14–150 PE), e a metamorfose e o *desprendemento en pinga* da post-larva (día 150–225 PE).

Os gloquidios de *M. margaritifera* ancoráronse inmediatamente ás branquias do peixe mediante a beliscadura do epitelio laminiñar, causando unha branquite proliferativa aguda, a cal foi esencial na rápida formación do quiste. De seguido, os gloquidios enquistados perderon a súa morfoloxía típica debido á desaparición do músculo adutor e a aparencia granular do manto. A pesar de que a larva ficou pechada dentro do tecido branquial, tanto as estruturas antixénicas internas localizadas dentro da cavidade paleal como aquelas localizadas sobre a superficie do periostraco poden activar esta resposta hiperplásica das branquias.

A pesar do enquistamento inicial dos gloquidios, durante os primeiros 14 días ocorreu un rexeitamento temperán na maioría dos peixes, relacionado cunha branquite erosiva acompañada dun infiltrado inflamatorio pleomórfico e dunha dexeneración epitelial, descritas por primeira vez nesta especie de hóspede adecuada como é o salmón atlántico. Namentres, as larvas inmaturas caeron das branquias mediante un mecanismo erosivo do tecido circundante, sufrindo en ocasións un dano no seu periostraco, e levando de tódolos xeitos a un fracaso no seu desenvolvemento e metamorfose en post-larva. Por mor da ruptura da barreira externa, a exteriorización das estruturas internas das larvas pode inducir unha resposta inflamatoria mais intensa e este nivel. Estes acontecementos secuenciais durante a gloquidiose temperá, apuntan cara unha resposta individual idiosincrática onde a resposta inmune do peixe está implicada, mais tamén se atopa completamente descoñecida.

A pesar de que todas as larvas foron rexeitadas nalgún peixe, outras larvas mantivéronse enquistadas nas branquias, continuando o seu desenvolvemento parasitario. Así, entre o período intermedio entre o día 14 e 150 PE, a larva persistente adquiriu un estadio intermedio, caracterizado pola presenza do *corpo en cogomelo* e da *membrana en cremalleira*, dúas estruturas transitorias e distintivas deste longo estadío parasitario intermedio. A membrana en cremalleira, localizada no espazo valvar, pode xogar unha función única na adquisición de nutrientes non particulados dende o peixe. Deste xeito, o troco de material non particulado a través da membrana en cremalleira ou das finas valvas destaca coma a hipótese máis plausible para explicar o intercambio de sustancias durante o parasitismo de *M. margaritifera*. Doutra banda, o corpo en cogomelo foi a estrutura máis prominente do manto larvario, constituído por células altamente globosas e vacuolizadas. Debido á prominencia do corpo en cogomelo e o seu potencial papel multifuncional durante o parasitismo de *M. margaritifera*, propúxose denominar a este estadio distintivo e intermedio como *larva cogomelo*. Unha vez que se desenvolveu a larva cogomelo coa súa membrana en cremalleira, as respostas branquiais

cronificaron. Esta simultaneidade de acontecementos suxire a potencial implicación desta estrutura membranosa no illamento fronte ao sistema inmune do peixe do posible material antixénico de dentro da cavidade paleal, reducindo así a activación do sistema inmune do peixe e modulando a resposta inflamatoria.

Transcorridos 200 días de parasitismo, *M. margaritifera* metamorfoseouse en post-larva, permitindo a descrición da morfoxénese e a adquisición do conxunto completo dos órganos que conforman a post-larva. Após o aumento da temperatura da auga, as post-larvas desprendéronse das branquias mediante a formación de estruturas pedunculadas que reduciron progresivamente a área de contacto entre os parasitos e as branquias. Debido ás semellanzas coa protrusión, a beliscadura e caída dunha pinga, este mecanismo, descrito aquí por primeira vez, propúxose denominarse como *desprendemento en pinga*. Este mecanismo non erosivo de desprendemento das post-larvas contrasta co rexeitamento das larvas inmaturas. Así, a morfoloxía da post-larva podería estar implicada activamente no desprendemento en pinga que é sincronizado polo aumento da temperatura da auga. Namentres, as valvas permanecían pechadas pola membrana en cremalleira que desapareceu tralo desprendemento. Finalmente, os órganos da post-larva serán esenciais para o asentamento e alimentación do material depositado no leito do río. Entre eles, o complexo do biso, localizado no pé da post-larva, foi descrito por primeira vez nos uniónidos ao remate do parasitismo.

A avaliación das lesións branquiais axuda a comprender o impacto da gloquidiose de *M. margartifera* na saúde do peixe. Ademais das lesións proliferativas da branquia que diminúen a capacidade respiratoria, as distintas respostas durante a gloquidiose temperá e tardía suxiren unha complexa interacción hóspede-parasito que leva tamén a distintas repercusións larvarias. O rexeitamento temperán das larvas inmaturas asociouse a edema intercelular, dexeneración e erosións epiteliais, os cales poden causar un compromiso adicional na respiración e osmorregulación. Doutra banda, a persistencia de larvas cogomelo e o desprendemento das post-larvas durante os estadios tardíos mantiveron a continuidade epitelial e minimizaron os cambios inflamatorios e dexenerativos. Estes diferentes patróns morfopatolóxicos teñen importantes implicacións na reparación e no regreso á morfoloxía e funcionalidade tisular normal, trala fin do parasitismo. Despois do rexeitamento temperán e erosivo, os procesos de cicatrización branquial consistiron na re-epitelización, a inflamación e o remodelado epitelial. Así e todo, a conservación da continuidade epitelial durante o desprendemento en pinga, apunta cara un anticipado proceso de remodelación epitelial, alcanzado por medio da apoptose, a polarización e a descamación celular. A pesar destes diferentes mecanismos de reparación, o salmón atlántico recuperouse completamente un mes despois da fin do parasitismo. Deste xeito, os peixes empregados como hóspedes de *M. margaritifera* poderían ser liberados a un hábitat axeitado ou incluso reutilizados noutros procedementos experimentais.

A comparación das dúas respostas branquias e os seus mecanismos de recuperación durante a complexa gloquidiose de *M. margaritifera* proporciona a primeira evidencia que indica un dano branquial menos severo naqueles peixes que permanecen parasitados ata o desprendemento das post-larvas, en comparación cos peixes que rexeitan prematuramente as larvas inmaturas. Esta relación entre a repercusión nas larvas parasitarias e a saúde do hóspede suxire unha superposición entre as estratexias de tolerancia e resistencia durante a gloquidiose

de *M. margaritifera*. Non obstante, cómpre unha caracterización exhaustiva do papel do sistema inmune para apoiar esta hipótese e entender a relación causal entre as respostas de peixe e a súa repercusión na larva.

O complexo parasitismo—con baixas supervivencias e desenvolvemento alterado das larvas parasitarias—evidencia a necesidade de implementar estratexias para solucionar as principais limitacións durante a fase parasitaria durante o cultivo do FPM. Así, propóñense aquí novas metodoloxías e ferramentas que poden integrarse cos procedementos rutineiros para monitorar e proporcionar unha visión panorámica máis completa da fitness das larvas e das post-larvas durante o cultivo do FPM. Deste xeito, as valvas semi-transparentes da larva permiten determinar e seleccionar correctamente as larvas mellor orientadas, mellorando a precisión de medida durante a avaliación do crecemento larvario. A pesar da súa transparencia, a coidadosa visualización das branquias inmersas mediante estereomicroscopía pode servir como unha ferramenta potencial de diagnóstico *in vivo* para cuantificar as cargas parasitarias do peixe durante o parasitismo dos FPM. Con todo, independentemente da técnica empregada para estimar o número larvas enquistadas nas branquias, futuros estudos deberían homoxeneizar a terminoloxía para expresar estas cargas parasitarias, e, idealmente, proporcionar a información detallada da metodoloxía empregada. Ademais do crecemento e da supervivencia larvaria, a acumulación de lípidos pode servir como un biomarcador específico da acumulación de enerxía durante o parasitismo nas branquias do peixe, e incluso tamén para predicir a fitness das post-larvas. Para facer isto a criosección dos tecidos en frescos e conxelados destacan coma unha técnica histolóxica complementaria que non só serve para tinguir os lípidos, senón que tamén reduce os frecuentes artefactos por encollemento derivados do uso de líquidos de fixación empregados de rutina en histopatoloxía. Cara á fin da etapa parasitaria, unha preocupación importante é a identificación da metamorfose e a determinación do momento óptimo para a recolleita das náiades xuvenís. Entre os cambios morfopatolóxicos, a visualización indirecta do músculo adutor nas preparacións das branquias en "squash" destaca coma un potencial marcador que serviría para identificar a metamorfose e comezar a sincronización da recolleita dos xuvenís nos criadeiros.

Os descubrimentos descritos nesta tese contribúen a unha mellor comprensión da patoxenia da gloquidiose, o cal pode axudar a xestionar os estadios parasitario durante o cultivo dos mexillóns de río. Este coñecemento básico contribuirá ao deseño de directrices para xestionar a etapa parasitaria durante o cultivo de *M. margaritifera*, onde o óptimo desenvolvemento larvario e a protección da saúde dos peixes son as dúas prioridades máis importantes. Ademais, este tres estudos abren novas liñas de investigación, onde a caracterización das respostas inmunitarias do peixe subxacentes está no punto de mira por atoparse potencialmente implicada tanto no curso da enfermidade coma no éxito no desenvolvemento larvario. Así a todo, estes esforzos quedarían en balde sen o compromiso do público xeral na protección dos mexillóns de río, e por iso cómpre un maior esforzo para facer comprender á sociedade por que os FPM son auténticas xoias dos nosos ecosistemas acuáticos.

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Palabras chave: *Margaritifera margaritifera*, *Salmo salar,* gloquidiose, histopatoloxía branquial, recuperación branquial, desenvolvemento larvario, gloquidio, interacción hóspede-parasito, desprendemento parasitario, corpo en cogomelo, glándula do biso.

LIST OF PUBLICATIONS AND MANUSCRIPTS

The development of the present Doctoral Thesis has led to **two scientific publications** (see Annex 1 for editorial permissions to employ the articles in this Ph.D. Thesis). The author had actively participated in the experimental design, the experimental procedures, the collection of the information, the data analysis, the redaction of the manuscript and the submission to the scientific journal of the two following scientific articles:

- **Study I:** Castrillo, PA, Varela-Dopico, C, Ondina, P, Quiroga, MI, Bermúdez, R. Early stages of *Margaritifera margaritifera* glochidiosis in Atlantic salmon: Morphopathological characterization. *Journal of Fish Diseases*. 2020; 43: 69–80. https://doi.org/10.1111/jfd.13100
  Journal Impact factor (2020): 2.767.
  Percentile Rank in the Category Veterinary Sciences (2020): 88.0%
  Percentile Rank in the Category Fisheries (2020): 70.0%
  Percentile Rank in the Category Marine & Freshwater Biology (2020): 75.9%
- **Study II:** Castrillo, PA, Varela-Dopico, C, Bermúdez, R, Ondina, P, Quiroga, MI. Morphopathology and gill recovery of Atlantic salmon during the parasitic detachment of *Margaritifera margaritifera*. *Journal of Fish Diseases*. 2021; 44: 1101–1115. https://doi.org/10.1111/jfd.13372
  Journal Impact factor (2020): 2.767.
  Percentile Rank in the Category Veterinary Sciences (2020): 88.0%
  Percentile Rank in the Category Fisheries (2020): 70.0%
  Percentile Rank in the Category Marine & Freshwater Biology (2020): 75.9%

The development of the present Doctoral Thesis has led **to one scientific manuscript**, waiting for acceptation in the scientific journal. The author had actively participated in the experimental design, the experimental procedures, the collection of the information, the data analysis and the redaction of the following manuscript:

- **Study III:** Castrillo PA, Bermúdez R, Varela-Dopico C, Quiroga MI, Ondina P. The parasitic travel of *Margaritifera margaritifera* in the gills of Atlantic salmon: from glochidium to post-larva. Unpublished study.

The main morphopathological findings during glochidiosis (Study 1 and 2) were chronologically integrated with the larval morphogenesis (Study 3), as briefly illustrated in a drop-down graphical abstract (Annex 2)

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LIST OF ABBREVIATIONS

FAO: Food and Agriculture Organization
FPM: Freshwater Pearl Mussel
H&E: hematoxylin and eosin
IUCN: International Union for Conservation of Nature
NASCO: North Atlantic Salmon Conservation Organization
OBM: opercular beats/min
PAS: periodic acid-Schiff
PAS-AB: PAS-Alcian Blue
PE: post-exposure
SEM: scanning electron microscopy

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General introduction



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GENERAL INTRODUCTION

1. FRESHWATER MUSSELS

1.1 General biology, life cycle and conservation

Freshwater mussels conform a large group of bivalves comprised of around 800 species that inhabit inland waters of all continents except Antarctica (Bogan, 2008; Graf & Cummings, 2007). Unionid adults are sessile and live semi-buried into the riverbed, digging into the substratum with their highly developed foot and filtering up to 50 L of water each day through their gills to obtain food and oxygen (Figure 1a) (Tankersley, 1996; Ziuganov, Zotin, Nezlin & Tretiakov, 1994). These animals tend to aggregate in dense communities (Figure 1b), hence they are capable of improving the quality of the riverbeds by bioturbation of sediments, deposit-feeding, filtering a huge amount of water volume and providing microhabitat for invertebrates that juvenile salmonids feed on (Boeker, Lueders, Mueller, Pander & Geist, 2016; Strayer, 2017; Vaughn & Hakenkamp, 2001; Vaughn, Nichols & Spooner, 2008). These functions improve dramatically the water quality by cleaning out algae, diatoms, organic particles and harmful bacteria, and absorbing heavy metals and other organic molecules (Bogan, 2008; Silverman, Cherry, Lynn, Dietz, Nichols et al., 1997). Due to this beneficial activity, unionids are considered keystone species and ecosystem engineers because they directly benefit other aquatic organisms and enhance aquatic biodiversity (Vaughn, 2018). Thanks to this protection to the water quality and the freshwater ecosystems, they were also termed as naiads, according to the mythological female nature deities presiding bodies of fresh water.

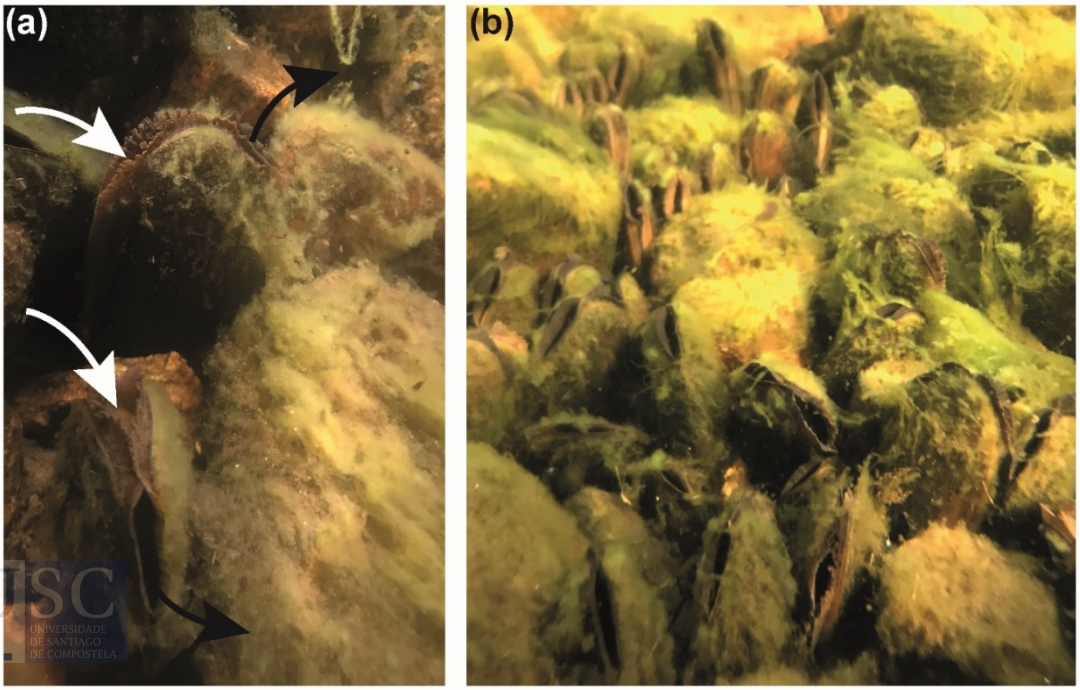
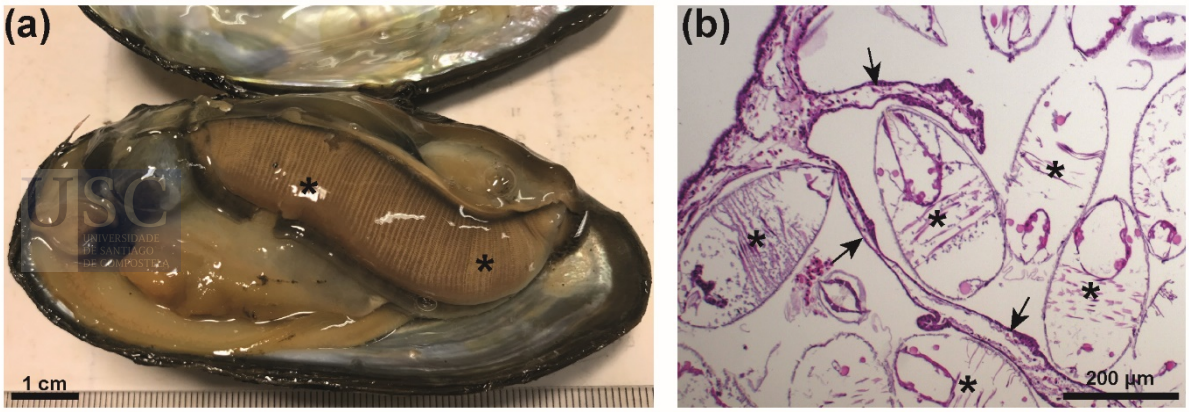


Figure 1. Image of freshwater mussels (*Margaritifera margaritifera*) located at the Lutto River (Lapland, Finland). (a) The water enters through the inhalant opening (white arrows) and, after being filtered, is expelled downstream through the exhalant opening (black arrows). (b) At the optimal regions of the river, mussels aggregate in dense communities lining completely the riverbed.

Unionids acquired the capacity to attach to a fish host to travel along the fluvial ecosystems, with a few species employing amphibians instead of fish (Wächtler, Dreher-Mansur & Richter, 2001). In addition to this dispersal, this interaction between freshwater mussel larvae and the fish host is regarded as a parasitic strategy for nutrition and protection, which allow the larvae to grow isolated from the external medium and metamorphose into juvenile mussels (Barnhart, Haag & Roston, 2008; Denic, Taeubert & Geist, 2015; Schwartz & Dimock, 2001; Skawina, 2021). This parasitic larva contrasts with the pelagic and veliger larva of most marine bivalves, which is passively dispersed within the plankton by the water currents (Bändel, 1988; Carriker, 2001), but it is also observed in other freshwater radiations of bivalves constituted by the families Sphaeriidae, Corbiculidae and Dreissenidae.

This unique life cycle starts with the release of the gametes from the gonad to a cavity located within the gills of the adult mussel, known as marsupium (Figure 2a). At this chamber, the eggs are fertilized with the sperm of a nearby male or even self-fertilized in those facultative hermaphrodite individuals (Kat, 1984). Protected from the outer environment, embryos are brooded in the marsupia for a very variable time period to develop and nourish from the egg yolk (lecithotrophy) or from the extra-vitelline supply of nutrients from their parents (matrotrophy) (Ostrovsky, Lidgard, Gordon, Schwaha, Genikhovich et al., 2016; Schwartz & Dimock, 2001; Watters & O'Dee, 2000). After completing their development into the marsupia, the gills, conformed by demibranchs, become enlarged and show a creamish appearance (Figure 2a) as a result of the presence of thousands of larvae within its lumen (Figure 2b). Afterward, the larva exits into the water column through the exhalant opening, attach and encyst into fish gills or skin and metamorphose into juveniles. Based on the large diversity among freshwater mussels, multiple studies reported a fantastic array of morphological adaptations and strategies to cope with this compulsory parasitic travel. Two main different larval types are described among unionids, the lasidium, restricted to approximately only 86 species of Etherioidea superfamily located in the southern hemisphere, and the glochidium, the most common larval type of unionids (Skawina, 2021; Strayer, 2008; Wächtler et al., 2001).

Figure 2. Macroscopical and histological appearance of a gravid freshwater mussel (*Anodonta anatina*). (a) During the necropsy, the outer demibranch was extremely enlarged all over its extension and showed a creamish coloration (asterisks). (b). Histologically, the marsupium, located between the ascendent and descendent lamellae, was completely filled with mussel larvae (asterisks) between the interlamellar junctions (arrows). H&E stain.



Due to the incubation in the marsupium, the glochidium was first described as an external gill parasite of freshwater mussels, termed as a different species *Glochidium parasiticum* by Rathke in 1797, cited by Lillie (1895). Long after, Leydig (1866) made the valuable discovery that the glochidium is the larval form of certain naiads which develops first in the marsupia of the adult bivalve and then attaches to a fish host to complete its development (Lillie, 1895). Despite the glochidium was discarded of being a parasite of the gill of freshwater mussels, it turned out to be a parasite of the fish gills and skin, assuming the hypothesis that glochidia attached to nourish from their fish host. To attach to a fish, the glochidium exhibits a pair of symmetrical valves articulated by a hinge, roughly similar to the general architecture of bivalves. Hence, valves clasp to the fish tissue by contraction of an adductor muscle (Figure 3). Nevertheless, there is a large morphological variability among different species regarding their sizes (from 70 up to 410 µm), and different valve shapes and modifications, allowing the successfully encystment into different hosts species and epithelial surfaces (Figure 3). Generally, hookless glochidia are only able to clasp into the delicate epithelium of fish gills by their valve flanges (Figure 3a and b), meanwhile, glochidia with hooked valves are also capable to anchor to a broader spectrum of epithelia of the fish fins, skin, or even the eye and the nasal openings (Figure 3c) (Araujo & Ramos, 1998; Barnhart et al., 2008; Hoggarth, 1999; Jansen, Bauer & Zahner-Meike, 2001; Lamand, Roche & Beisel, 2016; Lefevre & Curtis, 1910; Pekkarinen & Valovirta, 1996).

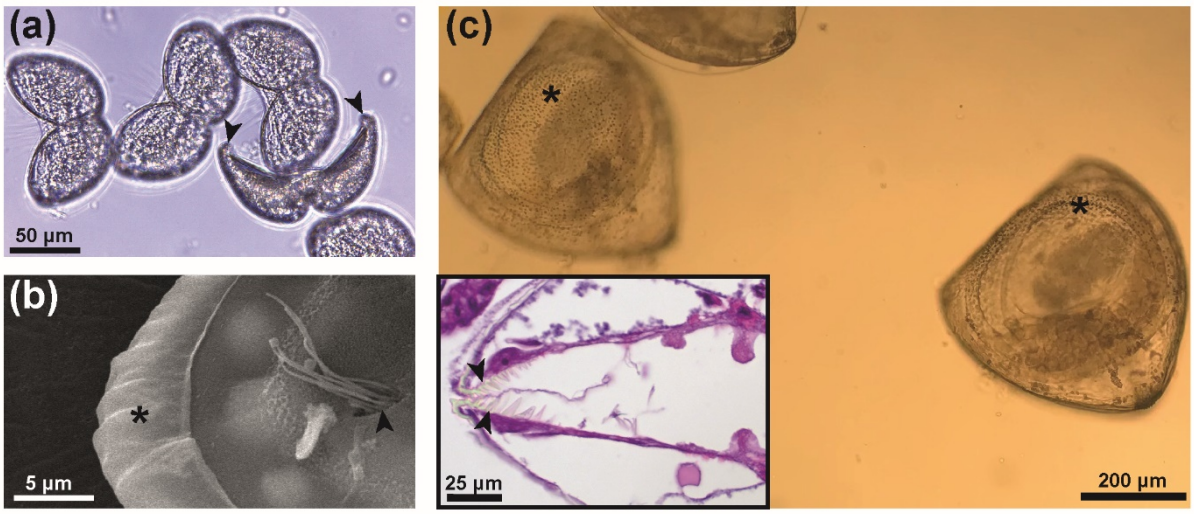


Figure 3. Microphotographs of glochidia of different freshwater mussel species under wet mount with marked morphological differences. (a) Glochidia of *M. margaritifera* are small and showed a pair of non-porous and hookless valves by light microscopy (arrowheads). (b) Detail of the ventral margin of the valves with a membranous flange conforming the periostracal flanges (asterisk) and a sensory hair tuft (arrowhead), observed by scanning electron microscopy. Source: Research group "Conservación de Peixes e Moluscos", authorized the use by Paz Ondina. (c) *Anodonta anatina* glochidia are large, triangular and with visible porous valves by light microscopy (asterisks). Inset. Detail of the valve margin with several hooks (arrowheads) towards the valve cleft, which serve to attach to the fish skin and gills. H&E stain.

Once encysted in the fish epithelium, larvae suffer a marked development and metamorphosis, acquiring a completely different morphology, termed as juveniles due to the morphological similarities to the adults. This morphogenesis was described through 2 to 6 sequential stages in only a few freshwater mussel species (Table 1). Despite metamorphosis is

one of the most distinctive life-changing events in many molluscan species (Joyce & Vogeler, 2018), there are only a few studies describing the morphogenesis during the parasitic development and metamorphosis of naiads within their host (Araujo & Ramos, 1998; Arey, 1932c; Blystad, 1923; Scharsack, 1994). A few species producing large glochidia (around 200–300 µm height) can quickly metamorphose *in vitro* after a few weeks in supplemented tissue culture with fish serum. This capability has provided further insight into the morphogenesis and the requirements of the larvae during this parasitism (Chumnanpuen, Kovitvadhi, Chatchavalvanich, Thongpan & Kovitvadhi, 2011; Fisher & Dimock, 2002; Lima, Kovitvadhi, Kovitvadhi & Machado, 2006).

Table 1. Morphological studies describing the larval development during the parasitic stage of different freshwater mussels.

Freshwater mussel	Technique	Employed fish host	Sequential stages	References
Anodonta cygnea	*In vitro*	-	-	Lima et al. (2006)
Utterbackia imbecilis	*In vitro*	-	2	Fisher and Dimock Jr. (2002)
Potamilus alatus	*In vitro*	-	2	Wen, Jin, Ma, Zheng, Xu et al. (2018)
Hyriopsis bialatus	*In vitro*	-	6	Chumnanpuen et al. (2011)
***Lampsilis* spp.**	*In vivo*	*Micropterus salmoides*	2	Lefevre and Curtis (1910)
Lampsilis ventricosa	*In vivo*	*Micropterus* spp. and *Stizostedion vitreum*	2	Lasee (1991)
Anodonta cygnea	*In vivo*	-	2	Bändel (1988)
Anodonta pyganodon	*In vivo*	*Lepomis punctatus*	3	Arey (1932c)
Pseudounio auricularius	*In vivo*	*Acipenser baeri*	6	Araujo, Camara and Ramos (2002)
Margaritifera falcata	*In vivo*	*Oncorhynchus* spp.	-	Karna and Millemann (1978)
M. margaritifera	*In vivo*	*Salmo trutta*	5	Scharsack (1994)

Nevertheless, the success of this metamorphosis is highly dependent on the host species where the mussel larva attaches to. As a result, certain larval species prefer only a few fish hosts to successfully develop, failing to metamorphose in many other fishes. This capacity of the parasitic larva to develop only in certain fish host species is attributed to a host specificity or suitability and, based on the number of fish hosts, freshwater mussels are defined as host generalists or specialists (Cummings & Graf, 2009; Marwaha, Jakobsen, Karlsson, Larsen & Wacker, 2021; Taeubert, Denic, Gum, Lange & Geist, 2010).

Once the metamorphosis is complete, the young mussels detach from the fish and drop to the bottom of the water column to quickly burrow into the benthos, acquiring an infaunal lifestyle. Despite the juvenile phase is considered one of the most critical stages, not much is known about this phase where the young mussels remain buried within the sediment, crawling and feeding on the algae and bacteria with the aid of the foot (Bradley, 2011; French & Ackerman, 2014; Yeager, Cherry & Neves, 1994). Sexual maturity is achieved after one to a few years, and the adults are semi-buried into the benthos, feeding mainly by suspension-feeding through their gills (Strayer, 2008). Adults can live several decades and, thanks to their seasonal growth, this huge life expectancy could be determined by measuring the conspicuous rings of the shell, similarly to the growth rings in trees, fish scales and otoliths (Geist, 2010; Haag, 2009).

Regarding the valves, their internal morphology shares many general common features among bivalves (Figure 4) (Cummings & Graf, 2009). The calcified shells provide attachment points for prominent muscles, i.e., the adductor and pedal retractor muscles. Internally, the visceral mass contains a pair of labial palps and ctenidia which serve to filter-feeding, separate and transport edible material from the suspended material in the water to the mouth. Moreover, ctenidia are involved in other functions, as respiration, osmoregulation and brooding the larval stages, as indicated previously. The visceral mass also contains the digestive apparatus, the gonads and the nephropericardial cavity, which is located dorsally where the heart pumps the hemolymph through an open circulatory system. At the ventral region of the visceral mass, a well-developed and muscular foot protrude to dig in the substratum. All the visceral mass is surrounded by the mantle, which extends over the inner face of the valves and inserts at the pallial line of the shell. The pallial margin extends up to the valve rims to secrete the shell.

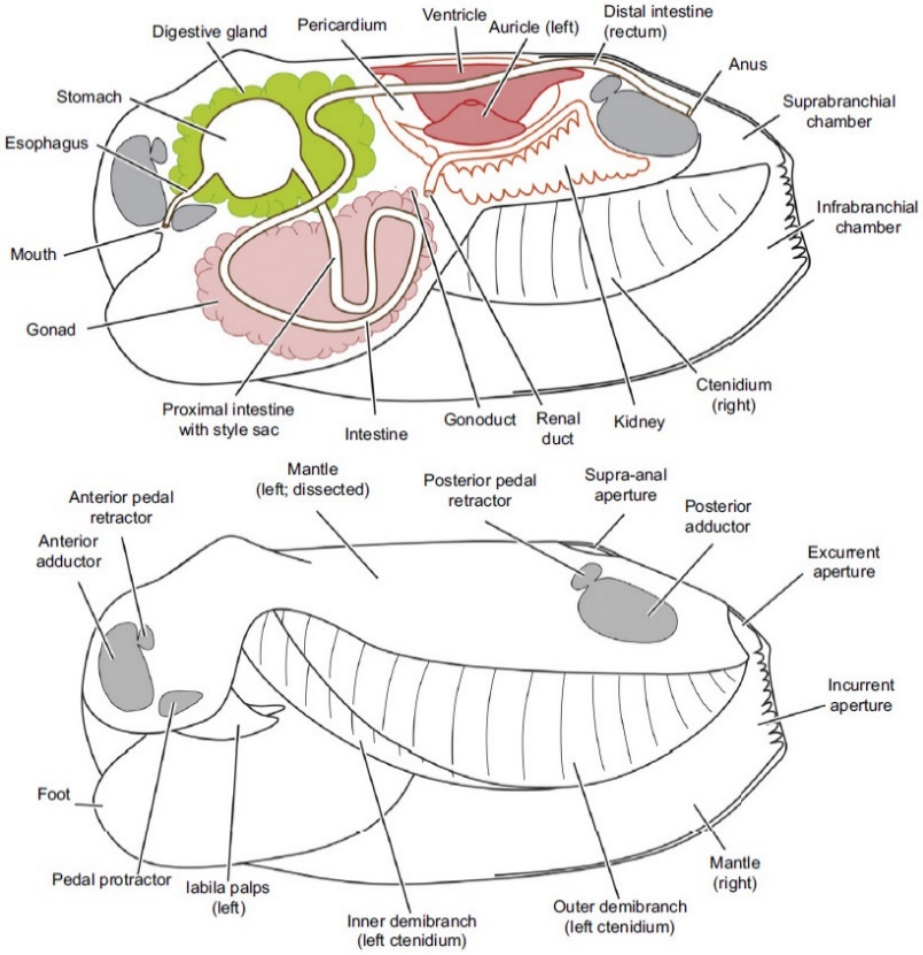


Figure 4. The internal morphology of a freshwater mussel. Source: Cummings and Graf (2009), authorized the use by Elsevier.

Despite the important role of naiads in freshwater ecosystems, unionid mussels are highly imperiled, reflected by the changes in their diversity and population structure. From the total of 634 species assessed by the International Union for Conservation of Nature (IUCN, 2021), 231 were categorized as *Near Threatened*, *Vulnerable*, *Endangered*, or *Critically Endangered*. Most of those species are located in North America, the region which accomplished with most of the global diversity of freshwater mussels (Lydeard, Cowie, Ponder, Bogan, Bouchet et al., 2004).

Although freshwater mussels in Europe are less diverse with 33 species from the families Margaritiferidae and Unionidae, 13 European species are classified as *Near Threatened*, *Vulnerable*, *Endangered*, or *Critically Endangered* (IUCN, 2021). Among them, the European margaritiferids, *Margaritifera margaritifera* (Linnaeus, 1758) and *Pseudunio auricularius* (Spengler, 1793), are classified as the two *Critically Endangered* species with almost functionally extinct populations in Southern Europe (Lois, Ondina, Outeiro, Amaro & San Miguel, 2014; Lopes-Lima, Sousa, Geist, Aldridge, Araujo et al., 2017; Orueta, Araujo & Ramos, 2001).

This overall dramatic decline is affected by the synergy of a wide variety of extrinsic factors comprised within the four main causes of extinction, known as the "evil quartet" or "the four horsemen of the ecological apocalypse": overexploitation; the occurrence of invasive species; habitat loss, fragmentation and degradation; and chains of linked extinctions, in which the loss of fish hosts exemplify the latter (Lois, Cowley, Outeiro, San Miguel, Amaro et al., 2015; Lopes-Lima, Sousa, et al., 2017). In addition, climate change is superimposed on all these threats, affecting at a global scale (Geist, 2011). Early in the 20th century, some freshwater mussel species became threatened in the upper Mississippi River because of the human exploitation due to pearl fishing and the manufacture of nacre buttons (Lefevre & Curtis, 1910). Since the '70s, when the modern extinctions were recognized and related to a multiplicity of factors, the conservation of the most endangered populations of freshwater mussels has been converted into a priority, focusing on the selection of priority species and populations, habitat restoration and captive propagation (Ferreira-Rodríguez, Akiyama, Aksenova, Araujo, Barnhart et al., 2019; Geist, 2010). These strategies depend on the stakeholder involvement and governmental regulations; therefore, further efforts are needed to outreach, educate and increase the social awareness towards the multiple benefits of the freshwater mussels to the society, i.e., ecosystem services (Ferreira-Rodríguez et al., 2019; Lydeard et al., 2004; Strayer, 2017; Vaughn, 2018).

1.2 The Freshwater Pearl Mussel

Margaritifera margaritifera (Linnaeus, 1758) is a freshwater mussel (Unionida: Margaritiferidae) whose name comes from Latin where *margarita* means pearl and *-fera* refers to bear. Hence, this bivalve is known as the Freshwater Pearl Mussel (FPM) due to its pearl formation, constituted by nacre, a highly resilient and iridescent mineral crystal of calcium carbonate which is also employed to form the inner layer of the shells (Figure 5a) (Araujo, 2011). This bivalve is protected by a thick and robust pair of valves up to 15 cm in length, identified by a kidney shape due to a mild compression at the middle region and two cardinal teeth at the left valve, and externally covered by a dark brown-black periostracum (Figure 5b and c) (Araujo, Reis, Machordom, Toledo, María et al., 2009). The extremely long life expectancy of *M. margaritifera*, from 45 up to 132 years, is easily manifested in the external layer of the valves with marked growth rings, and the umbonal region—the oldest region of the valves—frequently worn out (Figure 5b); but intact in young individuals (Figure 5c) (Bauer, 1992; San Miguel, Monserrat, Fernandez, Amaro, Hermida et al., 2004; Young & Williams, 1984a).

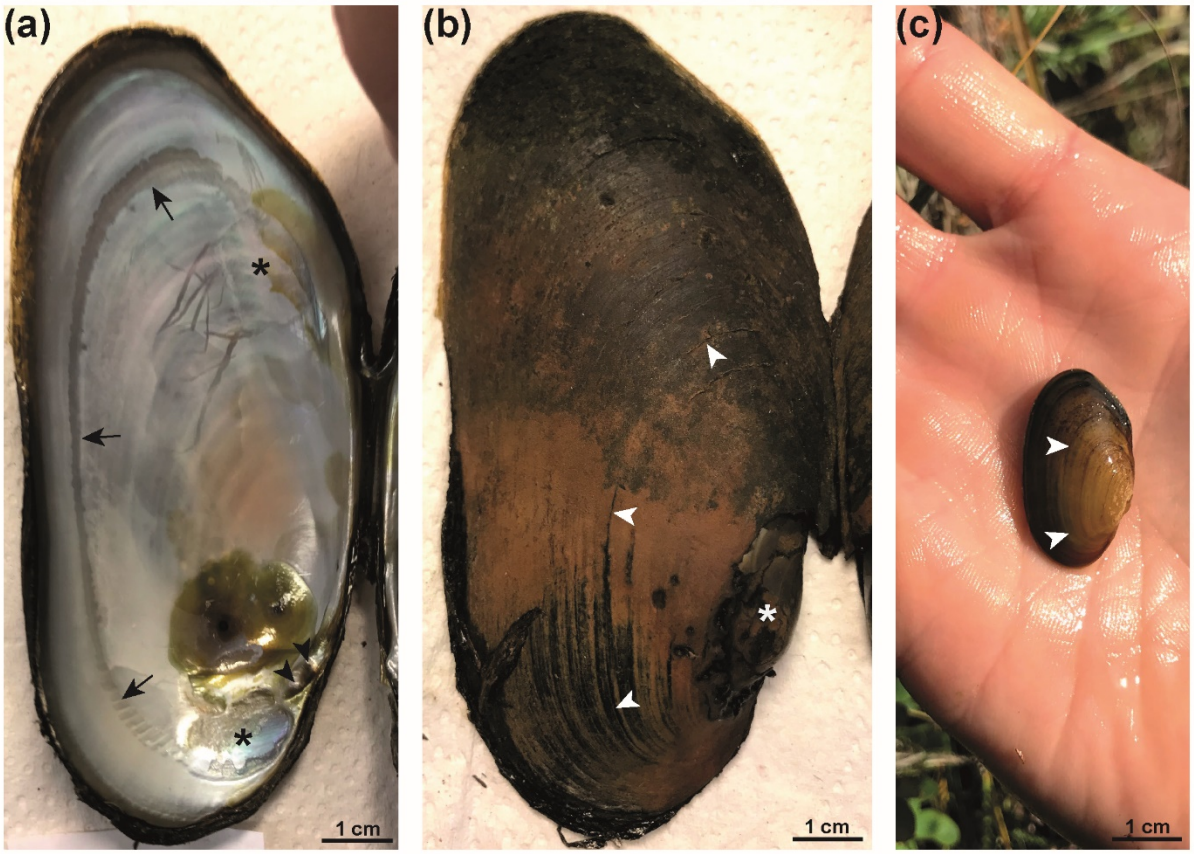


Figure 5. Freshwater Pearl Mussels at different ages at Lutto River, Lapland (Finland). (a and b) Adult mussel of approximately over 70 years old with the valves articulated by the hinge ligament, covered internally by a whitish and nacreous layer showing the cardinal teeth (black arrowheads), a whitish and iridescent appearance with the insertion of the pallial line (arrows), and the adductor muscles (black asterisks). Externally, valves are lined by a dark periostracum with concentric growth rings (white arrowheads), excepting a worn-out umbonal region (white asterisk). (c) Young mussel of around 10 years old displaying a smooth and fair periostracum with marked ring growths all over the valves (arrowheads).

Despite this longevity, the sexual maturity in this species is reached at around 6–20 years old and mature individuals display a long reproductive life of 30–60 years of average (San Miguel et al., 2004; Young & Williams, 1984a; Ziuganov et al., 1994). Adults are dioecious, but at low densities females become hermaphrodites and self-fertilization dominates, conferring the possibility of sparse populations to reproduce (Bauer, 1987b; Gomes-dos-Santos, Froufe, Amaro, Ondina, Breton et al., 2019). After fertilization, the eggs are brooded into the marsupia, within the two pairs of demibranchs, for a short brooding period of 1–2 months and hence named as tachytictic brooders (Bauer, 1994; Scheder, Gumpinger & Csar, 2011). As a result of this short incubation, *M. margaritifera* yields one of the smallest glochidia among naiads, with 60 µm length and 70 µm height (Bauer, 1992; Pekkarinen & Valovirta, 1996), in contrast with the majority of glochidia which measures around 200–250 µm length (e.g., Figure 3c). This small size suggests a low reproductive cost during larval production, allowing to produce up to 3–4 million glochidia per mussel, positively correlated with the body size (Barnhart et al., 2008; Bauer, 1994; Kat, 1984). Nevertheless, certain individuals skip the spawning season until they recover the body condition from the energetically demanding reproductive activity (Bauer, 1992). In mid- to late summer, when river flow is minimal and water temperatures are maximal,

glochidia are released in a synchronized manner during 1–4 weeks just after temperatures start to cool down (Bauer & Wächtler, 2001; Young & Williams, 1984a).

Due to glochidia`s need to encounter a potential fish host, mussels employ a complete array of mechanisms to attract fish during the glochidial release, for example by forming conglutinates of mucus and glochidia which might mimic a food prey (Barnhart et al., 2008). However, *M. margaritifera* releases their progeny in pale-yellowish and mucinous conglutinates which dissolve easily in water losing their attractive capacity for fish (Figure 6), a mechanism known as broadcast due (Barnhart et al., 2008). Because of these loose conglutinates and the small size of glochidia, they become quickly suspended in the water and must find passively a fish, as quick as possible since the larval survival decreases rapidly during the first hours, especially at high temperatures (Jansen et al., 2001). The glochidia of *M. margaritifera* flow through the mouth to the opercular chamber aided by their small size and pumped by the ventilatory movements of fish. Once within the opercular chamber, the sensory tufts of the glochidia (visible in Figure 3b) respond to the decrease in luminosity and increase in osmolarity, closing the hookless valves permanently by contracting the larval adductor muscle. As a result, glochidia clasp a small portion of gill tissue with the aid of small flanges located at the ventral margin of the valves (Pekkarinen & Valovirta, 1996).

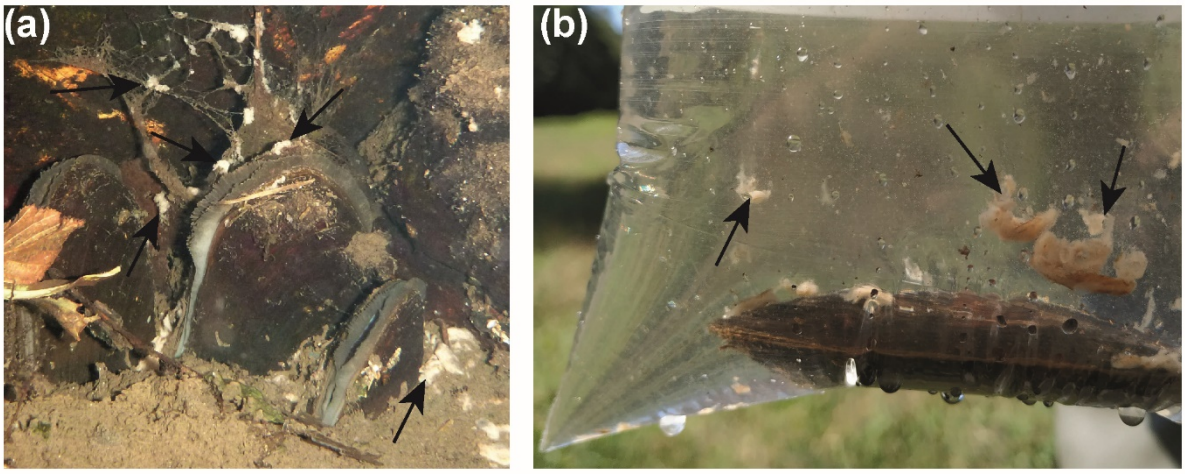
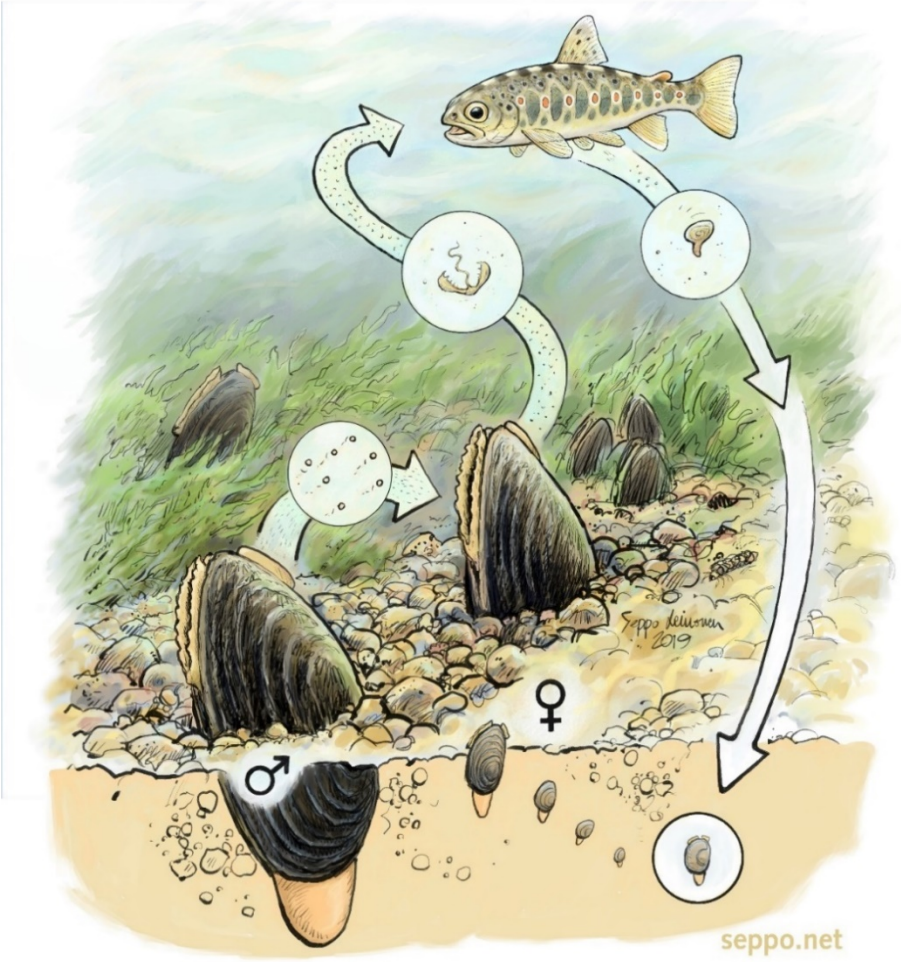


Figure 6. Freshwater Pearl Mussels releasing the microscopical glochidia in small and whitish conglutinates (arrows). (a) *In situ* adults releasing whitish conglutinates which remain deposited at the bottom of slow-flowing section of the Eo River, Galicia (Spain). (b) Captured gravid mussel at the Ulla River, Galicia (Spain), kept into small zip plastic bags to stimulate the broadcast of small brownish conglutinates. Note that conglutinates could be easily suspended in the water.

In contrast to the short incubation period, this small glochidium exhibits a long parasitic stage of several months, overwintering in the fish gills from late summer until the next spring (Bauer, 1994). To successfully develop and metamorphose into juveniles, larva needs to encyst in the gills of one of the two potential suitable hosts (Figure 7): the Atlantic salmon (*Salmo salar* Linnaeus, 1758), and both resident brown trout and migratory sea trout (*Salmo trutta* Linnaeus, 1758), described as the only native fish hosts in Europe (Bauer, 1987a; Young & Williams, 1984a). In the past, host age has been attributed an additional critical factor to this parasitism, in which only fish hatched in the same year (age class 0+) were considered the only suitable host for *M. margaritifera* (Bauer, 1987a). Conversely, more recent studies described

an opposite relationship in which the older *S. trutta* (age class 1+) became more heavily parasitized than the younger trout (age class 0+). This finding discards the age as a critical factor and points towards additional host specificities between *M. margaritifera* populations (Marwaha, Aase, Geist, Stoeckle, Kuehn et al., 2019; Salonen, Luhta, Moilanen, Oulasvirta, Turunen et al., 2017). Hence, the larval development was only successful between those mussels and Atlantic salmon or brown trout populations which have coevolved in the same geographic area (Clements, Thomas & Adams, 2018; Wacker, Larsen, Karlsson & Hindar, 2019).

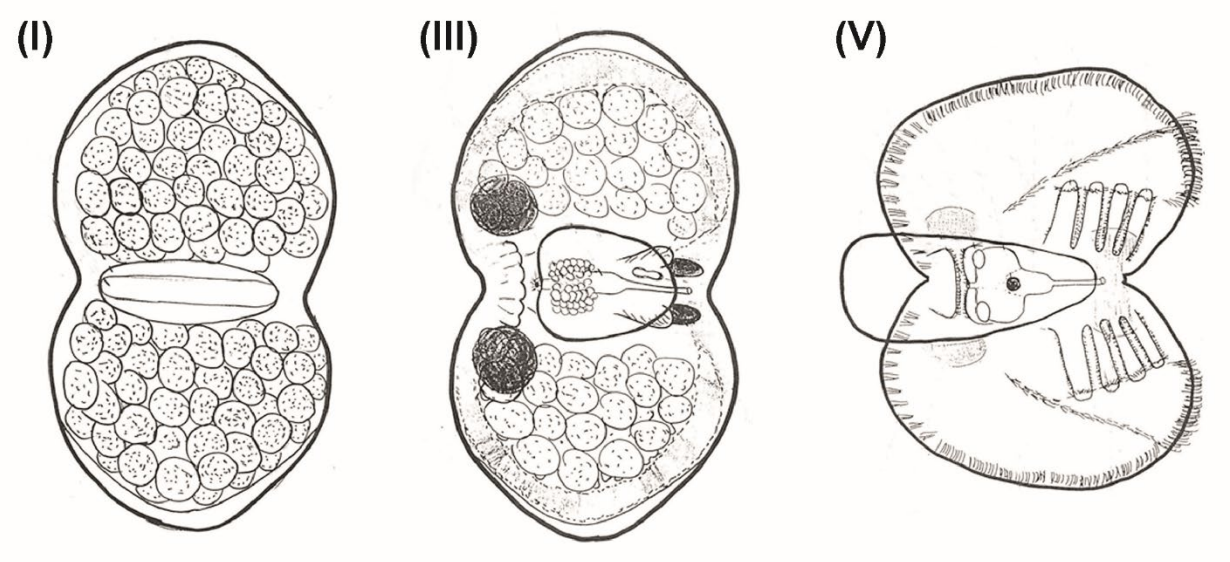
Figure 7. The life cycle of the Freshwater Pearl Mussel. Source: www.seppo.net; authorized the use by Seppo Leinonen.



Once attached to the gills, the larvae increase five times its size after several months, supposing one the most striking parasitic growths of unionids (Bauer, 1994). Despite the marked growth and long parasitism of the *M. margaritifera* larvae, there is still very little information about morphogenesis during the parasitic stage and the mechanisms of parasitic nutrition from the fish (Barnhart et al., 2008; Denic et al., 2015; Taeubert & Geist, 2017). Up to our knowledge, only Scharsack (1994) described this development and metamorphosis within the suitable host brown trout in five stages, by sectioning the parasitic larva and visualizing the outer surface mainly by scanning electron microscopy (SEM) (Figure 8).

Initially, the most prominent tissue was the larval mantle (Stage I), which afterward became partially replaced by the definitive mantle (Stage III), until its dissapearance at the end of the parasitism (Stage V). Simultaneously, the digestive apparatus, the adductor muscles, the gills and the foot were partially developed (stage III), until a large foot and 3–4 gill filaments were visible at the end of the parasitism (Stage V). However, there are no descriptions of the larval development concerning the morphopathological changes of the surrounding gill tissue. Besides this evident metamorphosis at the end of the parasitic stage, there is not a common terminology to refer to the recently detached mussels from the gills, termed indifferently as juvenile or post-larva (Schartum, Mortensen, Pittman & Jakobsen, 2016).

Figure 8. Summarizing diagrams of three out of the five developmental stages of *M. margaritifera* larvae during the parasitism of the brown trout gills. Note that the valves were manually opened to observe the internal morphology by scanning electron microscopy. Source: Scharsack (1994), authorized the use by the German



National Library of Science and Technology.

On the contrary, the post-parasitic stage had received increased attention, as juveniles undergo drastic morphological and functional changes during the first six months, shifting their alimentation from pedal to filter-feeding until reaching the critical size of 4 mm length (Araujo, Campos, Feo, Varela, Soler et al., 2018; Lavictoire, Ramsey, Moorkens, Souch & Barnhart, 2018; Schartum et al., 2016). In addition, during this transitional period, juveniles are completely buried into the riverbed, relying on coarse and well-aerated substratum but also becoming highly vulnerable to changes in the stream-bed quality (Geist & Auerswald, 2007). Alteration of the benthos has been pointed as a key issue linked to the lack of juvenile recruitment in most European populations, which have failed to successfully reproduce for the last 30–50 years (Geist, 2010). After the completion of the juvenile stage burrowed into the benthos, the FPMs locate semi-buried into the benthos, with their posterior region oriented towards the water flow, supporting the algal and macroinvertebrate diversity which cohabit with (Figure 9) (Vaughn, 2018). Adults live in cool and oligotrophic rivers with low-calcium and fast-flowing waters (Young & Williams, 1984a), with a preference for shallow river regions (0.5–2 m depth) (Ziuganov et al., 1994).

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Figure 9. One of the few remaining Freshwater Pearl Mussel colonies at the Ulla River, Galicia (Spain), constituted by a few extremely old individuals showing a worn-out shell (asterisk) with dozens of growth rings and a pair of membranous apertures (black arrows). The inhalant aperture is located ventrally and is characterized by short but arborescent papillae (white arrow), meanwhile, the exhalant aperture locates dorsally and exhibits a smoother surface (black arrow). Inset: Detail *of M. margaritifera* shells with the presence of Gastropods (encircled), case-building larvae of Caddisfly (white arrows) and Macrophytes (asterisks). Source: Research group "Conservación de Peixes e Moluscos", authorized the use by Paz Ondina.

1.3 Threats and conservation measures of the Freshwater Pearl Mussel

Historically, this species was widely distributed in the Holarctic region, found in North America, Europe and Siberia (Moorkens, 2011). In Europe, *M. margaritifera* populations extended from Artic Russia (70ºN) to the northwestern of the Iberian Peninsula (40ºN) (Bauer, 1986; Ziuganov et al., 1994). Nevertheless, this original distribution has dramatically receded up to 90% since the beginning of the 20th century and nowadays the FPM is considered one of the most endangered freshwater bivalves in the world (Geist, 2010). In Europe, this species is listed by the IUCN as *Critically Endangered* due to the highly fragmented populations and the lack of juvenile recruitment (Lopes-Lima, Bolotov, Do, Aldridge, Fonseca et al., 2018; Lopes-Lima, Sousa, et al., 2017; Moorkens, 2011). At the northwest of the Iberian Peninsula—the meridional limit of the species distribution—a serious recruitment failure was reported in a few populations with a significant number of juveniles (Geist, 2010; Lois et al., 2014; Reis, 2003; Sousa, Amorim, Froufe, Varandas, Teixeira et al., 2015).

M. margaritifera accomplished the criteria, not only for being considered as keystone but also of umbrella, indicator and flagship; thus it can be considered a target species for the conservation of freshwater ecosystems (Geist & Auerswald, 2007):

- The high habitat requirements of *M. margaritifera* convert this aquatic mollusc into an umbrella species, which means that its protection benefits indirectly many other species dependent on the same habitat, and hence, the mussel beds are considered as biodiversity hotspots (Vaughn, 2018).
- The high sensitivity to eutrophication and other changes as temperature, dissolved oxygen and water flow confer the condition of water quality indicator.
- The presence of this precious animal is related to pristine and healthy ecosystems; hence they have been used as a popular animal symbol, which is termed as a flagship.

All these specific terms are employed for making conservation-decisions, which allow focusing on those single species that define an entire ecosystem, and their conservation would imply the indirect protection of many other organisms. Among them, *M. margaritifera* contributes directly to humans by improving the quality of the water sources (Vaughn, 2017), but also favors other animals that we depend on. Thus, the conservation of *M. margaritifera* would benefit simultaneously human, animal and ecosystem health, promoting the One Health approach and the main goals of The Water Framework (Directive 2000/60/EC, 2000). The salmonids themselves, susceptible to the parasitic larvae of *M. margaritifera*, are also benefited because mussel beds improve the quality of the water and the spawning beds, provide a source of calcium leached from shells and support critical microhabitats for aquatic invertebrates upon juvenile salmonid feed (Hastie & Young, 2003; Ziuganov et al., 1994).

For these invaluable benefits, *M. margaritifera* has been protected since 1992 in the European Union under the Annex II and V of the Habitat's Directive (Directive 92/43/EEC, 1992), and included in Appendix III of the Convention of Bern (Convention of Bern, 1979), listing the fauna which requires additional regulation for their protection and exploitation. Hence, this endangered freshwater mussel became a target species of freshwater biodiversity conservation strategies in Europe (Geist, 2011).

In Galicia (NW of Spain), *M. margaritifera* was included within the category of *Endangered* within the Galician and Spanish Catalogue of Endangered Species regulated by the Decreto 88/2007 (2007) and the Real Decreto 139/2011 (2011), respectively. The inclusion as endangered species by the Ley 5/2019 (2019), implies its protection by a *Recovery Plan* (Ondina, Romero, Lois, Outeiro, Amaro et al., 2009). However, this proposed plan for *M. margaritifera* has not been adopted yet in Galicia, in contrast with another *Critically Endangered* margaritiferid, such as *P. auricularius*, which is already protected in Aragón (Decreto 187/2005, 2005).

Regardless of this devastating conservation status of *M. margaritifera* and the poor institutional support at a regional level, there are still good reasons for being optimist, based on the extreme longevity and reproductive capacity of the Freshwater Pear Mussel, even in polluted rivers and with extreme old age (Bauer, 1992). Different conservation strategies have been developed to ensure the survival of this valuable species and reinforce the most affected

populations. One of the most extreme actions relies on the captive breeding programmes, where Atlantic salmon and brown trout are used as suitable hosts to allow the parasitic larva to develop and metamorphose into juveniles (Gum, Lange & Geist, 2011; Lois et al., 2014).

1.4 The parasitic stage during the Freshwater Pearl Mussel culture

The FPM breeding consists of the production of juvenile naiads by replicating its complex life cycle in captivity. Thus, Atlantic salmon and brown trout are artificially exposed to glochidia and maintained in captivity for several months to collect the metamorphosed juveniles once they detach from the fish gills. This parasitic stage is the first step to obtain the juveniles of *M. margaritifera* and supposes a seasonal and year-round activity which has been carried out since 2012 by the Research Group "Conservación de Peixes e Moluscos" (CoPeMol) at the only FPM hatchery of the Iberian Peninsula in O Veral (Galicia, Spain), from the collection of glochidia at the end of summer to the detachment and posterior breeding of juvenile mussels in spring (Figure 10).



Figure 10. Freshwater Pearl Mussels hatchery of the Iberian Peninsula in O Veral (Galicia, Spain) throughout the four seasons. (a) When the water temperature starts to cool down at the end of summer, the gravid mussels release the glochidia and the salmon and trout are exposed to the glochidia. (b and c). In autumn (b) and winter (c), the parasitic larvae remain encysted, overwintering into the fish gills. (c) In spring, the juveniles detach from the gills when temperatures rise to start the free-living juvenile stages.

To culture the juvenile naiads, fish hosts are needed to complete the parasitic stage, hence it becomes necessary to preserve the fish welfare. Welfare is a concept created by humans to measure the physical and mental state of an individual, which assumes that an animal is in a good state of welfare when it is healthy, comfortable, well-nourished and able to express innate behaviour and not suffering from pain, fear or distress, as reported by the World Organisation for Animal Health (WHO, 2021). To preserve the fish welfare during the parasitic stages, the Research Group of "Grupo de Anatomía Patolóxica Veterinaria" (GapaVet) has monitored the fish health status during these conservation activities since 2014.

According to the literature, two different methods of gills infestations have been described in captivity to allow the encystment of *M. margaritifera* larvae into the fish gills. In the semi-natural method, fish cohabit in the same tank, or in communication with a tank, containing gravid FPMs (Preston, Keys & Roberts, 2007; Treasurer, Hastie, Hunter, Duncan & Treasurer, 2006). In the artificial infestation, fish are exposed by bath immersion with a moderate glochidial dose of ≤75,000 glochidia/L for 30–40′, based on preliminary recommendations (Österling, Ferm & Piccolo, 2014a; Taeubert & Geist, 2013). This artificial method is employed during the culturing procedures in O Veral hatchery station because it allows more controlled infestations and considers that bigger fish uptake more larva than smaller fish due to a larger gill surface (Young, Purser & Al-Mousawi, 1987). Therefore, additional studies standardized the parasitic loads with the fish size, and optimal glochidial loads of around 300 larvae/fish g were recommended to balance the juvenile culturing and the fish welfare (Taeubert & Geist, 2013).

Once encysted, the tiny glochidia exhibit a maximal diameter of 70 µm and growth up to 300–480 µm for 180–330 days (Table 2) (Bauer, 1987a; Taeubert et al., 2010). During this parasitism, which could last between 6 and 11 months, the larval growth is highly dependent on the external water temperatures; thus, the duration of the parasitic length has been measured in degree-days, reflecting the accumulated temperature throughout the parasitic period (Hruska, 1992). As a result, the larval growth and development rate are slowed down or even detained during the coldest months, between December and February, a period termed as a diapause stage (Cunjak & McGladdery, 1991; Murzina, Ieshko & Zotin, 2017; Scharsack, 1994). Despite this influence of water temperature on the larval development, very few studies described the duration of the parasitism, and highly variable periods were reported ranging between 1,700 and 3,400 degree-days, under different temperature conditions (Table 2) (Araujo et al., 2018; Hruska, 1992; Taeubert, Gum & Geist, 2013; Young & Williams, 1984b). Among all these studies, the extremely short parasitism of *M. margaritifera* of fewer than three months and 1,400 degree-days described by Hruska (1992) seems anecdotic in comparison with most parasitism lasting more than six months and 1700 degree-days (Table 2).

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Table 2. Studies describing the successful detachment *of M. margaritifera* juveniles after natural and/or artificial infestation of salmonid fish.

Duration of the parasitism					
Days	**Degree-days**	**Temperature (ºC)**	**Larval size (µm)**	**Fish species**	**References**
84	**1365**	15.5–17	-	*S. trutta*	Hruska (1992)
180	**-**	-	350	*S. salar*	Araujo et al. (2018)
244	**-**	1–18	298	*S. trutta*	Scheder, Lerchegger, Jung, Csar and Gumpinger (2014)
268	**-**	-	390	*S. trutta*	Young and Williams (1984b)
300	**-**	-	-	*S. trutta*	Scharsack (1994)
330	**1820**	1–16	-	*S. trutta*	Hruska (1992)
150–300	**1700–3400**	11.8 ± 0.2	-	*S. trutta*	Taeubert et al. (2013)
-	**-**	-	400	*S. trutta*	Bauer (1987a)
-	**-**	-	340–480	*S. salar*	Schartum et al. (2016)
-	**-**	-	379	*S. trutta* and *S. salar*	Marwaha, Jensen, Jakobsen and Geist (2017)
-	**1700**	-	320–380	*S. trutta*	Eybe, Thielen, Bohn and Sures (2015)
-	**2381**	-	-	*S. trutta* and *S. salar*	Thomas, Taylor and de Leaniz (2010)

After completion of metamorphosis, the encysted juveniles sloughed off, potentially aided by the internal movements of the foot (Kaiser, 2005; Waller & Mitchell, 1989). Juveniles are collected from the fish tanks by filtering the exiting water, being visible as small and whitish sand particles under naked eye (Figure 11). Temperature is a key factor promoting juvenile detachment in spring, when the river conditions are optimal for the survival of the recently detached juveniles (Marwaha et al., 2017). Hence, the water temperature can be artificially increased between 15ºC and 17ºC, and maintained constant for at least 15 days to synchronize the detachment of the metamorphosed larvae from the gills and facilitate the juvenile collection in a brief time period (Eybe et al., 2015; Scheder et al., 2014). Despite the synchronization of the juvenile detachment is a common procedure in captivity, there are no specific recommendations about the timing to initiate the juvenile detachment from the gills.

Wondering if the duration of the parasitic stage might affect the juvenile outcome, Marwaha et al. (2017) highlighted that the longest parasitism yielded in the acquisition of better resources and increased survival/fitness of the juveniles during the post-parasitic stages. This experience evidences that the parasitic development of *M. margaritifera* can affect juvenile survival and viability, as occurring during the early post-parasitic stage of *Unio crassus* and *Anodonta anatina* (Douda, 2015). However, no additional studies focused on the larval development and the impact that this parasitism might have in the acquisition of resources for the post-parasitic stage. In fact, before the first winter, juveniles need to overcome one of the most critical stages during the FPM captive breeding, suffering marked morphological and functional changes from pedal to filter-feeding (Marwaha et al., 2017; Thomas et al., 2010).

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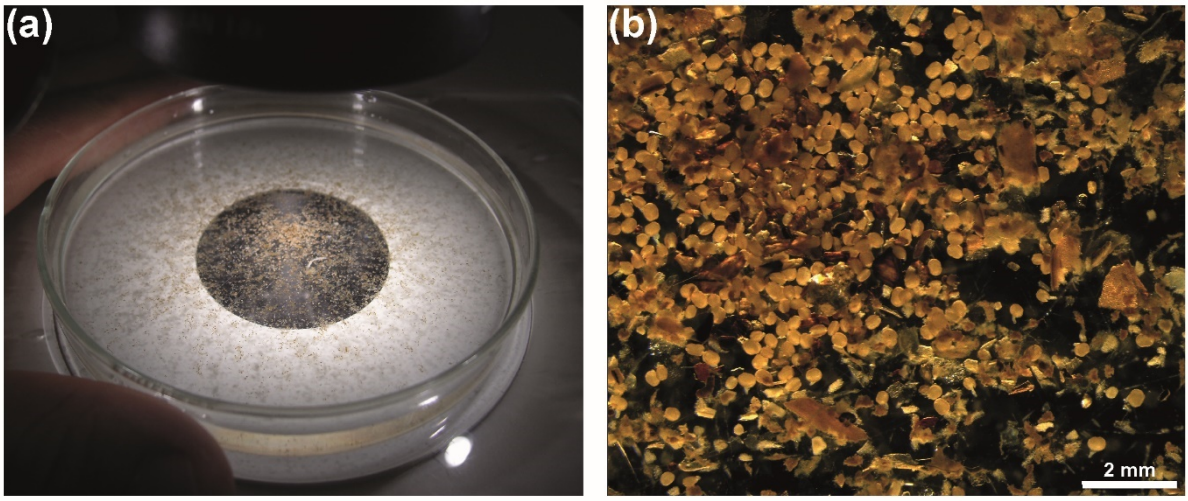


Figure 11. Recently collected *M. margaritifera* juveniles. (a) Thousands of juveniles in a Petri dish, macroscopically observed as whitish structures which resemble small sand particles. (b) Under the stereomicroscope, juveniles are interspersed between abundant organic particles and visualized as roundish structures with a pale appearance.

Besides this long-lasting parasitism, the culture of *M. margaritifera* suffers frequent larval developmental failures evidenced by a reduction in the prevalences of the parasitism, parasitic loads and larval growths (Table 3). Such decrease in the parasitic loads is known as larval survival, a term which refers to the percentage of the larvae that remain encysted in the gills during a certain period of time (Bauer, 1987a; Young & Williams, 1984b). The evaluation of the course of the larval growths and survival has been proposed as the only two measurable parameters to monitor the larval development (Marwaha et al., 2021), and are commonly employed to evidence a developmental failure of *M. margaritifera* larva (Table 3). Besides the evident reduced growth, very few studies described additional morphological changes of those larvae, only described as "unviable" larvae by Ieshko, Geist, Murzina, Veselov, Lebedeva et al. (2016) and Murzina et al. (2017). Due to this potential developmental failure, further studies

Table 3. Developmental failures of *M. margaritifera* larvae described during the parasitic stage in Atlantic salmon and brown trout.

Elapsed time (days)	**Larval fitness problems**	**Fish species**	**Factors involved**	**Techniques employed**	**References**
36–49	Reduced larval survival Reduced growth	*S. trutta*	Fish age	Light microscopy of the fresh gills	Bauer (1987a) and Bauer and Vogel (1987)
30–60	Reduced larval survival Reduced larval growth	*S. trutta* and *S. salar*	Mussel and fish populations	Light microscopy of the fresh gills (gill squash)	Salonen et al. (2017)
70	Reduced larval growth	*S. trutta*	Fish populations	Light microscopy of the fresh gills	Taeubert et al. (2010)
209	Unviable larvae	*S. salar*	-	Light microscopy of the fresh gills and histology	Ieshko et al. (2016) and Murzina et al. (2017)
293	Reduced larval survival	*S. trutta* and *S. salar*	-	Light microscopy of the fresh gills	Young and Williams (1984a) and Young and Williams (1984b)
300	Reduced larval survival Reduced larval growth	*S. trutta*	Fish age	Stereomicroscopy of the fresh gills	Marwaha et al. (2019)
-	Reduced larval survival	*S. trutta* and *S. salar*	Mussel genotypes	Stereomicroscopy of the fresh gills and genetic studies	Marwaha et al. (2021)

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are needed to thoroughly describe the normal morphogenesis, allowing to evaluate the morphology of the larvae with reduced growth and/or impaired development. Considering the potential involvement of the intraspecific suitability of fish in the larval rejection (Taeubert et al., 2010; Wacker et al., 2019), the fish responses will be further reviewed concerning the encysted larvae (see Section 3.2).

2. THE ATLANTIC SALMON

2.1 General biology, life cycle and conservation

The Atlantic salmon (*Salmo salar* Linnaeus, 1758) owes its colloquial name to their marine natural distribution through the Northern regions of the Atlantic Ocean, in contrast with the other five Pacific salmons restricted to the Pacific Ocean (genera *Oncorhynchus*). All salmon species are morphologically characterized by an elongated, laterally compressed and hydrodynamic body shape with a slender caudal peduncle. These species belong to the teleost fishes (Salmoniformes: Salmonidae) with an extremely remarkable life cycle due to its anadromous behavior; where most of the individuals smoltificate and migrate to the open ocean to grow up till achieving the reproductive size, and return to the natal river to mature and reproduce (Figure 12). This behavioral ability to travel back, exactly to the original hatching grounds where they were born, is known as *homing* and it is achieved by the combination of chemical and magnetic mechanisms.



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Figure 12. The life cycle of the Atlantic salmon. Adults return from the sea (top) to their natal rivers to spawn at the gravel nests (bottom). Fertilized eggs develop buried within the gravel until alevins are hatched in spring. Alevins grow and develop into fry, living close to the riverbeds. After smoltification, smolts migrate to the sea to feed and growth for several years, showing a silver coloration of the skin. Source: NASCO (2019) and authorized by the illustrator, Jennifer T. Proudfoot.

Once at their natal rivers, Atlantic salmon adults spawn from late autumn until mid-winter, when the water cools down. The fertilized eggs remain buried in gravel nests and hatch as alevins the following spring. The hatched alevins depend on the yok-sac and remain buried for several weeks until they reabsorb their vitelline and emerge as fry (age class 0+) (Figure 13). Afterward, they remain in fresh water for 1–5 years as parr, showing their typical roundish and darker colorations on the skin (Figure 13), until they migrate downstream and physiologically adapt to saline waters, an adaptation known as smoltification. During this process, salmon become silver-colored because of the deposition of guanine and hypoxanthine crystals at the skin and scales. Once in the sea, they travel thousands of kilometers to the northern feeding grounds and feed from plankton first, and then capturing small invertebrates and fish. Returning salmon are observed in rivers between early spring and autumn, acquiring a darker appearance and traveling against the water current. During this risky and energetically cost travel, salmon must overcome predators, waterfalls and diseases; and those survivors which arrive at the nesting sites are often with a low body condition and more susceptible to diseases, hence known as spent fish or kelts (Roberts, 1993). Due to the hormonal changes during sexual maturity, males develop a marked dimorphism of the jaws, especially the lower jaw elongates and bends upwards creating a hook, termed as “kype”. In contrast to the males, the females lack kype and show an abdominal distention as they develop their eggs. After spawning, most salmon die and only a few are able to survive and reproduce in the following season, unlike their Pacific salmon counterparts where the total of the population die (Klemetsen, Amundsen, Dempson, Jonsson, Jonsson et al., 2003). The same authors reported a considerably variable life cycle ranging from fully freshwater residents (as those precocious male parrs who are capable to mature and spent most of their lives in freshwater) to anadromous populations (growing in the sea for 3–5 winters and known as multi sea winter salmon).

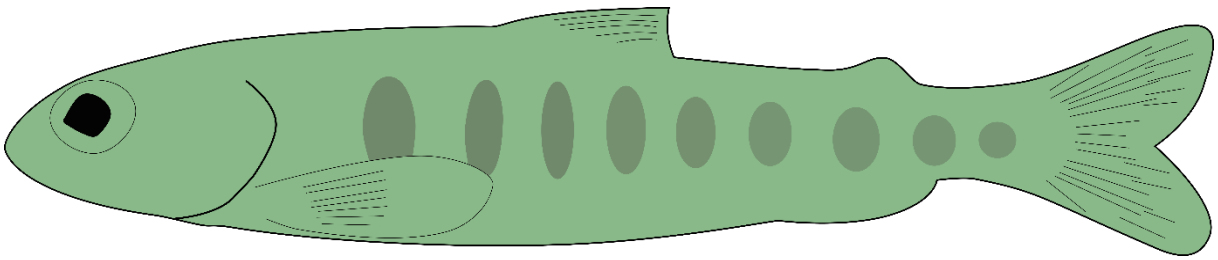


Figure 13. Atlantic salmon parr exhibits ventrally a pair of large pectoral fins which contact with the river substratum to stabilize their body, pelvic and anal fins, a large dorsal fin, a caudal and forked fin and a small adipose fin, the latter exclusive of salmonids. Note the well delimited “thumb prints” along its flanks.

Salmon plays an extremely important role in the functioning of their ecosystems with their anadromous behavior, providing food for predators and scavengers and also transporting nutrients from the marine to freshwater and terrestrial habitats. As a result of the long historical association with the society, the Atlantic salmon represent one of the most valuable fisheries in the world and many communities are dependent on salmon for their livelihoods, providing not only a food source but also other important economic, cultural and social values as reported by the North Atlantic Salmon Conservation Organization (NASCO) (NASCO, 2019). For centuries, local communities have sustained those returning salmon, making this species an integral part of their culture, diet, economy, identity, and social relations. Similarly, salmon

exhibits an important symbolism of good environmental quality, appears in art and spiritual and religious experiences (Worthington, Worthington, Vaughan, Ormerod & Durance, 2020). In addition, wild fish also supports the recreational angling industry with lucrative economic value in those areas where adult salmon migrate through (Butler, Radford, Riddington & Laughton, 2009). All these services evidence that wild Atlantic salmon will yield a larger economic impact than expected (World Wildlife Fund, 2001). Moreover, thanks to the parasitic interaction between the Atlantic salmon and the FPM, salmon exhibits an underestimated ecosystem function, serving as host to *M. margaritifera*, especially in those highly host-specific mussel populations dependent only on Atlantic salmon (Gum et al., 2011; Thomas et al., 2010; Watz, Aldvén, Andreasson, Aziz, Blixt et al., 2021).

During the freshwater stages, salmon distributes along the watercourses emptying into the Atlantic Ocean, from North Portugal to Northwest Russia in Eurasia, and from New England to Ungava Bay in Canada in Northern America (Verspoor, Stradmeyer & Nielsen, 2008; World Wildlife Fund, 2001). These rivers hosting Atlantic salmon show a high variety of habitats: from calm, less oxygenated estuarine conditions, where the salmon travels through, to upland rivers with well-oxygenated and oligotrophic water, the latter more suitable areas for spawning and juvenile nursery. At these upstream areas, underyearling salmon are commonly located in shallow riffles with a moderately coarse substrate. In spring and summer, salmon fry locates facing the water current and close to the bottom with the aid of the long pectoral fins which allow reducing the expenditure of energy at this fast-flowing habitat (Figure 13) (Gibson, 1993). For instance, at these optimal river sections, the density of salmon juveniles has been described around 0.3 individuals/m^2 at Galician rivers (Hervella & Caballero, 1998). When temperatures decrease in autumn, juveniles hide in chambers and deeper pools (Bjornn & Reiser, 1991). This timing in the preferential sites leads the salmon fry and parr to cohabit extremely close to the FPMs, especially when the glochidia are released in late summer and the juveniles detach in spring, as described in Section 1.2.

Globally, the Atlantic salmon is classified as *Least Concern* by the IUCN, but is acknowledged in the webpage that this classification needs updating (World Conservation Monitoring Centre, 1996). In Europe, wild populations are endangered in one-third of the rivers, especially at the species´ southern limit in France, Spain and Portugal (World Wildlife Fund, 2001); hence the Atlantic salmon is classified as *Vulnerable* in Europe (Freyhof, 2011). More recently, the NASCO (2019) claimed a more critical situation with 7% of populations extinct, 43% at risk, but in 36% of populations there is no data available. At the NW of the Iberian Peninsula, the Atlantic salmon is also a species in crisis like *M. margaritifera*, catalogued as *Endangered* (EN A1bd) in Spain (Álvarez, Ambrosio, Asensio, Doadrio, García et al., 2001). Here, salmon inhabits 20 river basins from the total of 50 that were inhabited in the 19th century, being extinct in 60% of the remaining rivers (Caballero, 2020). This situation was similarly reported by the World Wildlife Fund (2001), describing that 67% of those rivers which harbored salmon populations in the past are extinct, evidenced by a lack of successful reproduction for at least one salmon generation. This is also manifested in the average salmon captured at the Iberian Peninsula, which especially suffer a marked decrease between the '70s and the '90s (Álvarez, Antón, Azpíroz, Caballero, Hervella et al., 2010). In Galicia, 7 of the 16 salmon rivers

had disappeared before 1999 (Hervella & Caballero, 1998); nevertheless, the NASCO (2020) reported that the current status of 15 Galician populations of salmon is unknown nowadays. On the other hand, other studies are more optimistic, reporting a high genetic variability and adequate population structured in three of these rivers: Lérez, Ulla and Eo Rivers (Morán, Saura, Alvariño & Santamaría, 2008).

The Atlantic salmon is vulnerable to many threats, being considered an indicator species or a "canary in the coal mine". In Norway, worried about the impact of Atlantic salmon aquaculture in the wild populations, Bøhn, Gjelland, Serra-Llinares, Finstad, Primicerio et al. (2020) and Forseth, Barlaup, Finstad, Fiske, Gjøsæter et al. (2017) highlighted the escaped farmed salmon and sea lice from farms as the two most important threats to wild populations. The genetical and phenotypical divergence among wild and domesticated salmon, as a result of 50 years of breeding programmes within salmon aquaculture, supposes a risk of genetic introgression from farmed to wild salmon, as described in half of the Norwegian populations (Glover, Solberg, McGinnity, Hindar, Verspoor et al., 2017). Conversely, the wild populations also entail a sanitary and/or epidemiological risk due to the transmission of important pathogens, e.g., the notifiable monogenean *Gyrodactylus salaris* (Bakke & Harris, 1998; Hjeltnes, Bang-Jensen, Borno, Haukaas & Walde, 2019). This situation leads to a clear conflict of interests among the salmon industry and conservation programmes of Atlantic salmon and requires further research to guarantee a more sustainable aquaculture. Additional threats are also influencing to the decline of not only the Atlantic salmon, but also other diadromous fish (Limburg & Waldman, 2009). Among them, climate change has been pointed to cause physiological stress, depletion of energy reserves and increased susceptibility to disease in salmon (Bakke & Harris, 1998; Lorentzen, 2008).

In the European Union, the NASCO was created in 1982 to contribute to the conservation, restoration, enhancement and rational management of salmon stocks (Decision 82/886/EEC, 1982). Simultaneously, the Atlantic salmon was included in Appendix III of the Convention of Bern and the Annex II and V of the Habitat's Directive (Convention of Bern, 1979; Directive 92/43/EEC, 1992). To ensure their conservation and achieve the Europeans Resolutions, Agreements and Guidelines, the regional government of Galicia implemented, since 1996, certain conservation activities to improve the migration of salmon through the rivers, regulate the recreative capture, reinforce the populations with cultured juveniles and evaluate the impact of these actions (Caballero, 2020). Despite these activities, the latest evaluation of the NASCO (2021) reported that most of these measures are unsatisfactory, except for some of the actions regarding the Habitat Protection and Restoration and the Management of Salmon Fisheries. These circumstances reveal that both the Atlantic salmon and the FPM are insufficiently protected at the southern limit of their geographical distribution.

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2.2 The Atlantic salmon aquaculture

As a result of the overexploitation of wild Atlantic salmon, domestication and captive production of salmon became an obvious profitable alternative in the food market. Since the early '70s, salmon started to be cultured and genetically selected for economically important traits. But it was not until the '80s when salmon farming started to grow in Norway (Figure 14),

and nowadays the Atlantic salmon is the most profitable and technologically advanced fish production industry (FAO, 2020; Jones, 2004). With the increasing nutritional needs of the society, salmon farming followed an exponential growth, led by Norway and more lately by Chile, to ensure a continuous supply of salmon products throughout the year. Consequently, the worldwide production of Atlantic salmon in aquaculture reached its maximum during 2019, with a total production was of 2,618 million tonnes of live weight, in contrast with the insignificant amount of captured wild Atlantic salmon below 2,000 tonnes (Figure 14) (FAO, 2021). This current situation strikingly differs from that occurring only 40 years ago, when 10,000–15,000 tonnes of wild fish were captured every year as the unique production source of Atlantic salmon.

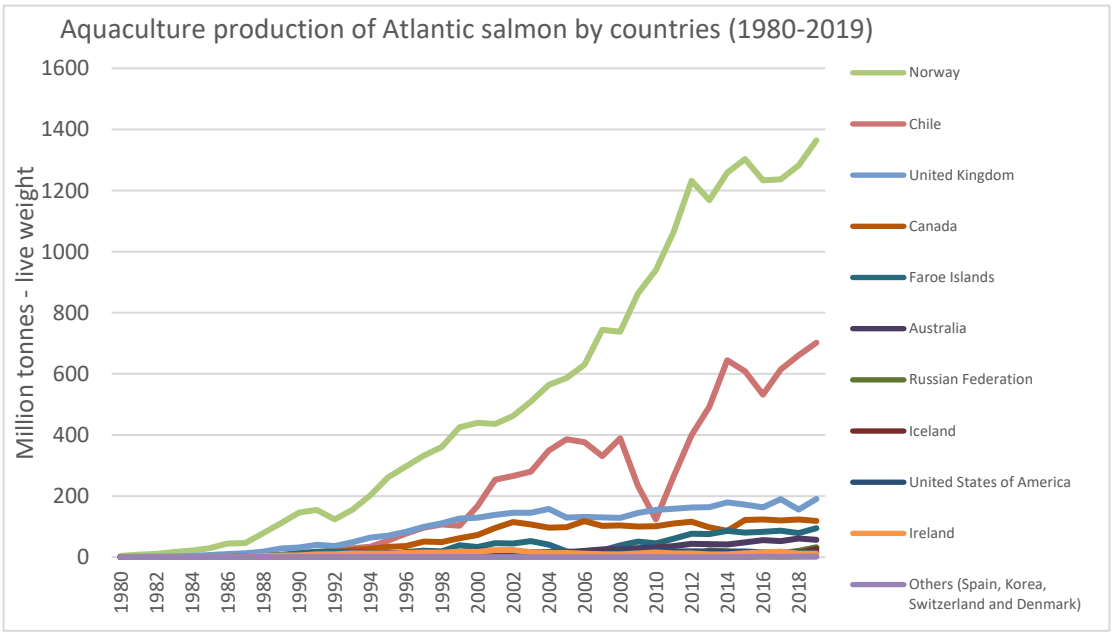


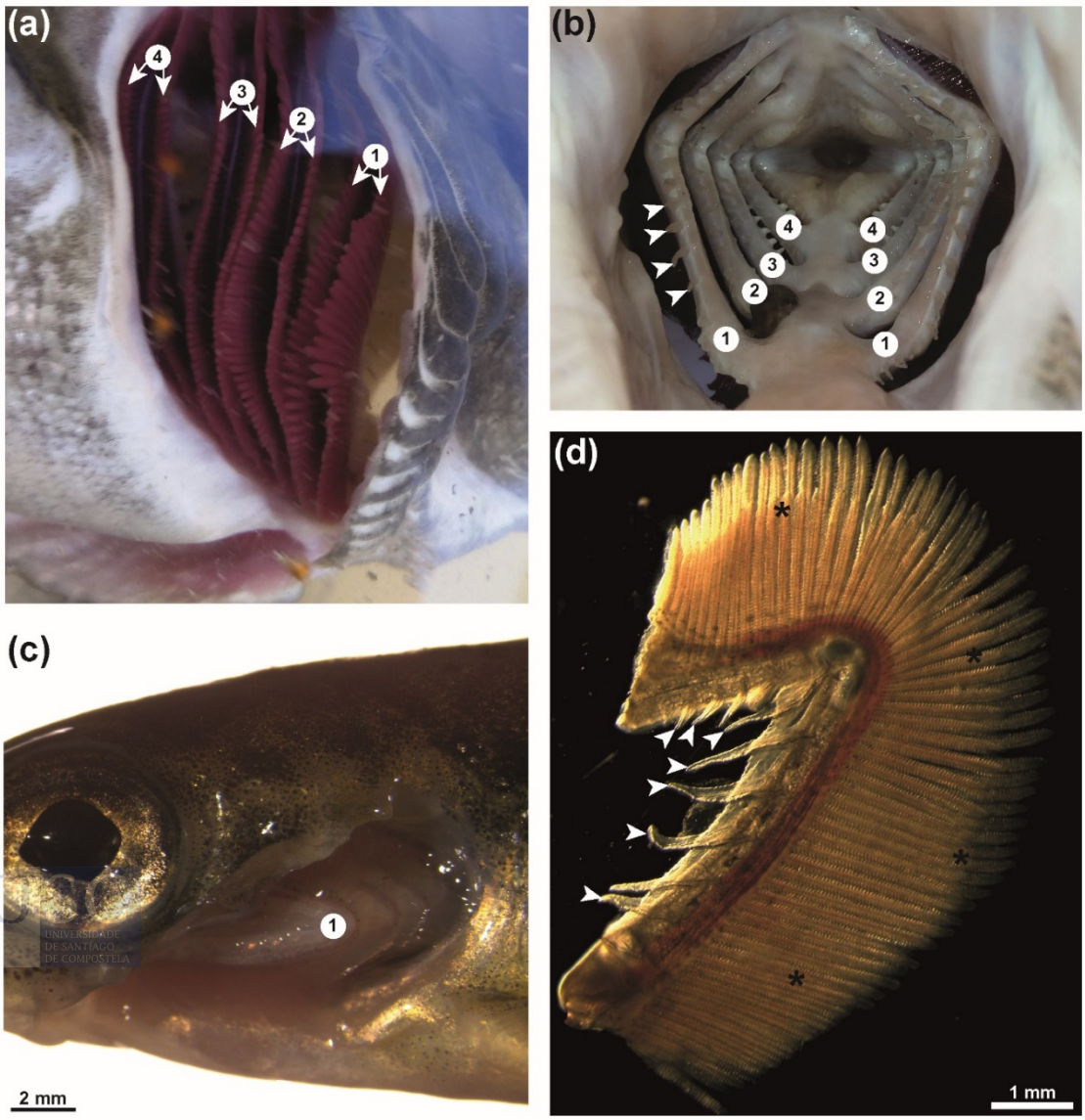
Figure 14. Evolution of the worldwide cultured amount of Atlantic salmon by country, between 1980 and 2019, elaborated with FishStatJ software (FAO, 2021). Note that Norway and Chile increased markedly their production during the last two decades in comparison with other countries, but also an irregular trend could be observed with temporary reductions of the total among of salmon produced.

The growth of Atlantic salmon aquaculture has been submitted to important sanitary and environmental shortcomings, as observed by the irregular trend in the salmon production of the leading countries during the last years (Figure 14). Norway is facing a parasitosis caused by the salmon-lice *Lepeophtheirus salmonis*, a copepod that became resistant to treatments during the last years, but also other viral diseases: e.g. Cardiomyopathy syndrome and Pancreas Disease (Hjeltnes et al., 2019). In the other hemisphere, in Chile, the spread of the Infectious Salmon Anemia virus, between 2008 and 2010, and the harmful algal blooms in 2016 caused devastating effects on the salmon productivity (Quiñones, Fuentes, Montes, Soto & León-Muñoz, 2019). The anatomical disposition of the gills, with a delicate epithelium in intimate contact with the external milieu, makes this organ highly exposed to harmful algae and suffering an acute gill pathology (Rodger, Turnbull, Edwards & Codd, 1994). Besides algal blooms, the increasing occurrence and severity of gill diseases attributed to infectious and non-infectious processes raises the concern on gill health (Boerlage, Ashby, Herrero, Reeves, Gunn et al., 2020).

2.3 Gill anatomy

As the glochidium of *M. margaritifera* develops exclusively at the branchial level, it becomes necessary to thoughtfully describe the gill anatomy and functionality to better understand the underlying mechanisms of host-parasite interaction. To do so, pathologists focus on what differs from the normal structure or function of the body to explain the pathogenesis of the disease, answering how disease develops and which actions can be taken to improve the animal status.

Gills are located halfway between the head and the abdominal cavity, protected by the opercula and composed of four pairs of holobranchs, located symmetrically at both sides of the pharyngeal cavity (Figure 15a and b). This position allows the water, that enters from the mouth, to flow from the pharyngeal cavity through the five gill slits, located between the holobranchs (Figure 15b). Thus, the water flows into the opercular chamber and outs through the opercular openings (Figure 15b and c), being actively pumped by the opercular movements which create a negative water pressure towards the opercular chamber. Moreover, when the mouth is open and the fish is in motion, the water current also flows passively through the gills.



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Figure 15. Macroscopical appearance of the salmonid gills conformed by four holobranchs at each side (1–4). (a) By the slight abduction of the lower jaw of an anesthetized adult brown trout, the holobranchs are externally observed at the posterior region of the head, protected by the opercula and composed each by a pair of hemibranchs which slightly separate in water (arrows). (b) With the mouth completely opened, the pharyngeal cavity shows at both sides the four gill arches with the five gill slits in between, where the water flows through. Note the gill rakers (arrowheads) arising from the gill arches. (c) After sectioning the opercula during the necropsy of a salmon fry, the first holobranch is visible inside the opercular chamber. (d) Stereomicroscopical photograph of a dissected holobranch with a "C" shaped and whitish gill arch which supports the gill rakers (arrowheads) and the delicate gill filaments oriented in the opposite direction (asterisks).

Holobranchs are supported by a bony gill arch with a "C" shape, where the gill rakers arise towards an anterior direction, protecting the gills from coarse suspended material (Figure 15d). On the other direction, each holobranch is constituted by two rows of macroscopical filaments which are known as hemibranchs (Figure 15a, 16). In salmonids, the proximal third of the hemibranchs of the same gill arch is fused by numerous elastic and muscle fibers, forming an interbranchial septum (Olson, 2000) (Figure 16b). At the distal region of the hemibranchs, filaments interdigitate with the neighbor filament from the adjacent hemibranch, forcing the water to flow unidirectionally through the gills: from the leading to the trailing edges of each filament (Figure 16b).

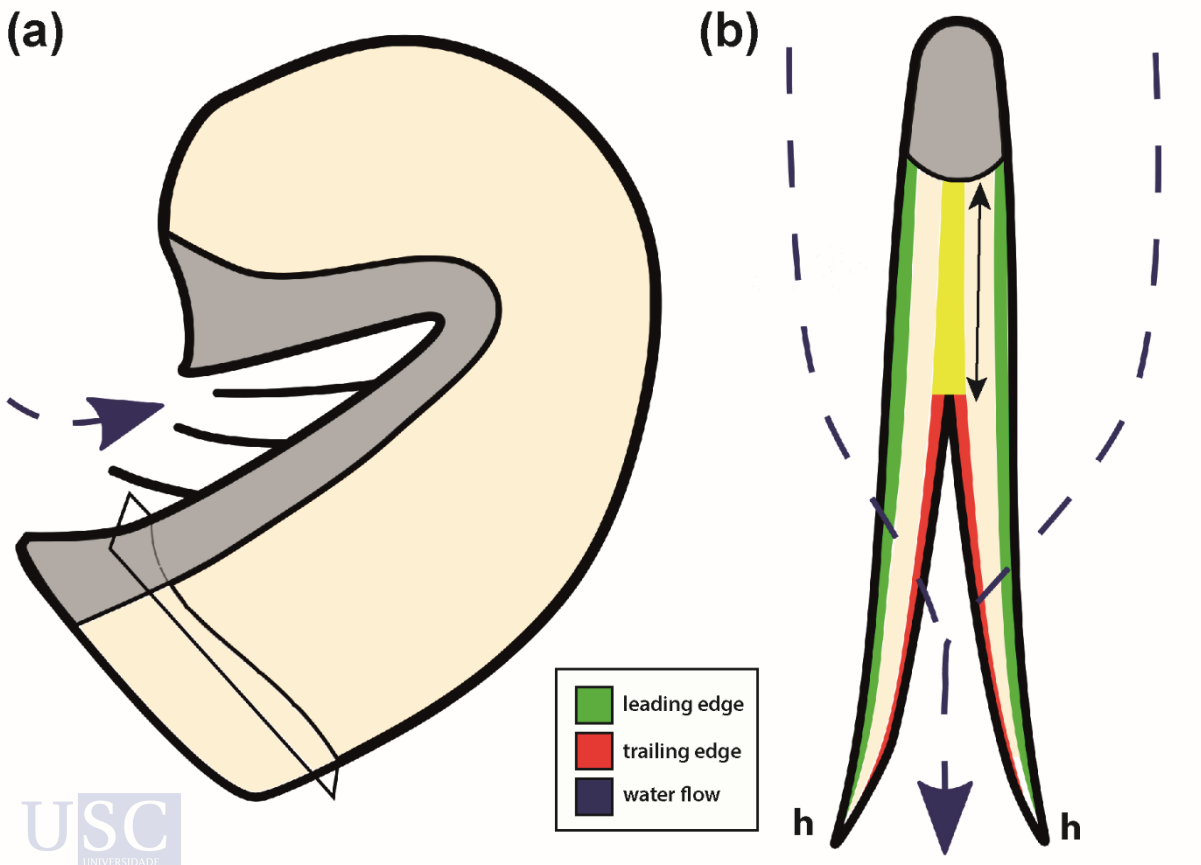


Figure 16. Schematic diagram of a holobranch and its two hemibranchs with the water flow (blue arrow). (a) Lateral view of a holobranch highlighting the direction of the gill flow entering from the pharyngeal cavity. (b) Transverse section of the holobranch, indicated in the lower region of Image (a), showing the water flow through the two hemibranchs (h), leading to the differentiation of the leading (green) and trailing edges (red) of the gill filaments. Note that both hemibranchs were fused at the proximal region by the interbranchial tissue (black arrows).

To increase the contact gill surface with the water, each filament is composed by thin plate-like structures, equidistantly spaced at both sides of the filaments, and termed as secondary lamellae (Olson, 2000) (Figure 17a and b). At the microscopical level, each lamella could be regarded as an envelope-like structure, covered externally by two layers of a simple and flat epithelium (Figure 18). Internally, the epithelium is supported by pilar cells which act as columns; allowing the blood to perfuse between both epithelial sheets of the lamellae. Due to this unique morphology, the lamellae are the primary site for respiration, where the transport of oxygen and carbon dioxide across the lamellar epithelium is favored by the reduced diffusion distance between the outer environment and the blood. Moreover, the microvillar surface increases, even more, the gill surface showing the typical interdigitate ultrastructural appearance of the apical epithelial surface (Olson, 1996).

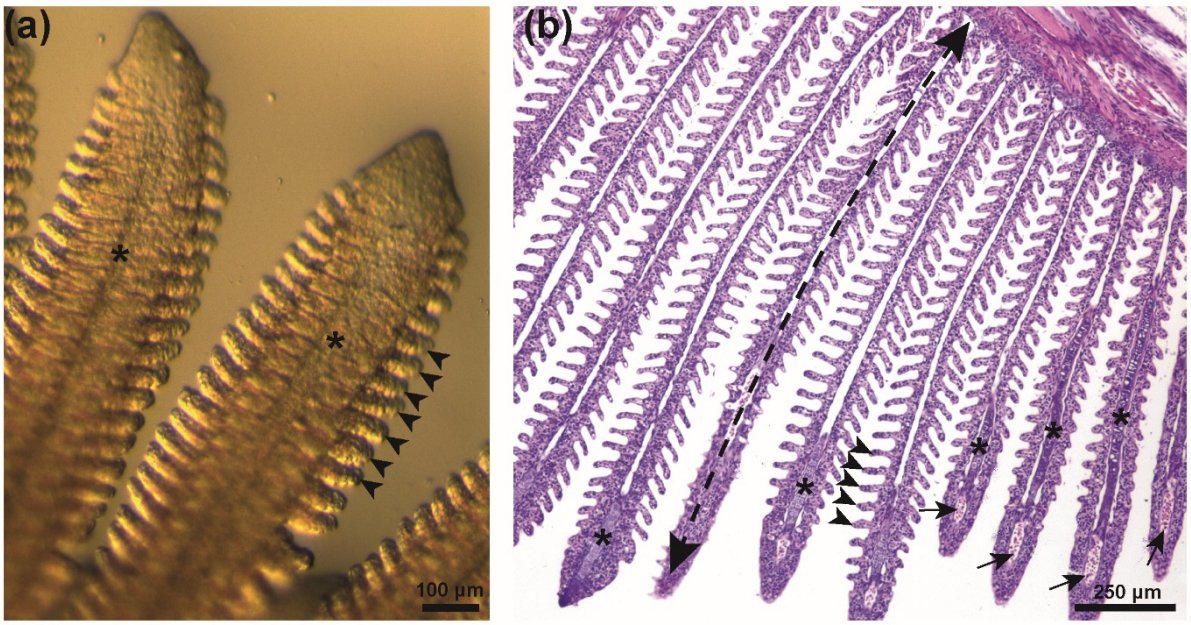


Figure 17. Microscopical morphology of the gill filaments. (a) Stereomicroscopy of the distal region of two filaments (asterisks), characterized by two rows of lamellae (arrowheads) at each side. (b) Histological section of a holobranch showing the disposition of filaments (dashed arrows) arising from the gill arch (right top), supported by gill rods (asterisks) and irrigated by arterioles (black arrows). The outer surface of each filament displayed abundant lamellae all over its length (arrowheads). H&E stain.

To maximize the exchange of substances among the water and the blood, lamellae are perfused by an intricate arterio-arterial system which flows in counter-current direction of the water: from the trailing to the leading edge of the filaments (Hughes, 1984; Olson, 2002). Thus, the blood irrigates the lamellae entering from the trailing edges of the gill filaments and flows back through the leading edge (Olson, 2002). Meanwhile, an small proportion of the blood diverges towards the arteriovenous sinus, an intricate structure whose anatomy and functionality are not completely understood, potentially related with the nutritive support, blood shunt and/or the removal of the interstitial fluid, waste products and cellular debris (Evans, 1987; Laurent & Dunel, 1980; Olson, 2002; Rummer, Wang, Steffensen & Randall, 2014).

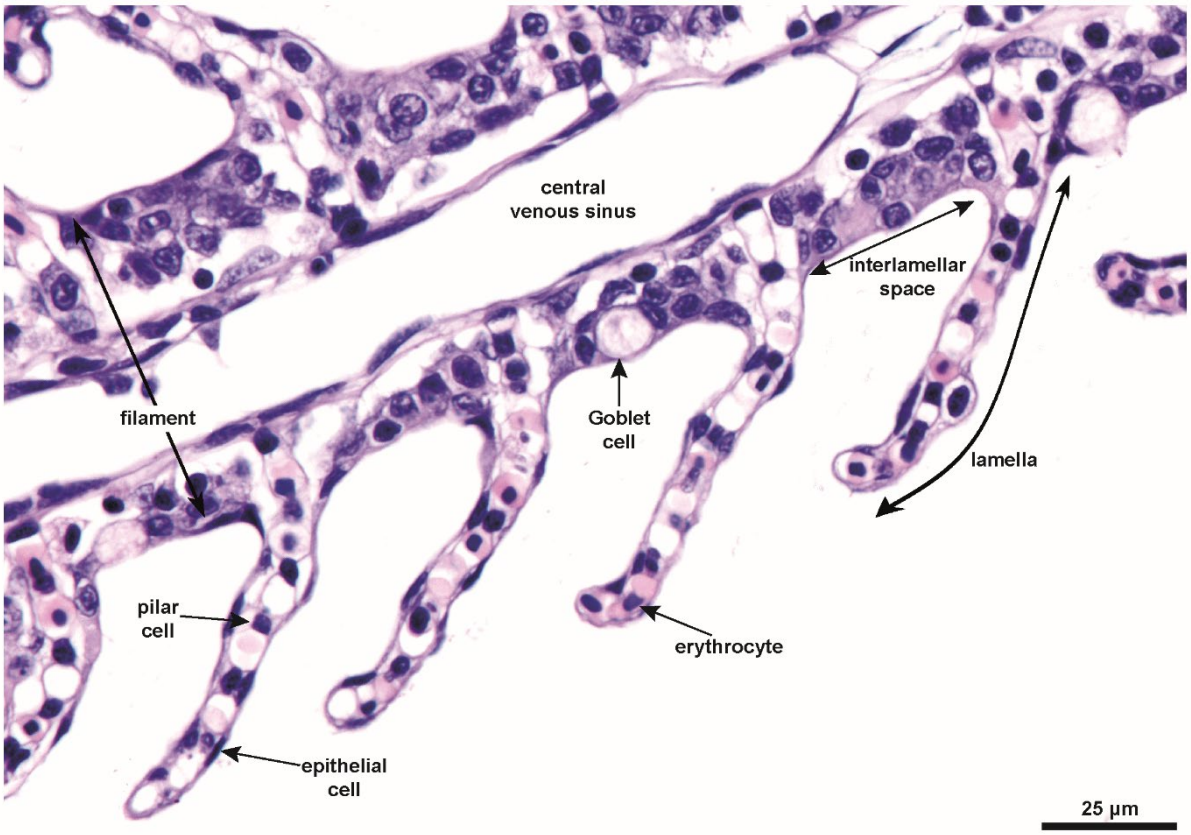


Figure 18. Morphological detail of a gill filament with equidistant secondary lamellae, spaced by the interlamellar spaces which lay over the central venous sinus. Each lamella is lined externally by extremely thin epithelial cells, separated by pilar cells in between. Interlamellar cells involved undifferentiated epithelial cells and other specialized cell types, e.g., Goblet cells. H&E stain.

Lamellae are separated by the interlamellar spaces, a region where the undifferentiated epithelial cells proliferate and repopulate the branchial epithelium (Figure 18). Other specialized cells, as chloride and Goblet cells, are also located frequently at these regions. Chloride cells are involved in osmoregulation. During the smoltification of Atlantic salmon these cells become hyperplasic (increase in the number of cells) and hypertrophic (increase of the cell size), and exhibit a marked mitochondrial Na^+/K^+-ATPase activity to expel large amounts of Na^+ and Cl^-; counteracting the passive diffusion of salts towards the fish (Laurent & Dunel, 1980; Wilson, Laurent, Tufts, Benos, Donowitz et al., 2000). Goblet cells produce mucus which interacts with the glycocalyx located at the outer surface of the epithelial cells (Koppang, Kvellestad & Fischer, 2015; Olson, 1996). This mucus creates a tissue-water interface that reduces water turbulences and traps bacteria and debris; essential for the mechanical and immunological protection (Marcos-López & Rodger, 2020), and the exchange of substances among the gill and the external environment (Ferguson & Speare, 2006; Shephard, 1994).

The gills are also an important defensive organ related to the mucosal immune response of fish (Bjørgen & Koppang, 2021; Salinas, 2015). Gills exhibit an important quantity of diffuse lymphoid tissue, described for the first time in 2008 in Atlantic salmon by Haugarvoll, Bjerkas, Nowak, Hordvik and Koppang (2008); lately termed as interbranchial lymphoid tissue due to the abundant T-cells dispersed between intraepithelial cells at the dorsal region of the

interbranchial tissue and all over the trailing edges of the filaments (Dalum, Austbø, Bjørgen, Skjødt, Hordvik et al., 2015). Based on gene transcription and morphological data, this lymphoid structure is related to the recruitment of immune cells (Dalum, Griffiths, Valen, Amthor, Austbø et al., 2016). Besides lymphocytes, many other cell types could be found on the gills based on their morphology and molecular markers, e.g., granulocytes, mononuclear cells and eosinophilic granular cells (Koppang et al., 2015).

This morphology evidence that gills exhibit a large surface area with a small diffusion distance with the external environment, high ventilation rates and high perfusion by the totality of the cardiac output. These features justify how the gills are involved with other physiological processes involved in maintaining systemic homeostasis when facing internal and environmental changes. Thus, not only respiration but also osmoregulation, acid-base balance and nitrogenous excretion are important functions carried out by the gills, which therefore could be considered as a multifunctional organ (Evans, Piermarini & Choe, 2005).

2.4 Gill responses to disease

In response to physiological and environmental changes—and also pathogens—, fish gills exhibit high plasticity with curious adaptations of the epithelium (Gjessing, Steinum, Olsen, Lie, Tavornpanich et al., 2019; Nilsson, 2007). As a result of the branchial responses, their normal structure and functionality can be altered at a certain point, leading to a disease status. Different mechanisms can initiate the pathological processes of the gill: the colonization of the biofilm located on the epithelial interface (e.g., bacterial gill disease), the entrapment of pathogens distributed systemically by the phagocytic or endothelial cells of the gill (e.g. microsporidia), the interaction of water-borne toxins with the gill epithelium (e.g. algal blooms) or the direct damage to the gill tissue (e.g. monogenean trematodes) (Rodger et al., 1994; Speare, Ferguson, Beamish, Yager & Yamashiro, 1991a; Weli, Dale, Hansen, Gjessing, Rønneberg et al., 2017).

Despite the marked epithelial plasticity and the variety of initiation mechanisms, the understanding of gill diseases is still in its infancy, and the connection between the gill lesions and the pathophysiological effects remains unanswered (Gjessing, Krasnov, Timmerhaus, Brun, Afanasyev et al., 2020; Speare, 2011). In fact, epithelial hyperplasia and lamellar fusions, frequent gill lesions, could reduce extensive regions of the gill but also minimize the available area for further damage, enhancing the survival of those fish challenged by a disease (Ferguson & Speare, 2006). For these reasons, further studies are required to understand the impact of the gill lesions on the multiple functionalities of the gills and hence the fish health (Foyle, Hess, Powell & Herbert, 2020). Moreover, the reparative mechanisms of the gill after injury have been scarcely studied, with a few studies describing an effective healing process when the basement membrane of the gill epithelium was preserved intact; recovering after 3 to 8 weeks after removal of the inciting cause (Daoust & Ferguson, 1986; Ferreira Sales, Enes dos Santos, Rizzo, de Azambuja Ribeiro, dos Santos et al., 2017; Kudo & Kimura, 1983; Speare, Carvajal & Horney, 1999).

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3. GLOCHIDIOSIS

3.1 General features

After the discovery of the glochidium capacity to parasitize fish at the end of the 19th century, researchers approached this host-parasite interaction from different perspectives. First studies focused on the most relevant features of the parasitic interaction, from the mechanism of attachment to the fish to the potential mechanisms of nutrition (Figure 19) (Arey, 1932c; Blystad, 1923; Lefevre & Curtis, 1910; Young, 1911). Based on further histopathological studies, this interaction was considered a fish disease, named glochidiosis (Meyers & Millemann, 1977). During the '80s and '90s, when the salmon aquaculture began to rise exponentially, the wild and dense *M. margaritifera* populations in Scotland were regarded as a harmful gill parasite to the salmon aquaculture (Bruno, McVicar & Waddell, 1988).

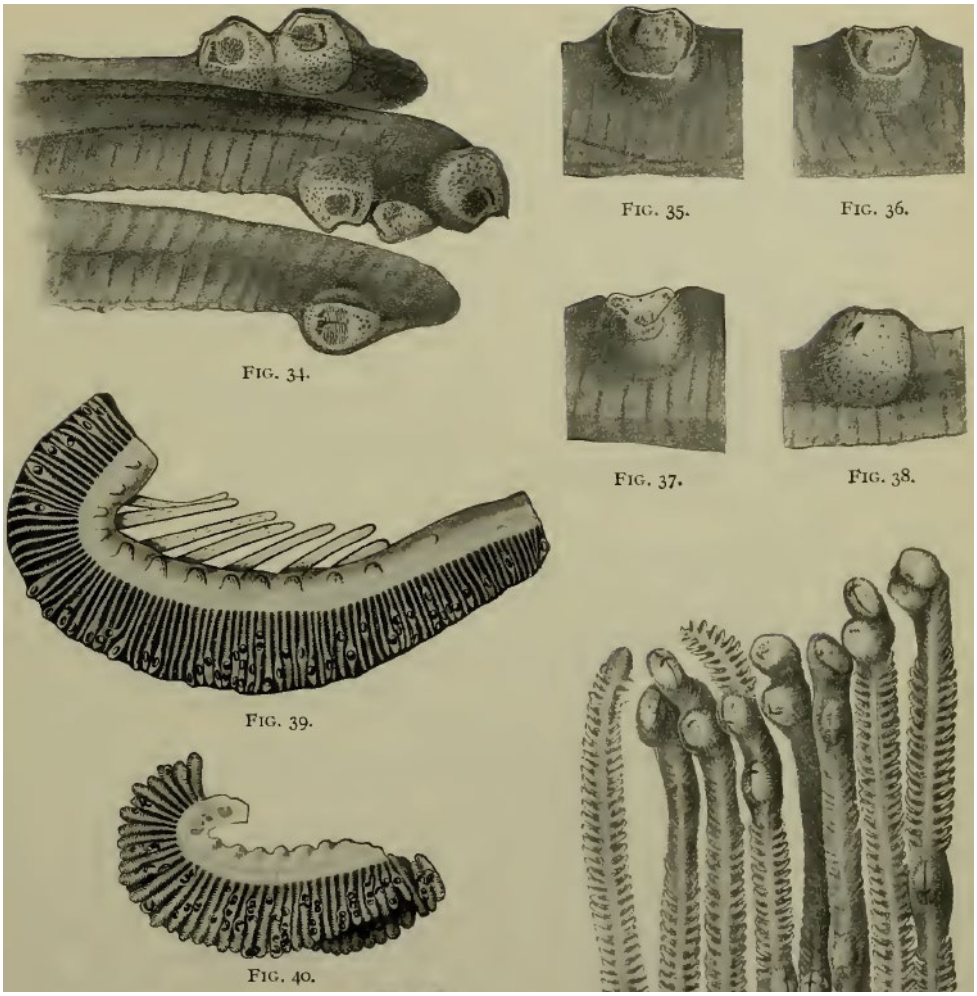


Figure 19. One of the first images representing the parasitic stage of freshwater mussels encysted in the fish gills. Source: Lefevre and Curtis (1910).

Later, the dramatic decline of freshwater mussels within the last 30 years had changed completely the importance of this interaction, and mussel propagation programmes arise as one of the last-time measures to protect the most affected populations. Concerned about fish welfare, researchers have started to investigate the impact of glochidiosis on the fish, aiming to establish a balance between mussel production and fish health (Chowdhury, Marjomäki &

Taskinen, 2019; Taeubert & Geist, 2013). Despite glochidia can attach to different epithelia, most of the studies focused mainly on the impact that this parasitism might cause on the gills, the primary target organ of many freshwater mussels larvae (Jansen et al., 2001).

During the parasitic stage of the freshwater mussel life cycle, the glochidia attach to the fish gills by closing their valves. Once encysted, as represented in Figure 19, they develop and metamorphose into mussel juveniles to detach from the fish gills. To understand the impact of glochidiosis on the fish, multiple studies described the branchial lesions associated with the glochidia of different freshwater mussel species. It is well-known that glochidia cause an epithelial hyperplasia of the gill epithelium causing the formation of a parasitic cyst, which becomes progressively more extensive, involving and fusing adjacent lamellae (Bruno et al., 1988; Treasurer & Turnbull, 2000). In addition, a complex gill response could be inferred because of the migration of epithelial cells surrounding the larvae and the presence of inflammatory cells at deeper regions of the filaments (Fustish & Millemann, 1978; Nezlin, Cunjak, Zotin & Ziuganov, 1994; Reis, Collares-Pereira & Araujo, 2014). In the most severe cases, necrosis and hemorrhages also accompanied the highly parasitized areas, being indicative of certain vascular damage in the gills (Bruno et al., 1988; Howerth & Keller, 2006). Those branchial changes may cause a reduction of the exchange surface area between the water and the fish gills, leading to respiratory distress manifested as hyperventilation (Crane, Fritts, Mathis, Lisek & Barnhart, 2011; Kaiser, 2005), reduced swimming capacity (Thomas, Taylor & Garcia de Leaniz, 2013) or even the death by asphyxia in heavily infested fish (Howerth & Keller, 2006; Karna & Millemann, 1978; Meyers & Millemann, 1977). Besides respiration, the osmoregulatory capacity of the gills could also be affected based on the increased levels of Cl^- and K^+ (Slavík, Horký, Douda, Velíšek, Kolářová et al., 2017; Treasurer & Turnbull, 2000).

Despite the main pathological effects of glochidia are detected at a branchial level, these parasitisms induce systemic changes as the increase of the haematocrit, which could be regarded as a compensatory mechanism to minimize the respiratory impairment (Filipsson, Brijs, Näslund, Wengström, Adamsson et al., 2017; Marwaha et al., 2019; Methling, Douda & Reichard, 2019). Other studies suggest the induction of a stress response because of the increased standard metabolic rate (Filipsson et al., 2017; Methling et al., 2019), the reduced condition factor and even a delayed growth, worsened by the increased energy demands due to the parasitic nourishment (Cunjak & McGladdery, 1991; Chowdhury et al., 2019; Douda, Velíšek, Kolářová, Rylková, Slavík et al., 2017; Ooue, Terui, Urabe & Nakamura, 2017; Treasurer et al., 2006). On the other hand, the respiratory capacity of fish was positively correlated with the parasitic loads of the larval stages of *Sinanodonta woodiana*, explained by a potential tolerance strategy to cope with, or even overcompensate, glochidiosis (Methling et al., 2019).

Once the larvae metamorphose into juveniles and detach from the gills, the capacity of the gill to recover has been overlooked despite the marked gill lesions associated with this transient parasitism. In fact, Arey (1932a) highlighted the parasitic encystment as a great opportunity for the study of wound healing in fish. The only observations after the parasite "excystment" described a partial repairment of the gill lesions after 50–90 days (Kaiser, 2005; Treasurer & Turnbull, 2000).

Nevertheless, an early detachment of the larva also occurs when the glochidia of a host specialist freshwater mussel attach to a non-suitable host. In these cases, a more complex morphopathological pattern is suggested in view of the apparition of "irregular" aggregates of epithelial cells (Rogers-Lowery & Dimock, 2006; Scharsack, 1994) and a marked proliferative and/or inflammatory gill responses (Fustish & Millemann, 1978; Waller & Mitchell, 1989). These morphopathological, physiopathological and behavioral changes point towards a more complex pathogenesis of glochidiosis in which both defense, compensatory and reparative mechanisms of the gills simultaneously interplay.

3.2 The *M. margaritifera* glochidiosis

During the parasitism of *M. margaritifera* in their suitable salmonid hosts (Atlantic salmon and brown trout fry), the fish gills displayed a similar morphopathological pattern to those described in other gill glochidiosis, characterized by a proliferative response of the epithelium accompanied by lamellar fusions and inflammatory infiltrates (Bruno et al., 1988; Ieshko et al., 2016; Nezlin et al., 1994; Scharsack, 1994; Treasurer & Turnbull, 2000). Despite the larvae remain encysted for several months in the fish gills, there are no studies that evaluate the evolution of the gill lesions alongside the parasitism in this mussel species.

Regardless this proliferative branchitis, no mortalities were associated with infestations below approximately 1000 larvae per fish, in both salmonid hosts. In captivity, salmon fry parasitized with less than 1000 larva per fish did not suffer mortalities (Bruno et al., 1988; Treasurer & Turnbull, 2000), similarly to brown trout that can harbor parasitic loads between 500 and 1600 larva per fish (Preston et al., 2007; Young et al., 1987). Under natural conditions, wild salmon and brown trout fry cohabiting with mussel densities around 1.3–30 mussels/m^2 were caught alive with maximal glochidial densities up to 1000 larvae/fish (Cunjak & McGladdery, 1991; Treasurer & Turnbull, 2000; Young & Williams, 1984a). Occasionally, salmon parr exhibited higher parasitisms over 1000 larvae/fish in Russian rivers with mussel densities between 6 and 8.5 mussels/m^2 (Ziuganov, 2005).

As described in other freshwater mussels, a physiopathological impairment was also evidenced in *M. margaritifera* glochidiosis, including respiratory and osmoregulatory disbalances, compensatory mechanisms (e.g., polycythemia), and other secondary effects manifested as behavioral changes and reduction of the body condition (highlighted in red in Table 4). In contrast to this negative impact, other studies described a beneficial impact, evidenced by an increased condition factor in brown trout and increased survival rates in older spawner salmons infested with 1,500–2,000 larvae (highlighted in green in Table 4) (Chowdhury, Roy, Auvinen, Pulkkinen, Suonia et al., 2021; Marwaha et al., 2019; Ziuganov, 2005). However, additional experimental studies are needed to demonstrate the potential specific and direct impacts on the fish physiology and health (Makhrov & Bolotov, 2010), and therefore prove this hypothetical benefits of the *M. margaritifera* larvae.

Taking into consideration the fish weight, as employed during the *M. margaritifera* breeding programmes (see Section 1.4), most of the standardized parasitic loads calculated during these infestations were below 25 larvae/fish g (Table 4), far away from the previously recommended parasitic loads which were set around 300 larvae/fish g (Taeubert & Geist, 2013).

Despite no mortalities were associated with glochidiosis below 1000 larvae per fish, a more precise evaluation of the physiopathological effects is needed to optimize the culturing efforts.

Table 4. Physiopathological and behavioral effects of *M. margaritifera* glochidiosis in its suitable fish host alongside the course of the parasitism. Positive and negative effects on fish health are highlighted in green and red, respectively. The parasitic loads were expressed in larvae/fish g or estimated by dividing the intensity of the parasitism (larvae/fish) by the fish weight; the latter indicated with asterisks.

Elapsed time/month	Effect of glochidiosis	Fish species	Fish age	Fish weight (g)	Larvae/ fish g	References
12–24 hr	Higher mortality and reduction of critical swimming speed	*S. trutta*	> 1+	-	>906	Taeubert and Geist (2013)
0–14 days	Altered thermoregulation behavior	*S. trutta*	> 1+	181	-	Horký, Slavík and Douda (2019)
30 days	Splenomegaly	*S. trutta*	0+	10	20*	Thomas et al. (2013)
57 days	Decreased foraging behavior	*S. trutta*	0+	2.4	25.1	Österling, Ferm and Piccolo (2014b)
90–120 days	Delayed mortality to *Flavobacterium* disease	*S. trutta*	1+	-	-	Chowdhury et al. (2021)
105 days	Reduced weight	*S. salar*	0+	21	2.4–5.3*	Treasurer et al. (2006)
160–167 days	Slow recovery of ventilatory rates after exercise	*S. trutta*	0+	12–55	1.0–3.1*	Thomas et al. (2013)
270 days	Higher mortality	*S. trutta*	0–1+	-	-	Marwaha et al. (2021)
300 days	Increased condition factor and haematocrit	*S. trutta*	1–2+	-	-	Marwaha et al. (2019)
310 days	Increased blood Cl^- after transfer to sea water	*S. salar*	0–1+	65	7.9*	Treasurer and Turnbull (2000)
315 days	11% reduction of the increase in weight	*S. trutta*	1–2+	150	20*	Chowdhury et al. (2019)
june-november	Increased survival	*S. salar*	spawner	-	-	Ziuganov (2005)
october	Reduced foraging and activity, and subordinate behavior	*S. trutta*	0+ and 1+	-	0–23	Filipsson, Petersson, Höjesjö, Piccolo, Näslund et al. (2018)
january-february	Reduction of condition factor	*S. salar*	0+	-	-	Cunjak and McGladdery (1991)
april	Increased metabolic rates and haematocrit	*S. trutta*	0+	4	0–11	Filipsson et al. (2017)

During the long parasitism of *M. margaritifera*, the parasitic loads are not constant and marked glochidial rejection, frequently observed even in the suitable fish species, especially during the first 40 days (Bauer, 1987a; Jung, Scheder, Gumpinger & Waringer, 2013; Young & Williams, 1984b). Despite this early detachment affects larval survival during the culturing programmes (see Section 1.4), there is a paucity of data for explaining this premature detachment in Atlantic salmon and brown trout; and no studies described the morphopathological changes occurring in both larva and host during these critical stages. Up to our knowledge, only Scharsack (1994) described a quick rejection of the larvae during the first 24 h of the parasitism in the unsuitable rainbow trout *Oncorhynchus mykiss*, associated with epithelial depressions after the larval rejection, using scanning electron microscopy (SEM). The subjacent mechanisms related to the premature rejection of the parasitic larvae are completely unknown, and certain studies hypothesize the involvement of the acquired immune response of fish, based on the splenomegaly suffered by infested brown trout (Thomas et al., 2013) and reduced parasitic loads obtained after secondary exposure to glochidia (Chowdhury, Salonen,

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Marjomaki & Taskinen, 2018). However, these results are not sufficient to understand the factors and mechanisms involved in the early larval rejection in fish not previously exposed to glochidia, and how this rejection could affect the fish health.

This early rejection differs from the detachment of the metamorphosed juveniles, after several months encysted into the gills. At these late stages, the juvenile mussels achieved a marked size in the gills around 400 µm (see Section 1.4) before detaching from the fish to bury in the riverbed. Very few studies focused on the detachment from the gills, only describing signs of "opening up" of the gill epithelium surrounding the juveniles by SEM (Scharsack, 1994; Wächtler et al., 2001). As reported in other glochidiosis, there is little information about how the gills recover from the infestation, only describing a partial repairment after 50 days, when farmed salmon were transferred int to saltwater (Treasurer & Turnbull, 2000).

Since the dramatic recession of FPM, many researchers have focused on culturing the juvenile mussel and optimizing the parasitic stage of the *M. margaritifera* larvae in its suitable salmonid hosts. These efforts need a deep understanding of this host-parasite interaction which involves both the development of the parasitic larvae within the fish gills and the evolution of the fish gill response during glochidiosis. On one hand, encysted larvae should undergo a metamorphosis into juveniles, but frequently an early developmental failure hinders the juvenile collection. Simultaneously, there are many other important knowledge gaps regarding its morphogenesis, the acquisition of nutrients from the fish and the completion of metamorphosis. On the other hand, there are very few descriptions of the evolution of the gill lesions alongside the parasitism, essential to understand the physiopathological impact of glochidiosis on fish and the gill healing capacity to recover after the parasitism. Despite it seems obvious that both larvae and fish mutually interplay, there are no studies that integrate the larval development with the fish response, essential to provide a better understanding of the pathogenesis of glochidiosis.

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Objectives



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OBJECTIVES

The present Doctoral Thesis was held within the conservation programme of *M. margaritifera,* focused on the artificial propagation of the most affected populations at the NW of the Iberian Peninsula. The main aim of this study is to characterize the host-parasite interaction between the FPM and the Atlantic salmon. Therefore, the gill morphopathological changes during the most critical stages, and the morphogenesis of the parasitic larva were simultaneously described by the combination of stereomicroscopy, light microscopy, histology, histopathology and/or scanning electron microscopy. Hence, the specific objectives of this Doctoral Thesis are:

1. To comprehensively characterize the lesions suffered by the fish during the critical stages of glochidiosis.

2. To describe the larval development and metamorphosis of the FPM.

3. To assess the gill healing process and the potential sequelae after the parasitism.

4. To deepen into the pathogenesis of glochidiosis by integrating both gill lesions and larval development during the parasitic period.

5. To find tools that might help to design future strategies to preserve both the FPM and fish welfare during the parasitic stage of the culture of freshwater mussels.

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General methodology



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GENERAL METHODOLOGY

To morphologically evaluate this host-parasite interaction, a set of experimental glochidiosis was designed and performed within the FPM culture, carried out by the Research Group COPEMOL at the hatchery located at the "Centro Ictiogénico de O Veral". These *in vivo* procedures were authorized by the competent authorities (reference number 02/17/LU-002; Annex 3) and were carried out by authorized personnel, following the regulations for the protection of animals used for scientific purposes (Directive 2010/63/EU, 2010; Real Decreto 53/2013, 2013). The author declares that he has no competing interests.

Alongside the parasitosis, fish were reared under controlled conditions, necropsied and sampled, to evaluate and describe qualitatively both the morphopathological changes suffered by the fish and the morphogenesis of the parasitic larva at the "Facultade de Veterinaria" by the Research Group GapaVet. As a result, the combination of routinary techniques employed in Freshwater Mussel Conservation and Veterinary Pathology would provide a novel approach to experimentally assess fish glochidiosis. Since this Ph.D. thesis employed a common and sequential methodology, a brief and comprehensive overview of the experimental design and the techniques will be summarized and illustrated here, meanwhile more detailed information is described in each study.

1. *IN VIVO* PROCEDURES IN THE SALMON HOST

1.1 Experimental infestation

Wild *M. margaritifera* mussels located at different rivers of Galicia (NW of the Iberian Peninsula) were selected by the careful opening of the valves and rapid visualization of the gravid demibranchs (Figure 1a). Gravid mussels were kept individualized to stimulate the broadcast and collect thousands of glochidia (Figure 1b). Once abundant mature and viable glochidia were collected, Atlantic salmon fry (*S. salar*) of age class 0+ were exposed by bath immersion to glochidia to induce an artificial infestation on fish, conforming the exposed group (Figure 1c). These fish were reared for several months in open system tanks to allow the development of the encysted larvae in the fish gills (Figure 1d). A group of non-exposed fish from the same batch served as a control group. Replicates of both groups were employed to evaluate fish mortalities.

1.2 Selection of infested fish and synchronization of the post-larval detachment

Although a number of fish prematurely rejected the encysted larva, fish that remained infested after six months contained large larva encysted in the gill tissue, visible macroscopically by manual immobilization and abduction of the opercula. This technique allowed us to the *in vivo* diagnosis and selection of the infested fish employed for the Study 2. Thereafter, infested fish were introduced in a recirculating system to synchronize the post-larval detachment by progressively increasing the water temperature up to 17ºC, and collecting the detached post-larva, also known as juveniles, from the tank outlet using sieves (Figure 1e). To reflect the temperature evolution throughout each trial, the water temperature was monitored

every three hours with a temperature data logger (UX100, Onset HOBO®), and the elapsed accumulated temperature was calculated as the sum of the average daily water temperature between the maximal and minimal values, expressed in degree-days.

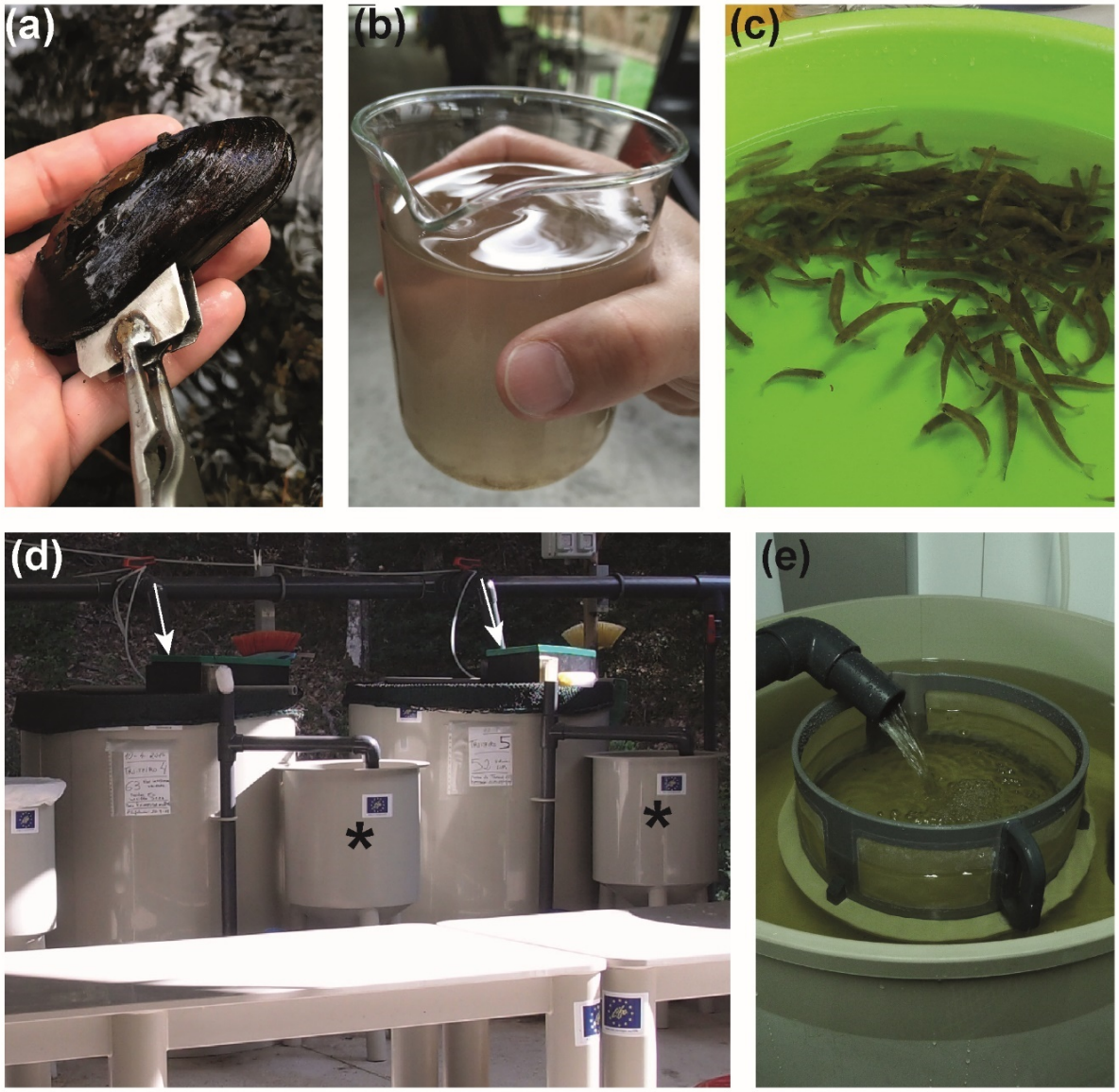


Figure 1. Some of the steps during the artificial infestation of fish within the Freshwater Pearl Mussel culture. (a) A wild adult mussel is being carefully inspected by opening its valves to a fixed gape up to 8 mm by modified retaining reverse pliers (or tongs) to evaluate the gravity status of the demibranchs. (b) After the broadcast, glochidia are suspended in a water solution to determine the glochidial concentration. The cloudy appearance of the water is due to the presence of hundreds of thousands of glochidia dissolved in this crystal flask. (c) Salmon are transferred to small and temporary water containers to expose them by bath immersion to a known concentration of glochidia for 30 min. (d) One section of the fish rearing facilities with two open system tanks, each with a water inlet (arrows), a water capacity of 500 L and a water outlet which opens into adjacent smaller tanks (asterisks) where the juvenile mussels could be collected. (e) Upper view of an adjacent tank where the water exists from the tank with fish and is filtered by a sieve to daily collect the juvenile mussels recently detached from the fish.

2. SAMPLING FOR LIGHT AND SCANNING ELECTRON MICROSCOPY

Based on the proportion of infested fish in the tank, 6–10 exposed and control fish (Figure 2a) were sampled randomly and periodically during the parasitism and even after the detachment of juveniles from the fish gills. This period comprised between day 0 and day 246 post-exposure (PE). Each fish was anesthetized with an overdose of Tricaine methane-sulfonate (MS-222, Sigma-Aldrich®) and a bicarbonate solution (1:2); and the euthanasia was confirmed by sectioning cranially the spinal cord. Immediately, the infested animals during the Study 2 were immersed individually in water in lateral recumbency, the operculum was abducted and the left holobranchs were stereomicrophotographed (Figure 2b). Holobranchs were excised and left gills were observed and photographed under the stereomicroscope and light microscope (Inset, Figure 2c). During early and late glochidiosis, two of the most critical stages, the standardized parasitic loads were expressed in "larvae/fish g" by counting the number of larvae in the left gills of infested fish, multiplying by two and diving by the fish weight. Additional squashes of small portions of the gill tissue, without the supporting arch, were visualized directly by light microscopy. Larvae morphologically oriented in a lateral view were photographed with a light microscope to measure the larval shell length in the antero-posterior axis, and to statistically evaluate the growth rate alongside the parasitism. Subsequently, complete necropsy of animals was performed, and right holobranchs and other organs (skin, thymus, digestive tract, heart, kidney and spleen, Figure 2d) were sampled and immediately fixed or cryopreserved, for histology and/or electron microscopy.

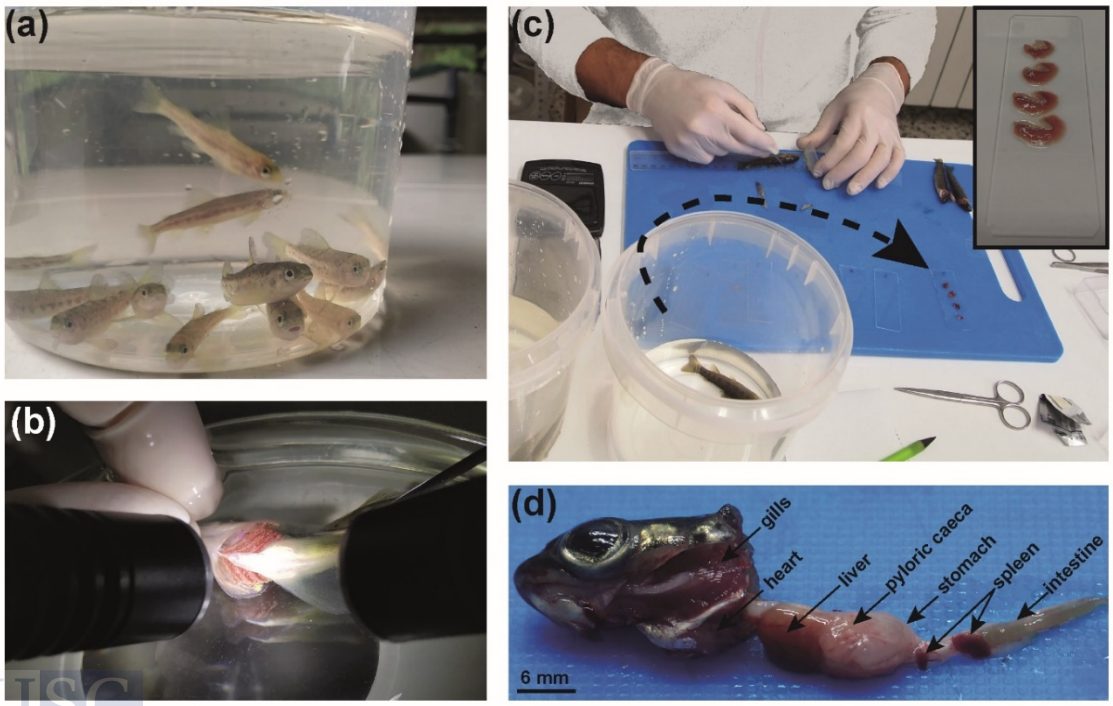


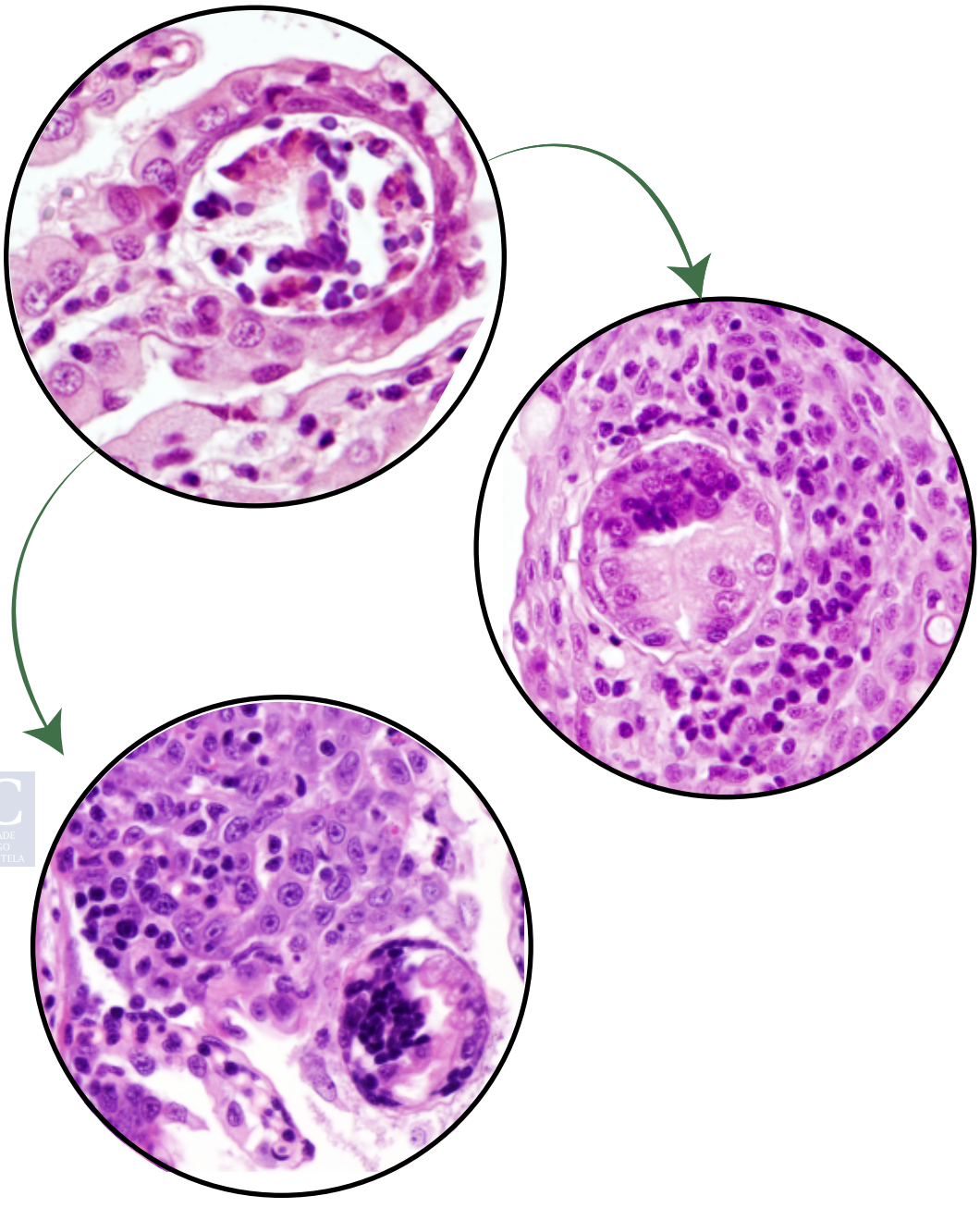
Figure 2. Sampling procedure of Atlantic salmon during the experimental trials. (a) Fry were periodically sampled from the rearing tanks. (b) Technique used to observe the left gills by stereomicroscopy with the fish immersed in water within a Petri dish and two good light sources. (c) Photography showing the sequential steps during the necropsy (arrow): 1. fish anesthesia and euthanasia, 2. measurement of the fish weight and length and 3. dissection of the gills first, and then the remaining organs. Inset: Detail of the four left holobranchs ready to observe by stereomicroscopy and light microscopy. (d) Methodology employed to dissect and collect altogether almost all organs and tissues sampled.

Once the detachment from the fish gills was initiated, viable and unviable post-larvae were photographed under the stereomicroscope to measure and statistically compare their length. Then the viable ones were sampled for the histological analysis.

Immediately, right holobranchs were fixed in Bouin´s, Dietrich´s and Davidson´s fluids to compare the effect of different fixatives on the encysted larvae and minimize the shrinkage artifacts due a potentially high osmolarity. After fixation for 18 h, right holobranchs were decalcified for 6 h in a 10% ethylenediaminetetraacetic acid solution (Osteodec, Bio-optica®) and processed for histopathology by routine methods. Additional fresh frozen gill tissue were sampled for cryosectioning, to avoid the dissolution of lipids during the routinary fixation and dehydration protocols. Sections were stained with hematoxylin and eosin (H&E) and other histological sections were additionally stained with additional histochemical techniques to highlight more specific structures (Horobin, 2019; Suvarna, Layton & Bancroft, 2019). On the other hand, the sampled juveniles were fixed in Davidson´s fluid for 3 h, rinsed in 10% ethanol, stained with a light solution of eosin, wrapped in a lens paper inside the cassettes and embedded by pippeting them, prior being dehydrated and processed as indicated for the fixed gills. Slides were observed and photographed using an BX51 light microscopy equipped with an EP50 digital camera and a polarized light (Olympus®). On the other hand, samples for scanning electron microscopy (SEM) were dehydrated in ethanol solutions and routinely processed for examination under an scanning electron microscope .

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Study 1: Early stages of glochidiosis



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STUDY 1: EARLY STAGES OF GLOCHIDIOSIS

Article I: Early stages of *Margaritifera margaritifera* glochidiosis in Atlantic salmon: Morphopathological characterization

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ABSTRACT

Freshwater mussels of the order Unionida encyst into the fish mucosa to metamorphose and complete their life cycle, causing a parasitic disease known as glochidiosis. This parasitic stage represents a bottleneck for the survival of naiads, particularly for critical endangered species as *Margaritifera margaritifera,* however, little is known about the events occurring during this critical stage. Therefore, this study aimed to histologically characterize the development of *M. margaritifera* glochidiosis in Atlantic salmon to get insight into the pathogenesis of this interaction. Fish exposed to glochidia were sampled during the first 44 days post-exposure and organs were observed by stereomicroscopy and light microscopy. Glochidia attached to the gills by pinching the lamellar epithelium, whereupon an acute proliferative branchitis engulfed most of the larvae. However, during the first 14 days, a severe detachment of unviable glochidia occurred, associated with the presence of pleomorphic inflammatory infiltrate and epithelial degeneration. In the cases where larvae remained attached, a chronification of the lesions with none to scarce inflammation was observed. These results provide key information to better understand the complex host-parasite interaction during the early stages of glochidiosis and provide valuable information to optimize artificial rearing of naiads in conservation of threatened freshwater mussel populations.

Keywords: gill histopathology, Freshwater Pearl Mussel, glochidium, parasite, *Salmo salar*.

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1. INTRODUCTION

Freshwater mussels from the order Unionida are bivalves which play important and pervasive functions in the riverine ecosystems due to their biofiltration capabilities and retention of matter and energy from the water to the benthos (Vaughn, 2018). They are commonly referred to as naiads and characterized by a compulsory parasitic stage where larvae need to temporarily attach to a suitable fish host. Thus, the larval stage—known as glochidium—clasps and encysts into the skin or gill mucosa, metamorphoses and detaches from the fish as a juvenile mussel, establishing itself within the river substrate (Karna & Millemann, 1978; Rogers-Lowery & Dimock, 2006; Scharsack, 1994).

However, this interaction has also been considered as a larval adaptation for nutrition and transport upstream in the fluvial ecosystems (Barnhart et al., 2008; Denic et al., 2015; Terui & Miyazaki, 2015); but on the other hand, it has been considered a parasitic disease, known as glochidiosis. Once the glochidia reach the gills, a parasitic cyst develops around the larvae, formed by a well-localized epithelial hyperplasia and lamellar fusions (Bruno et al., 1988; Howerth & Keller, 2006; Ieshko et al., 2016; Nezlin et al., 1994; Treasurer & Turnbull, 2000). Those tissue changes, in the case of intense gill parasitisms, may lead to the impairment of fish welfare (Thomas et al., 2013), related to behavioral changes (Filipsson et al., 2018; Horký et al., 2019), respiratory distress (Kaiser, 2005; Taeubert & Geist, 2013; Thomas et al., 2013) and growth reduction (Ooue et al., 2017); although a complete gill recovery would be expected following glochidia detachment (Treasurer & Turnbull, 2000). Nevertheless, in non-suitable hosts, a rejection of the parasite attributed to the host specifity occurs before concluding its metamorphosis. In these cases, a more complex morphopathological pattern is suggested since the formation of irregular aggregates of epithelial cells (Rogers-Lowery & Dimock, 2006; Scharsack, 1994) as well as marked proliferative and/or inflammatory gill responses were reported (Fustish & Millemann, 1978; Waller & Mitchell, 1989).

Margaritifera margaritifera (L., 1758) constitutes one of the most imperiled naiad species because of their small, fragmented and functionally extinct populations. Thus, it is listed as "*Critically Endangered*" in Europe (Lopes-Lima, Sousa, et al., 2017), and protected under the Habitats Directive. Therefore, different conservation strategies have been developed to ensure the survival of this valuable species and reinforce the populations, some of them relying on captive breeding programmes, where fish are used as hosts for the parasite stage. However, *M. margaritifera* possess an extremely delicate larval phase that feeds over 6 months on the two suitable hosts, Atlantic salmon (*Salmo salar* L.) and trout *(S. trutta* L.) juveniles (Denic et al., 2015). Those facts, along with the premature detachment of larvae during the first month of artificial culture, jeopardizes the success of conservation programmes. Nonetheless, there is a paucity of data regarding the most critical stage of the *M. margaritifera* life cycle and on the host factors that influence the larval survival (Clements et al., 2018; Marwaha et al., 2019; Wacker et al., 2019). In order to elucidate the underlying mechanism involved in the larval attachment and rejection, the purpose of this study is to histologically characterize the gill lesions in *S. salar* juveniles, together with larval development, during the first weeks of *M. margaritifera* glochidiosis. This knowledge may contribute to the design of future strategies to preserve both freshwater mussels and fish welfare during aquaculture activities.

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2. MATERIAL AND METHODS

2.1 Experimental infestation

Offspring of wild Atlantic salmon captured at the north-west of the Iberian Peninsula, were hatched in "Centro Ictiogénico de O Veral" (Lugo, Spain) in spring of 2015. Fish health status was confirmed by virological and histopathological studies prior to the beginning of the trial.

Mature glochidia were collected from 6 gravid mussels located in Eo basin, north-west of the Iberian Peninsula, in September 2015. In order to obtain a considerable amount of glochidia, adult mussels were stressed by variations of water temperature and dissolved oxygen. By light microscopy, larvae were observed to confirm the absence of ectoparasites and the density of viable forms were estimated using a Neubauer counting chamber.

An exposed group composed of 500 salmon fry of age class 0+ (mean weight of 3.1 g) were immersed for 30 min in a bath at 15ºC containing 1,000 glochidia/fish g corresponding to 30,000 glochidia/L, based on previous experiences (Meyers & Millemann, 1977; Taeubert & Geist, 2013). Air supply and permanent stirring of water was provided to ensure oxygen supply and glochidial resuspension. Additionally, a control group, composed of 500 salmon fries immersed in a bath without glochidia, was kept under identical conditions. Replicates of both groups were employed to evaluate fish mortalities.

After exposure, fish were kept in freshwater circular tanks of 0.5 m^3 volume maintaining a similar fish stocking density (0.7–1.1 fish/L) and exchange rate (9–12 renewals/day/100 fish). All animals were fed with a commercial dry pellet (AquaPro, Skretting®, Norway) at about 1% body weight, three times per week. Fish mortalities were checked daily. Water quality was monitored by measuring total ammonia, nitrite and nitrate with a multiparameter photometer (HI 83203, HANNA instruments®, US) five times per week and temperature was measured with a temperature data logger (UX100, Onset HOBO®, US) every 3 h (Supplementary table S1).

Supplementary table S1. Water quality mean values at different interval days, grouped to represent the periods between sampling times. Nitrogen compounds are total ammonia (N-NH3), nitrite (NO^{-2}) and nitrate (NO^{-3}) are expressed in mg/L; temperature is expressed in Celsius degrees (ºC). Sample mean and standard error are provided.

Interval day	N-NH^3	NO^{-2}	NO^{-3}	ºC
0–3	0.24 ± 0.05	0.01 ± 0.01	1.83 ± 0.47	11.9 ± 1.0
4–10	0.29 ± 0.08	0.01 ± 0.01	2.12 ± 2.27	12.8 ± 1.0
11–14	0.12 ± 0.04	0.02 ± 0.01	3.43 ± 1.63	12.7 ± 0.8
15–21	0.21 ± 0.05	0.02 ± 0.01	1.10 ± 0.98	11.5 ± 0.8
23–28	0.20 ± 0.16	0.03 ± 0.05	1.74 ± 2.31	11.7 ± 0.8
41–44	0.11 ± 0.11	0.04 ± 0.04	2.29 ± 3.12	12.6 ± 0.6

2.2 Sampling procedure

Ten control and 10 exposed fish were randomly sampled at 40 min, 2 hr, 6 hr, 24 hr, 3, 10, 14, 21, 28 and 44 days post-exposure (PE). Fish were anaesthetized and euthanized individually by overexposure in a solution composed of 200 mg/L of tricaine methanesulfonate (MS-222, Sigma-Aldrich®, USA) buffered with 400 mg/L of sodium bicarbonate. Euthanasia was confirmed by sectioning of the spinal cord. Weight (± 0.1 g), fork length (± 0.1 cm) and mean standardized weight (100×weight/$length^3$) were calculated and standard deviation expressed

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(±). Complete necropsy of animals was performed and right holobranchs and other organs (skin, thymus, digestive tract, heart, kidney and spleen) were immediately fixed in Bouin´s fixative for 18 h.

Left holobranchs were dissected out and fresh mounts were examined and photographed under the stereomicroscope and light microscope (Paling, 1968). At 40 min, 14, 28 and 44 days PE, the intensity of infestation was assessed according to Taeubert and Geist (2013). Briefly, the sum of glochidia on left holobranchs was counted and multiplied by two and divided by the fish weight to standardize the intensity of infestation. Additional photographs were obtained by microscopy to calculate the glochidial size, measuring the longest axis of glochidia (n = 50, ± 0.1 µm) with ImageJ software (Rueden, Schindelin, Hiner, DeZonia, Walter et al., 2017).

2.3 Histological procedure

After fixation, right holobranchs were decalcified for 6h in a 10% ethylenediaminetetraacetic acid solution (Osteodec, Bio-optica®, Italy). Decalcified holobranchs and remaining organs were processed for histopathology by routine methods. Sections (3 µm) from paraffin-embedded gill tissue were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), PAS-Alcian Blue (PAS-AB), Masson´s trichrome and Gram´s method, and slides were observed using an Olympus® BX51 light microscopy equipped with an Olympus® DP72 digital camera and CellÂ software (Matrix Optics®, Malaysia). Finally, to evaluate the tridimensional distribution of parasites in the branchial filament, transverse, frontal and sagittal sections of 40 min PE fish were evaluated.

All procedures were carried out at the facilities of "Centro Ictiogénico de O Veral" (Xunta de Galicia, Spain) and followed the Directive 2010/63/EU, on the protection of animals used for scientific purposes (Directive 2010/63/EU, 2010), national (Law 6/2013 and RD 53/2013, on the protection of animals used for scientific experiments) and institutional regulations (USC Review Board).

3. RESULTS

After 40 min PE, all exposed fish were highly infested with an average of 527 glochidia/fish g (Table 1), meaning that 53% of the initial doses of infecting glochidia had attached to the gills.

By stereomicroscopical observation of the holobranchs, a homogeneous distribution of the glochidia pinching the branchial lamellae could be observed throughout the different regions of all holobranchs (Figure 1a and b). On light microscopy, the different histological orientations of the holobranchs along with the identification of the cartilage rod and the interbranchial septum demonstrated that glochidia were clasped mostly at the outer area of the filaments (Figure 1c and d).

Table 1. Evolution of prevalence, intensity of infestation and glochidial size during four representative sample points. Sample mean and standard error are provided.

Days post-exposure	0	14	28	44
Prevalence (%)	100	60	30	10
Intensity (glochidia/fish g) †	527 ± 114	184 ± 145	88 ± 98	106
Glochidial size (µm)	71.1 ± 6.0	70.3 ± 4.6	92.4 ± 0.7	132.9 ± 24.7

† Calculated only using the infested fish.

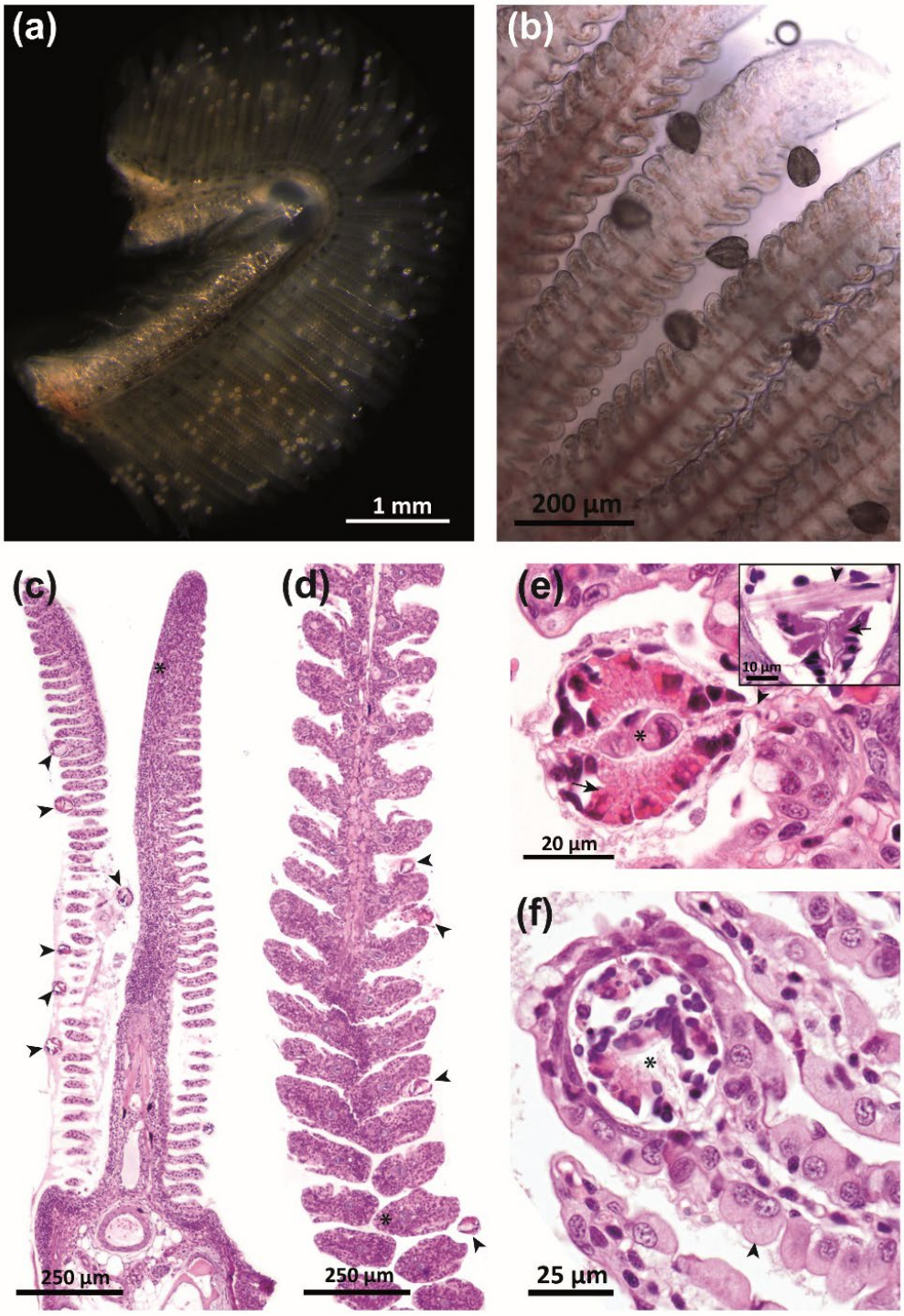


Figure 1. Distribution of recently attached glochidia throughout the gills (a–d) and the branchial response during the first 6 h post-exposure (PE) (e and f). (a) Stereomicroscopy of a fresh gill mount showing the random distribution of larvae through the different regions of the holobranch. (b) Stereomicroscopical detail showing the glochidia superficially attached on the gill lamellae by closing their valves. (c and d) Transversal (c) and frontal (d) histological sections of the holobranch showing the preferential distribution of glochidia (arrowheads) towards the outer area of the gill filaments. H&E stain. (e) Trapped lamellar gill tissue (asterisk) clasped between the valve clefts (arrowhead) and surrounded by mantle cells containing eosinophilic granules (arrows). H&E stain. Inset: detail of the larval adductor muscle (arrowhead) and the PAS positive granules of the mantle cells (arrow). PAS stain. (f) At 6 hr PE, a marked epithelial hypertrophy (arrowhead) covered completely the glochidia, with a few cell debris inside the mantle cavity (asterisk).

Glochidia were superficially attached to the gills due to the contraction of the adductor muscle, which clasped the branchial epithelium of lamellae inside the pallial cavity between both valve clefts (Figure 1e). The cavity was lined by mantle cells, characterized by a high number of eosinophilic and PAS positive granules in their cytoplasm (inset, Figure 1e). No other morphopathological evidence was observed either in gills or in other tissues at 40 min PE (Figure 1c–e).

At 2 h PE, the gill epithelial cells related to the parasite became slightly hypertrophic and hyperplastic, showing a pale cytoplasm, a large and oval nucleus with a small central nucleolus (Figure 1f). Mitotic figures were commonly observed at the interlamellar spaces close to the parasitized areas. Concurrently, multifocal fusions of adjacent lamellae surrounded partially the glochidia and progressed to completely circumscribe each larva at 6 h PE. During this period, the clasped gill tissue inside the pallial cavity disappeared almost completely (Figure 1f).

At 24 h PE, in addition to the hyperplastic tissue covering the parasites, epithelial cells also displayed focal necrotic/apoptotic changes, mainly located at the superficial layers (Figure 2a), and associated with a mild increase of intercellular spaces (Figure 2b). This spongiosis occasionally led to the formation of rare microvesicles of 5–20 μm width containing fluid and degenerating cells, characterized by a shrunken nucleus, and phagocytic figures (Figure 2b and c).

At 3 days PE, parasites became surrounded by an intense gill hyperplastic response which created nodular structures easily observed by stereomicroscopy (Figure 2d). Histologically, these nodules corresponded to severe and extended epithelial hyperplasia and lamellar fusions that entirely engulfed the glochidia, creating a parasitic cyst (Figure 2d). At the apical regions of the cysts, edema became more extensive and the superficial spongiotic microvesicles contained some degenerated epithelial cells (Figure 2e). Associated with the parasitized areas, diffuse and severe inflammatory infiltrates compounded by a moderate number of perivascular granulocytes and scarce mononuclear cells were often present at the center of the filaments (Figure 2e). In addition, the central venous sinus contained a high number of granulocytes (Figure 2f). Regarding the larva, at 3 days PE, they had lost the granular appearance of the mantle cells and the host tissue previously clasped inside the pallial cavity disappeared (Figure 2g and h). Moreover, the larval adductor muscle became atrophied, observed by the less extended cytoplasm of the myofibers (Figure 2g and h), which completely disappeared at the next stage.

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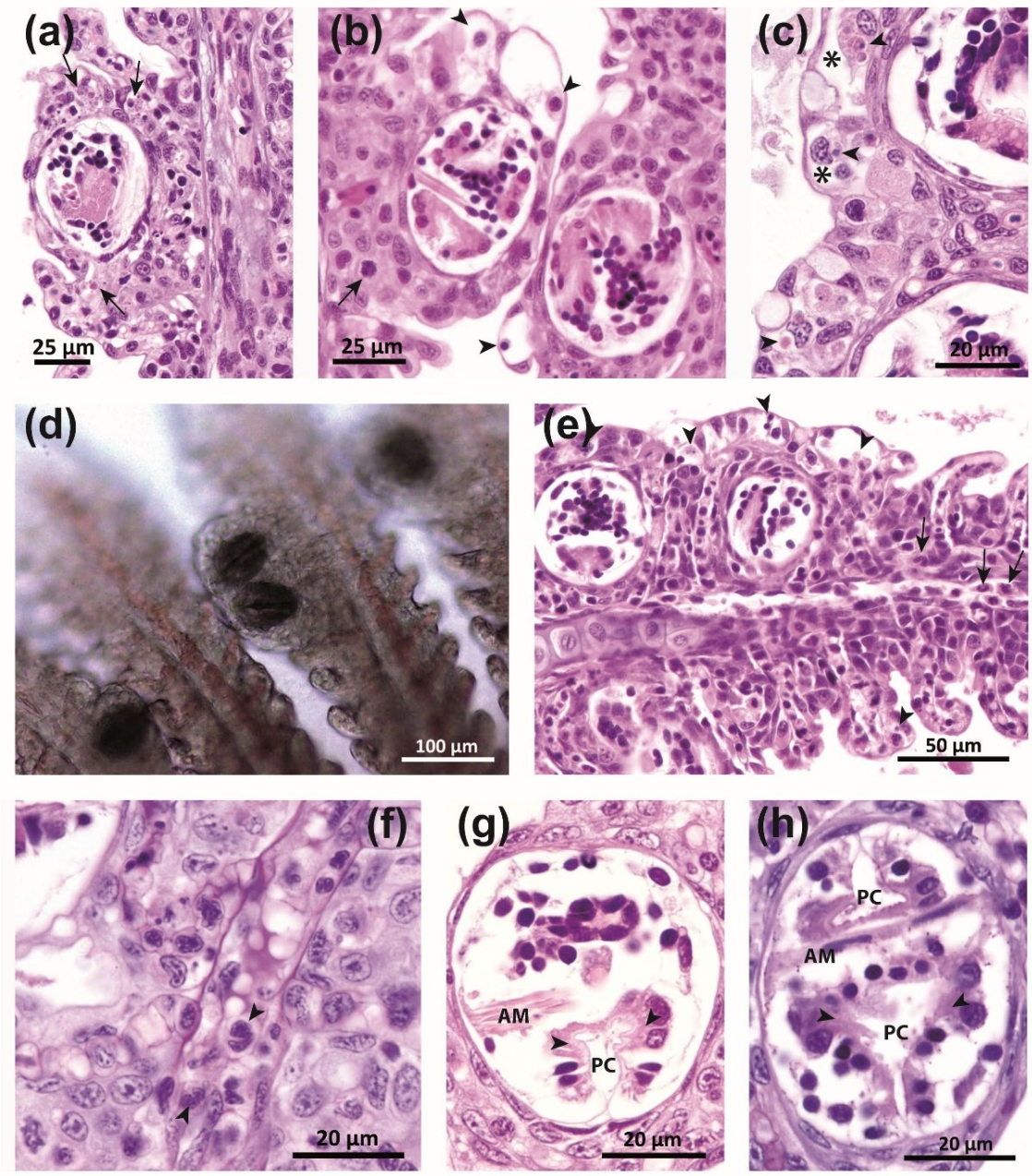


Figure 2. Images of gill response and glochidial development during the first 3 days post-exposure (PE). (a) Focal necrosis of the hyperplastic epithelium (arrows) associated with the encysted larvae at 24 hr PE. H&E stain. (b) Superficial epithelial layers surround glochidia displayed a moderate intercellular edema at 24 hr PE, characterized by buoyant intraepithelial micro-vesicles with degenerated cells inside (arrowheads). The presence of mitotic figures was frequently observed (arrow). H&E stain. (c) Detail of those apical areas of the parasitic cysts displaying spongiosis (asterisks) closely related to phagocyted figures (arrowheads). H&E stain. (d) Stereomicroscopical photograph showing a parasitic cyst at 3 days PE. (e) Extensive spongiosis at 3 days PE was characterized by superficial micro-vesicles containing fluid and cellular debris (arrowheads) and polymorphonuclear granulocytes infiltrating at deeper layers (arrows). H&E stain. (f) Detail of the central venous sinus delimited by endothelial basement membranes containing granulocytes (arrowheads). PAS stain. (g and h) At 3 days PE parasites showed an atrophied larval adductor muscle, mantle cells without granules (arrowheads) and an empty pallial cavity. H&E and PAS stain respectively. Abbreviations: AM, adductor muscle; PC, pallial cavity.

Unexpectedly, at 14 days PE, a dramatic decrease in the intensity of parasitism was observed (Table 1). The microscopic visualization of fresh gills at 10 and 14 days PE evidenced a detachment of glochidia and a "patchy appearance" of the gills due to the presence of some nodules of 100–150 μm size constituted by fused lamellae lacking glochidia (Figure 3a), in comparison with the parasitized ones (Figure 3b). On light microscopy, several stages could be established to better characterize this intense early expulsion of immature glochidia. On one hand, there were parasitic cysts where the gill proliferative lesions predominated with few if any evident degenerative changes. On the other hand, other cysts showed erosive lesions with loss of apical epithelial covering and sloughing glochidia, suggesting the mechanism of rejection (Figure 3c). In those cases, a pleomorphic inflammatory response, mainly compounded by granulocytes and lymphocytes, arose from the base of the lamella (Figure 3c). Epithelial cells surrounding the parasite displayed different degrees of degeneration, from hydropic degeneration to necrosis, accompanied by phagocytic figures (Figure 3c and d). Moreover, moderate spongiosis was observed by the increase of the intercellular spaces surrounding these degenerating cells (Figure 3d). Eventually, the gill fused areas with no glochidia showed protuberant and irregular borders with superficial or crescent-shape erosions, related to epithelial necrotic areas and lymphocytic infiltrates at deeper levels (Figure 3e).

Regarding the morphology of the parasites, the normal glochidia possessed a thin and PAS positive periostracum in intimate contact with the concentrically disposed hyperplastic gill tissue (Figure 3f). The extrapallial cavity was located underneath the periostracum and surrounding the cellular structures which were composed of undifferentiated cells located dorsally and mantle cells ventrally (Figure 3f). Mantle cells faced each other, creating a lumen that corresponded to the pallial cavity. The cells contained an elongated nucleus, marginated chromatin with one to few evident nucleoli and protruding cytoplasmic borders (Figure 3f). In addition, larvae with altered morphology were found at 10 and 14 days PE, associated with gill areas with marked epithelial degeneration (Figure 3g). In those cases, the periostracum appeared fragmented, the pallial and extrapallial cavities were collapsed and mantle cells displayed a more eosinophilic cytoplasm and loss of cytoplasmic borders (Figure 3g). Sporadically, a few of these distorted larvae also showed hydropic degeneration of their cellular structures being surrounded by severe epithelial necrosis of the host covering epithelium (Figure 3h).

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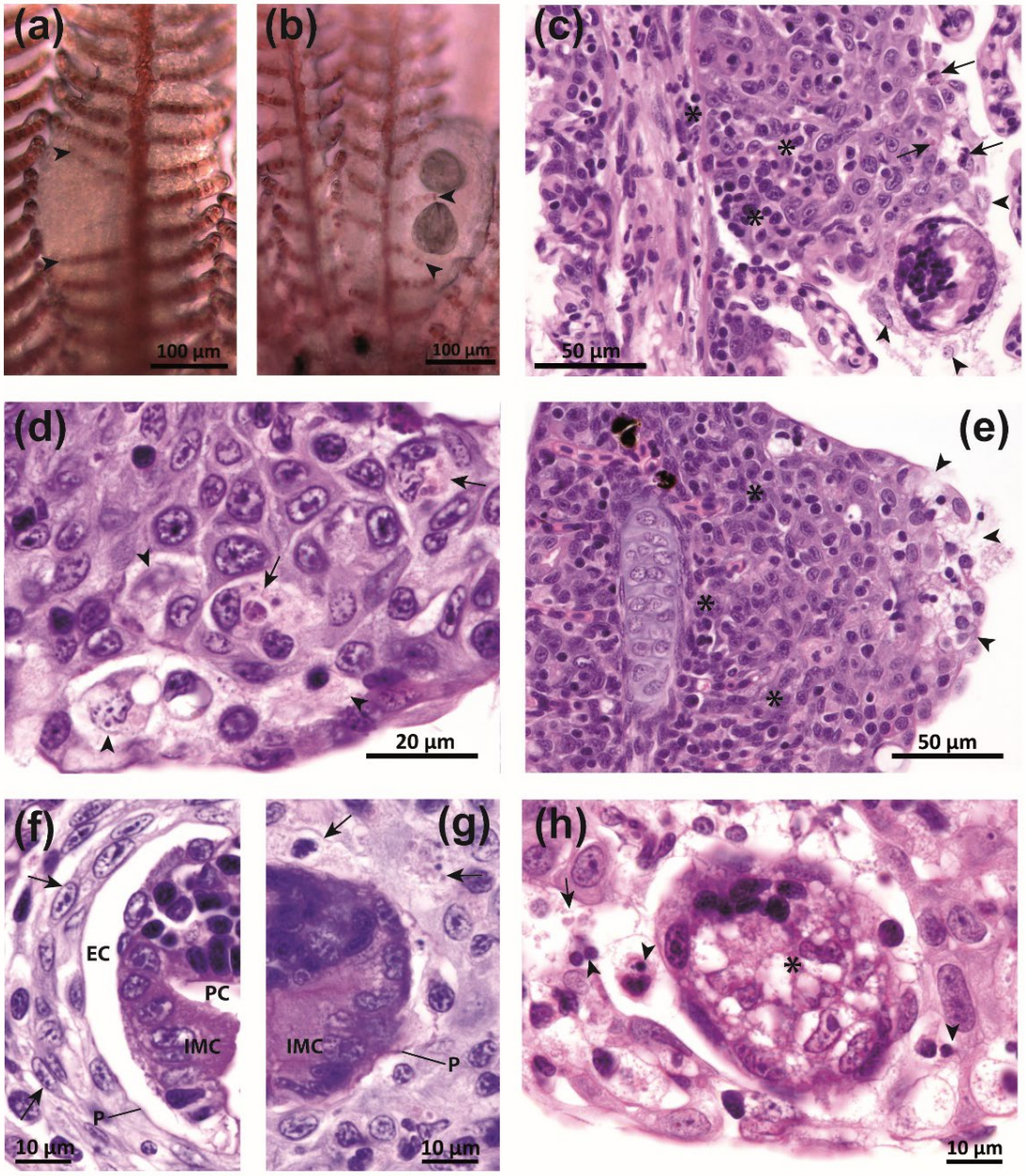


Figure 3. Images of the intense early expulsion of immature glochidia at 10 and 14 days post-exposure (PE). (a and b) Stereomicroscopy images showing gill nodules without any parasite (a) or with larvae completely embedded by fused lamellae (arrowheads) (b). (c) In situ detaching glochidia were related to epithelial erosions (arrowheads) and necrotic epithelial cells (arrows). Moreover, a pleomorphic inflammatory infiltrate was observed at deeper regions (asterisks). H&E stain. (d) High magnification of spongiotic areas displaying degenerated epithelial cells with vacuolized cytoplasm (arrowheads) and phagocytic figures with eosinophilic necrotic debris (arrows). H&E stain. (e) Proliferative nodular area without parasites highlighting superficial erosion with a high number of loosely attached and degenerated epithelial cells (arrowheads) and a lymphocytic infiltrate close to the core of the filament (asterisks). H&E stain. (f and g) Specular image of two histological transverse hemi-sections of a glochidium. PAS stain. The normal larval morphology surrounded by flat epithelial cells (arrows) (f) contrasts with the collapsed cavities and fragmented PAS positive periostracum of other larvae enclosed by degenerated epithelial cells (arrows) (g). (h) Degenerated glochidia with highly vacuolized cells (asterisk) embedded into a localized and severe epithelial necrosis characterized by pyknotic cells (arrowheads) and cellular debris (arrow). H&E stain. Abbreviations: EC, extrapallial cavity; IMC, inner mantle cells; P, periostracum; PC pallial cavity.

By visualization of fresh tissues at 21, 28 and 44 days PE, and as a consequence of the previous glochidial loss, only a few fish remained parasitized (prevalence of 10–30%) which harbored a moderate number of parasites (88–106 glochidia/fish g). Glochidia almost doubled their initial size, reaching 133 μm (Table 1); and they were covered by a thick epithelial covering and displayed a whitish appearance (Figure 4a). In relation to the distribution of the parasitic cysts over the gills, the previous multifocal distribution shifted towards a distribution in clusters of 5–20 individuals without any preferential gill area (Figure 4a).

On light microscopy, gill tissue response at 21, 28 and 44 days PE evolved towards more intense proliferative lesions and lamellar fusions around the glochidia, which compressed adjacent filaments (Figure 4b). Epithelial degenerative changes were mild to absent, and the inflammatory infiltrates shifted to a mononuclear population with predominance of lymphocytes, (Figure 4b and 4c). Moreover, large numbers of interlamellar cysts related to proliferative changes occurred at this time, giving the gills a cribriform appearance (Figure 4b). These cysts were roundish, with variable size (20–50 μm) and were either empty of content or contained cell debris and mucus, which was secreted by peripheral goblet cells (inset, Figure 4b).

The remaining larvae appeared roundish and turgid by stereomicroscopy. The larval development was confirmed histologically, where the digestive primordium was visible and the inner mantle cells acquired their characteristic mushroom shape: hypertrophied cells with basal nucleus and extended, vacuolated and protruding cytoplasm (Figure 4c) with acidic glycoproteins located in the apical part of the cytoplasm (Figure 4d). Moreover, from 21 days PE onwards, a novel non-cellular scleroproteinaceous structure was visualized at the distal region of mantle margin, joining both valve rims. This proteinaceous barrier, located at the median plane, is described here for the first time, filling completely the cleft between valves. At different orientations, it displayed a C-shaped morphology which was intensely stained light green with Masson´s trichrome stain and dark blue with PAS-AB (Figure 4c–f). Moreover, in some cases and particularly at 44 days PE, a moderate number of glochidia displayed a distorted and shrunken morphology, characterized by a wrinkled periostracum, displacement of inner structures, loss of their cavities and invagination of host surrounding tissue, considered as a potential shrinkage artifact (Figure 4g).

Processes of gill recovery occurred through the experiment, as the branchial nodules lacking glochidia were progressively smaller and less abundant from 21 days PE onwards (Figure 4h). On light microscopy these areas were linked to a reduction of the amount of hyperplasic epithelium, lamellar fusions and interlamellar cysts. Therefore, at 28 and 44 days PE, lesions were scarce with a predominance of lamellar synechia and slight epithelial hyperplasia (Figure 4i).

Throughout the assay 3 individual fish died in each group exposed to glochidia (accumulated mortality of 0.06%) and none in the control group.

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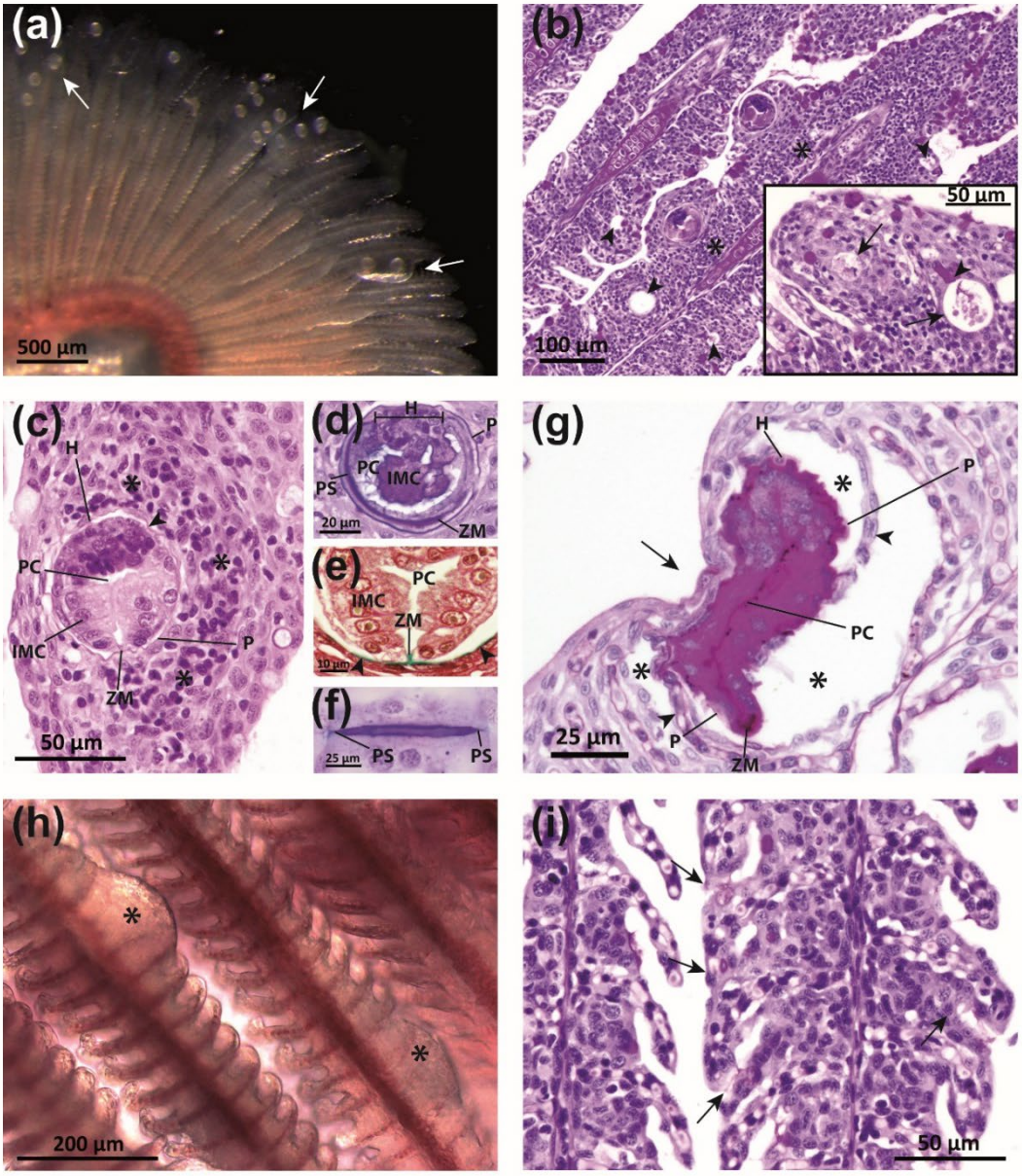


Figure 4. Images of parasitized gills (a–g) in comparison to others that lost all the glochidia (h and i) at 21, 28 and 44 days post-exposure (PE). (a) Multifocal distribution of parasites grouped in clusters (arrows) photographed by stereomicroscopy at 21 days PE. (b) Clusters of parasitic cysts infiltrated by lymphocytes (asterisks) and associated with interlamellar cysts located in adjacent areas (arrowheads). PAS stain. Inset: interlamellar cysts (arrows) containing cell debris and PAS positive mucus secreted by goblet cells (arrowhead). PAS stain. (c) Moderate intraepithelial lymphocytic infiltrate (asterisks) surrounded a transversely sectioned glochidium with normal morphology. H&E stain. (d) Sagittal section emphasizing acidic glycoproteins located in the apical part of the inner mantle cells and the C-shaped zip membrane all over the valve rim. PAS-AB stain. (e) Detail of the ventral region of the larvae in a transverse section showing the zip membrane, filling completely the valve cleft, and the periostracum (arrowheads) stained intensely light green. Masson´s trichrome stain. (f) Coronal section of the ventral region of a larva showing the zip membrane. PAS-AB stain. (g) Shrunken glochidia where parasitic structures and surrounding host tissue were displaced (arrow), creating artefactual-like cavities (asterisks). Note that epithelial cells from adjacent lamellae are detached (arrowheads). (h) At 44 days PE, mild nodules without any parasitic structure were associated to lamellar fusions (asterisks). (i) Scarce mild hyperplastic areas and lamellar synechia (arrows). PAS stain. Abbreviations: H, hinge; IMC, inner mantle cells; P, periostracum; PC, pallial cavity; ZM, zip membrane.

4. DISCUSSION

A thorough morphopathological description of larval development and fish gill lesions during the first weeks of *M. margaritifera* glochidiosis was performed in order to shed light on this critical stage of the life cycle of this endangered freshwater mussel.

The success rate of the infestation procedure was 53%, similar to other experiences which obtained glochidial uptake rates up to 43% (Taeubert & Geist, 2013), although multiple factors may influence this rate, such as the health status of the larvae, its developmental stage and the concentration of larvae in the bath (Lynn, Doerder, Gillis & Prosser, 2018; Meyers & Millemann, 1977; Scheder et al., 2011), or the respiratory rate of fish (Paling, 1968). Regarding the distribution of larvae observed by stereomicroscopy, no preferences were detected for any of the holobranchs as described in other *M. margaritifera* infestations (Clements et al., 2018; Treasurer & Turnbull, 2000; Young & Williams, 1984b).

Using light microscopy, the different orientation of holobranchs in the paraffin blocks allows to observe for the first time the tridimensional distribution of the parasite over the gill tissue, where most of the glochidia were anchored to the outer area of the filaments, as already suggested by previous studies (Blazek & Gelnar, 2006; Wootten, 1974). This distribution may be explained by the unidirectional water flow, thus, each hemibranch functions as a sieve (Hughes, 1984) where the outer area first faces the water current containing the glochidia, so most of the larvae would attach to this area. In addition, the tridimensional evaluation through the different histological orientations was also useful to accurately identify anatomical references (Dalum et al., 2015), contributing to a better understanding of this host-parasite interaction.

Once the glochidia attach to the gill epithelium by closing their valves, the clasped gill epithelial cells rapidly disappear. This fact has been attributed to anoxic necrosis by compression of the lamellar circulation (Waller & Mitchell, 1989), followed by an enzymatic disintegration by the granule content of the mantle cells (Arey, 1932c; Scharsack, 1994).

During the first 12 h epithelial cells adjoined to the parasites underwent hypertrophy. This cellular adaptation, not described previously for glochidial parasitism, was referred as "rounded, swollen and migrating epithelial cells" (Nezlin et al., 1994; Reis et al., 2014; Waller & Mitchell, 1989). In addition to hypertrophy, epithelial hyperplasia, synechia and subsequent lamellar fusion occurred, leading to the formation of the parasitic cyst (Howerth & Keller, 2006). These epithelial responses constitute a common gill response against several gill injuries (Ferguson & Speare, 2006), and some parasites have taken advantage of it by embedding and growing in the hyperplastic epithelium (Ventura & Paperna, 1985).

From 24 h PE and during the first two weeks, an acute proliferative branchitis was associated with the parasites, with the presence of granulocytic infiltrates jointly with localized epithelial spongiosis and degeneration. Similar branchitis are widely described in fish aquaculture and it is considered an unspecific response to a wide range of parasites such as metazoans (González-Lanza, Alvarez-Pellitero & Sitjá-Bobadilla, 1991), amoeba (Adams & Nowak, 2001; Daoust & Ferguson, 1985; Roubal, Lester & Foster, 1989), protozoa (Goldes, Ferguson, Daoust & Moccia, 1986; Speare, Brackett & Ferguson, 1989; Ventura & Paperna, 1985) and microsporidia (Matthews, Richards, Shinn & Cox, 2013). However, inflammatory

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infiltrates associated with freshwater mussel infestations are seldom described and, up to our knowledge, only Waller and Mitchell (1989) described an intense necrosis and inflammation associated to *Lampsilis radiata* glochidial infestation.

Such gill inflammatory and degenerative responses may lead to the tissue disruption and superficial epithelial erosions observed in the outer surface of parasitic cysts. The consequent early larval detachment matches a dramatic reduction of the glochidial load observed from the first 14 days PE, similar to that described by Jung et al. (2013) during the first month PE in the suitable host *S. trutta*. These kind of lesions were also reported around the glochidia in non-suitable hosts, in this case showing incomplete capsules or irregular aggregates of epithelial cells (Rogers-Lowery & Dimock, 2006; Waller & Mitchell, 1989). Nevertheless, a slightly different pathogenesis should be considered in non-suitable fish species, as rejection occurs as soon as in the first 2 days and deeper erosions are observed after the glochidial leakage (Reis et al., 2014; Scharsack, 1994).

Many larvae, including those in sloughing processes, displayed an altered morphology at 14 days PE, thus, a potential active participation of host immune response may be inferred during the glochidial rejection in some fish. The permanent valve closure suggests that putative antigens located on the surface of the periostracum might elicit an acute branchitis, as similar avoidance responses were reported against membrane surface antigens of ciliated parasites (Dickerson & Findly, 2014). However, the exposition of the internal structures by damage of the periostracum together with the epithelial necrosis may exacerbate regional responses, as occurs after degradation of other parasitic covers (Lovy, Wright & Speare, 2007; Wouda, Snoep & Dubey, 2006).

Regarding the gill response around persistent glochidia, a chronification of the branchitis was reflected by the shift of the inflammatory infiltrates from a pleomorphic to a lymphocytic component. It is known that T-lymphocyte responses play a key role against other tissue-dwelling parasites, leading to the expulsion or to the chronification of the parasitism (Wang, Johansson, Abós, Holt, Tafalla et al., 2016; Zaph, Cooper & Harris, 2014).The involvement of specific immunoglobulins (IgM) has been reported during parasite expulsion in other glochidiosis (Dodd, Barnhart, Rogers-Lowery, Fobian & Dimock, 2006; Rogers-Lowery, Dimock & Kuhn, 2007), although there is a paucity of data regarding *M. margaritifera*. Therefore, further studies are required to characterize the lymphocytic population and the role of potential specific immunoglobulins involved in the response against *M. margaritifera,* as well as the characterization of other immune cells (Waller & Mitchell, 1989).

After glochidial expulsion, the gill healing process at the site of detachment was complete by 44 days PE, with a normal lamellar appearance in these areas, as has been suggested in previous studies in this species (Treasurer & Turnbull, 2000). The fact that glochidiosis did not cause deep lesions and the basement membrane underneath the epithelium was preserved, might be a key feature to facilitate gill healing (Daoust & Ferguson, 1986). Regarding the disapearearance of the hyperplastic lesions and return to the normal gill architecture, since apoptosis was not a prominent feature in this study, other remodelation mechanisms should be considered such as the reduction of mitotic rate or the detachment of interlamellar cell mass (Nilsson, 2007).

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Despite no clinical signs in infested fish being observed in this study and the connection between gill lesions and pathophysiological effects remaining poorly understood (Ferguson & Speare, 2006), our results would suggest some negative consequences on fish performance. The proliferative lesions and edema observed in this study increase the diffusion distance and reduce the surface area of the water-blood barrier, compromising the gas exchange (Hvas, Karlsbakk, Mæhle, Wright & Oppedal, 2017). In addition, the epithelial degeneration and erosion related to the parasite detachment could increase the epithelial permeability and consequently it might enable secondary infections or osmoregulatory disbalances, as described in other gill diseases (Hvas et al., 2017; Powell, Carson & van Gelderen, 2004). However, as a result of the early larval rejection, the parasitism was localized in clusters and below 300 glochidia/fish g, following the recommendations for artificial breeding of this naiad (Taeubert & Geist, 2017). Thus, the impaired respiratory function might be balanced and alleviated due to redistribution of blood flow to non-affected gill regions (Booth, 1979) or other compensatory mechanisms (Filipsson et al., 2017; Powell, Nowak & Adams, 2002).

Regarding the glochidial development, the parasitic cysts grew from the 14 to 44 days PE similar to previous studies (Nezlin et al., 1994; Young et al., 1987; Young & Williams, 1984b). The lack of growth of the glochidia during the first two weeks may be attributed to the employed methodology where the longest axis is measured due to the loss of the typical axe-shape (Barnhart et al., 2008).

During the morphogenesis of the mantle margin, mantle cells develop as described by Scharsack (1994). Moreover, a novel structure, located at the mantle margin, delimited the pallial cavity by joining both valve rims. To the best of our knowledge, only Treasurer and Turnbull (2000) mentioned the presence of a "*hyaline membrane*" related to the periostracum but no further descriptions were mentioned about this structure . Its proximity to the periostracum-forming zone and its similar staining properties suggests that the secretory activity at the mantle margin might be involved also in the accumulation of such material at the distal region of the mantle margin. This structure, different from other mucoid structures (Chumnanpuen et al., 2011), could be regarded rather a different adaptation of the mantle margin of molluscans (Audino, Marian, Wanninger & Lopes, 2015) and we propose to term this proteinaceous structure as "*zip membrane*" based on its anatomic features. However, further studies are needed to characterize its composition and function, which could give insight to the exchange mechanisms of metabolic substances through the pallial cavity during this long-lasting larval parasitic stage (Arey, 1932c; Denic et al., 2015).

From 21 days onwards, special care should be taken during the histopathological evaluation of parasitized gills due to the presence of interlamellar cysts, this being the first description of this lesion in glochidiosis, although Treasurer and Turnbull (2000) observed similar "s*paces*" in late stages of *M. margaritifera* glochidiosis. Those interlamellar cysts observed by histology should not be confused with parasitic cysts, since they do not contain parasitic forms, and may constitute a chronic response due to the combination of epithelial hyperplasia and exfoliation failure, trapping cellular debris and secreted mucus inside the cyst (Ferguson & Speare, 2006). Concurrently, some larvae suffered a shrinkage histological artifact characterized by displaced larval organs and invagination of surrounding structures, already reported by Araujo et al.

(2002) and Pekkarinen and Valovirta (1997). This artifact may occur during fixation in well-developed larvae due to the high osmolarity of the fixative and the low diffusion coefficient of the periostracum, similar to other hard tissues, such as cartilage, eye and certain fish tissues (Margo & Lee, 1995; Nassar, Luke, Luke, Kamal, Soliman et al., 2015; Speilberg, Evensen, Bratberg & Skjerve, 1993). Therefore, an accurate evaluation of the integrity of parasite periostracum and internal structures is essential to distinguish this artifact from non-viable glochidia with altered morphology (Ieshko et al., 2016; Treasurer & Turnbull, 2000).

The metamorphosis of the larval internal organs at 44 days PE was uncomplete (Scharsack, 1994) suggesting a long and slow organogenesis, which contrasts with the much shorter and quicker development of other margaritiferids (Araujo et al., 2002; Karna & Millemann, 1978). This difference might be influenced by the initial size of glochidia (Bauer, 1994) and the seasonal dynamics of water temperature which defines mostly the development rate of *M. margaritifera* larvae (Hruska, 1992; Murzina et al., 2017).

In conclusion, the morphopathological characterization of both fish lesions and larval development performed in this study provides a better comprehension of the pathogenesis of glochidiosis. In particular, fish displayed a transient proliferative branchitis leading to the early glochidial expulsion together with epithelial degeneration. However, in some cases, chronification of gill lesions with none to scarce inflammation, leads to the persistence of the glochidia. Future research on the immune mechanisms involved in the glochidial expulsion, persistence and gill healing is needed, which would be essential for *in situ* and *ex situ* conservation of threatened mussel species.

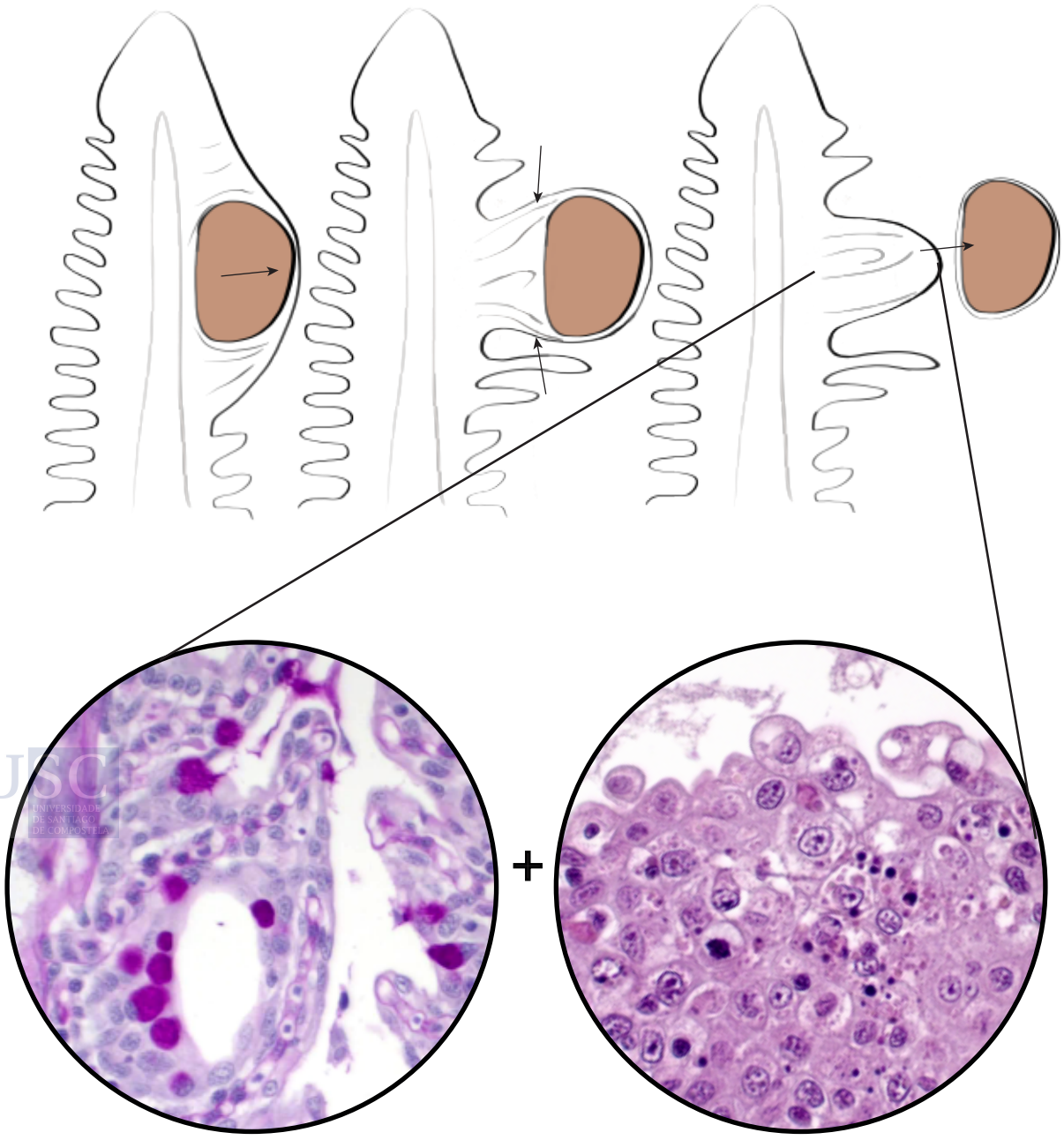
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Study 2: Late stages of glochidiosis



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STUDY 2: LATE STAGES OF GLOCHIDIOSIS

Article II: Morphopathology and gill recovery of Atlantic salmon during the parasitic detachment of *Margaritifera margaritifera*

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ABSTRACT

During the conservation aquaculture of the freshwater mussel *Margaritifera margaritifera*, fish health has become a concern due to the need of mussel larvae (glochidia) to parasitize the salmonid gills and metamorphose into juveniles. However, there is lack of information about the impact on fish during the juvenile detachment and the subsequent gill healing. To evaluate the morphopathological changes and gill recovery after the parasitism of *M. margaritifera*, 51 Atlantic salmon fry (*Salmo salar*), infested with around 22 larvae/fish g, were necropsied during the synchronized detachment of the mussel juveniles, and gills were assessed by stereomicroscopy, and by light and scanning electron microscopy. Salmon showed no clinical signs during the trial and gills recovered their normal morphology almost completely in a short time, suggesting a minimal impact on fish health after glochidiosis. In this sense, the non-erosive droplet detachment and the goblet cell hyperplasia favored an effective gill remodeling mediated by apoptosis, polarization and cell shedding of the gill epithelia, providing insights to the defense, clearing and healing mechanisms of the gill. These morphopathological techniques could also be implemented to preserve fish welfare and to optimize the artificial breeding programmes of endangered freshwater mussels.

Keywords: gill healing, Freshwater Pearl Mussel, fish pathology, parasite detachment, *Salmo salar*, glochidiosis

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1. INTRODUCTION

Freshwater mussels are bivalves with extraordinary capacities of biofiltration and burrowing, thus, they provide significant ecosystem services and also indirectly protect many other species (Boeker et al., 2016; Vaughn, 2018). Despite its importance, certain naiads such as *Margaritifera margaritifera* (L., 1758) are categorized in the European Red List of the International Union for Conservation of Nature as *Critically Endangered* due to the serious decline of the populations (Cuttelod, Seddon & Neubert, 2011), and they have become target species of freshwater biodiversity conservation strategies (Geist, 2011). To protect the most affected populations, which lack natural recruitment (Lois et al., 2014), one emergency conservation strategy is to implement artificial breeding programmes and a European standardized monitoring protocol (Boon, Cooksley, Geist, Killeen, Moorkens et al., 2019; Gum et al., 2011).

The culturing techniques of freshwater mussels depend on the compulsory larval parasitic stage on Atlantic salmon (*Salmo salar* L.) and brown trout (*Salmo trutta* L.) fry, regarded as a mechanism of dispersal, nutrition and protection (Barnhart et al., 2008; Denic et al., 2015; Taeubert & Geist, 2017). In particular, the larva of *M. margaritifera* clamps and encysts into the salmonid gills for several months until it detaches in spring—once temperature rises over 15 ºC—as a free-living juvenile (Hruska, 1992; Taeubert et al., 2013). Accordingly, fish suffer a multifocal proliferative branchitis, which leads to a disease status known as glochidiosis, given by the name of the infesting larvae, the glochidium (Karna & Millemann, 1978).

This host-parasite interaction supposes a bottleneck for freshwater mussel aquaculture and a concern towards the welfare of the host fish. In an attempt to optimize the culturing efforts several studies had established certain recommended glochidial loads (Taeubert & Geist, 2013), based on the impact on fish survival, growth, respiration, metabolism, swimming capacity and fish behaviour (Cunjak & McGladdery, 1991; Chowdhury et al., 2019; Filipsson et al., 2017; Österling et al., 2014b; Preston et al., 2007; Thomas et al., 2013). However, there is a lack of data about the pathogenesis of the disease, in which the morphopathological evaluation of the gill lesions remain overlooked, mostly focused on the premature rejection of unviable larvae, which is associated with an erosive branchitis during early stages of glochidiosis (Castrillo, Varela-Dopico, Ondina, Quiroga & Bermúdez, 2020). This contrasts with the extensive knowledge of other gill ectoparasites which compromise the fish welfare in aquaculture, for example *Neoparamoeba perurans* and *Ichthyophthirius multifiliis* (Powell, Leef, Roberts & Jones, 2008; Tumbol, Powell & Nowak, 2001). Lastly, gills are able to recover after removal of the inciting cause as chemicals or infectious agents (Daoust & Ferguson, 1986; Ferreira Sales et al., 2017; Kudo & Kimura, 1983; Speare et al., 1999); nevertheless, very few studies refer to the recovery of lesions after glochidiosis (Kaiser, 2005; Karna & Millemann, 1978; Scharsack, 1994; Treasurer & Turnbull, 2000).

Employing this artificial glochidiosis as a model of parasitic gill disease and recovery in Atlantic salmon, the purpose of this study was to perform a comprehensive morphopathological evaluation during the late stages of glochidiosis including the juvenile detachment and the subsequent gill healing process. Hence, this study may also provide insights to understand this

complex host-parasite interaction and to preserve fish welfare during the rearing of these important and endangered freshwater mussels.

2. MATERIAL AND METHODS

2.1 Experimental gill infestation and selection of infested fish

Experimental fish came from an artificial glochidiosis trial performed in September 2015 (Castrillo et al., 2020). Briefly, 1000 salmon fry (mean weight of 3.1 g) were exposed by bath immersion to *M. margaritifera* glochidia (1,000 glochidia /fish gram) collected from gravid wild mussels. A group of non-exposed fish from the same batch served as control group. At day 14 post-exposure (PE) an early detachment of inviable glochidia occurred and successfully infected fish could be only identified by means of light microscopy, due to the larval size at this stage (approximate diameter of 70 μm).

After this early rejection, the remaining salmon were reared for six more months to allow the development of the encysted larvae and became macroscopically visible on the gill (approximate diameter of 350 μm). Thus, after 202 days PE, each exposed fish could be *in vivo* diagnosed as infested or non-infested by manual immobilization, abduction of the opercula and gill visualization (Salonen & Taskinen, 2017). This diagnostic procedure was performed in less than four seconds. As a result, 51 out of 600 exposed fish were selected as infested (prevalence of 8.5%). The same exploratory procedure was performed on 51 non-exposed fish. Moreover, to ensure an optimal fish health status and confirm the absence of any other pathological processes prior to the *in vivo* procedure, five exposed and non-exposed fish were necropsied and processed for histopathology.

2.2 Synchronization of juvenile detachment

Infested and control fish (n = 51, mean weight 5.8 g and length 8.1 cm) were relocated into a recirculating system to synchronize the detachment of *M. margaritifera* juvenile mussels by exposing fish to high water temperatures for several weeks (Hruska, 1992). Thereby, the temperature was heated daily 1º up to 17ºC, and thereafter maintained constant until the end of the experiment (± 0.2; Supplementary table S1) by employing a temperature control system (Aquarium Controller Evolution, Aquatronica®) and a thermostat (Ako®). Moreover, water was pretreated by mechanical filtration and ultraviolet irradiation, and the exchange rate was set at 2,000 L/h with a degree of recirculation of 98% (± 0.2). Water quality was periodically monitored in each tank by measuring ammonia, nitrite, and nitrate with a photometer (HI83203, HANNA instruments®), and pH and dissolved oxygen by a pH/dissolved oxygen meter (PD 650 meter, Oakton®) (Supplementary table S1). Both batches were fed with a commercial dry pellet (AquaPro, Skretting®) at about 0.5% body weight to minimize the input of ammonia into the system and the fish mortalities were checked daily.

The detached mussel juveniles were collected daily from the tank outlet by sieves with a mesh size of 150 μm. Later on, they were pipetted, counted under the stereomicroscope, and classified as viable or unviable for culturing based on the presence or absence of pedal and valve movements, respectively. Additionally, viable juveniles were photographed with a Leica® M125 stereomicroscope and a M170HD digital camera. Since the number of

experimental fish was progressively reduced due to the sampling procedure, each daily mussel juvenile count was standardized by the number of remaining fish left in the tank in juveniles/fish.

Supplementary table S1. Physico-chemical parameters of water in the control and infested fish groups between day 202 and 246 post-exposure (PE) after the glochidial infestation. Nitrogen compounds are expressed in mg/L and temperature (± 0.2) is expressed in Celsius degrees (ºC).

		Control group						Infested group					
Days PE	ºC	NH_3 & NH_4^+	NH_3	NO_2^-	NO_3^-	pH	DO (%)	NH_3 & NH_4^+	NH_3	NO_2^-	NO_3^-	pH	DO (%)
202	11	0.14	0.0002	0.03	2.8	6.8	90	0.11	0.0001	0.04	1.2	6.8	86
204	13	0.15	0.0002	0.04	4.6	6.7	87	0.11	0.0003	0.02	5.3	7.0	90
208	17	0.16	0.0003	0.04	3.0	6.8	85	0.15	0.0004	0.04	4.1	6.9	84
212	17	0.08	0.0002	0.04	1.3	6.9	88	0.14	0.0005	0.05	1.2	7.0	91
219	17	0.09	0.0002	0.03	4.3	6.9	93	0.07	0.0002	0.05	2.3	7.0	93
221	17	0.14	0.0005	0.04	1.6	7.0	91	0.09	0.0004	0.04	0.4	7.1	86
225	17	0.18	0.0005	0.03	2.0	6.9	91	0.13	0.0004	0.05	3.1	7.0	90
232	17	0.20	0.0007	0.06	1.6	7.0	92	0.04	0.0001	0.05	3.8	6.9	88
239	17	0.15	0.0003	0.11	5.4	6.8	87	0.05	0.0001	0.03	3.5	6.9	87
246	17	0.10	0.0003	0.06	3.8	6.9	90	0.13	0.0003	0.04	3.3	6.8	91

2.3 Sampling procedure: determination of the opercular rate and necropsy

Eight infested and control fish were randomly sampled at day 203, 221, 225, 232 and 239 PE, focusing on the juvenile detachment. At day 246 PE, the remaining eleven fish of each group were sampled to conclude the study. After hand-netting, fish were individually held into individual transparent buckets with 1 L of water, and the opercular movements were measured for 20 s twice (3 and 15 min after net capture) to calculate the mean opercular rate of each fish, expressed in opercular beats/min (OBM).

Afterward, salmon were individually anesthetized and euthanized by overexposure to a solution of 200 mg/L of tricaine methanesulfonate (MS-222, Sigma-Aldrich®) buffered with 400 mg/L of sodium bicarbonate. Euthanasia was confirmed by sectioning the spinal cord. Weight (± 0.1 g), fork length (± 0.1 cm) and Fulton´s condition factor (100 × weight [g]/length [cm] ^{3}) were calculated and standard deviation expressed (±). Immediately, the whole animal was immersed in water in lateral recumbency, the operculum was abducted and the left holobranchs were stereomicrophotographed employing the previous equipment.

Subsequently, complete necropsy of animals was performed, and right holobranchs and other organs (skin, thymus, digestive tract, heart, kidney and spleen) were sampled and immediately fixed in Bouin's fixative for 18 hr. For scanning electron microscopy (SEM), a small portion of gill tissue was fixed first in 2.5% glutaraldehyde with 0.1 M cacodylate buffer (pH 7.3) and then in 1% osmium tetroxide. Left holobranchs were dissected out and examined to estimate the larval load of infested fish, standardized by fish weight (larvae/fish g) according to Marwaha et al. (2019). Lateral stereomicrophotographs of each left holobranch were obtained using the previous equipment to evaluate the larval distribution in the gill.

All procedures were carried out at the facilities of “Centro Ictiogénico de O Veral” (Xunta de Galicia) and followed the Directive 2010/63/EU, on the protection of animals used for scientific purposes (Directive 2010/63/EU, 2010), national (Law 6/2013 and RD 53/2013, on

the protection of animals used for scientific experiments) and institutional regulations (USC Review Board).

2.4 Light and scanning electron microscopy (SEM)

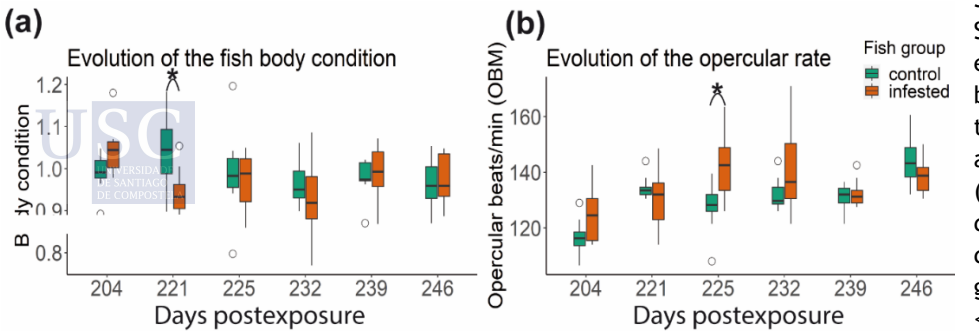
After Bouin´s fixation, right holobranchs were decalcified for 6 h in a 10% ethylenediaminetetraacetic acid solution (Osteodec, Bio-optica®). Decalcified holobranchs and remaining organs were processed for histopathology by routine methods and sections (3 µm) from paraffin-embedded tissue were stained with hematoxylin and eosin (H&E). Gill tissue was additionally stained with periodic acid-Schiff (PAS) and Masson-Goldner trichrome. Slides were observed and photographed using an Olympus® BX51 light microscope equipped with an DP72 or EP50 digital camera. On the other hand, samples for SEM were dehydrated in ethanol solutions and routinely processed for examination under a JEOL JSM-6360LV scanning electron microscope.

2.5 Statistical analysis

The quantitative variables, body condition and opercular rate, were statistically compared between groups employing the Wilcoxon-Mann-Whitney U test. The strength of the relationships between body condition and opercular rate with the parasitic loads were calculated during the first three samplings (day 203, 221, 225 PE) employing the Spearman´s Correlation Coefficient. The significance level was 95% in all cases (p-value < .05). All the numeric data were analyzed by RStudio software (R Core Team, 2019).

3. RESULTS

During the late glochidiosis, no mortalities were observed and fish displayed a mean body condition of 0.98 ± 0.08 and a mean opercular rate after hand-netting of 135 ± 13, with similar values between the infested and the control group throughout most of the samplings (Supplementary figure S1). Moreover, the juvenile detachment was successfully synchronized between day 203 and 238 PE, yielding a total of 13,891 juveniles of *M. margaritifera.* During this period, the detachment displayed a left-skewed distribution with a peak of 38 detached juveniles/fish at day 226 PE (Figure 1a). Based on the curve of this detachment, three stages could be established to better describe the evolution of the juvenile detachment in relation with the fish parasitosis and the histopathological changes (Figure 1a and b): the rising detachment stage (day 203–226 PE), the declining detachment stage (day 227–238 PE) and the post-detachment stage (day 239–246 PE).



Supplementary figure S1. Comparison of the evolution of the fish body condition (a) and the opercular rate after hand-netting (b). The asterisks (*) denote the significant differences between groups (p-value <0.05).

3.1 The rising detachment stage (day 203–226 PE)

At the necropsy, 96% of fish were infested (Figure 1b) and harbored a median parasitic load of 22.4 ± 47.4 larvae/fish g, in which highly infested fish in the upper quartile showed a mean of 102 larvae/fish g; meanwhile, the remaining fish below the upper quartile displayed a mean of 12.6 larvae/fish g (Figure 1c). No significant relationships were detected in the correlation analysis between the parasitic load and the body condition nor the opercular rate after hand-netting (Figure 1c).

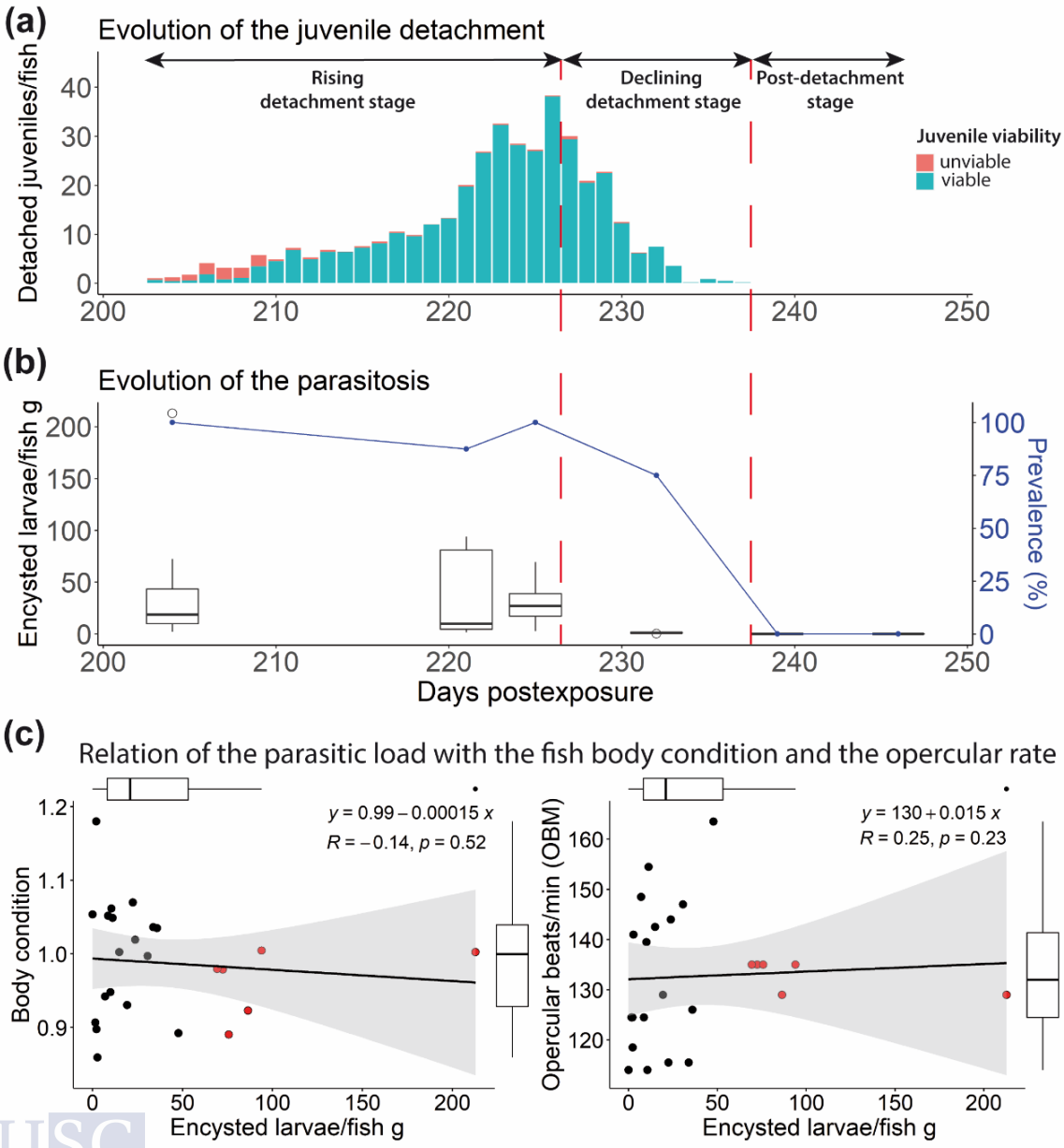


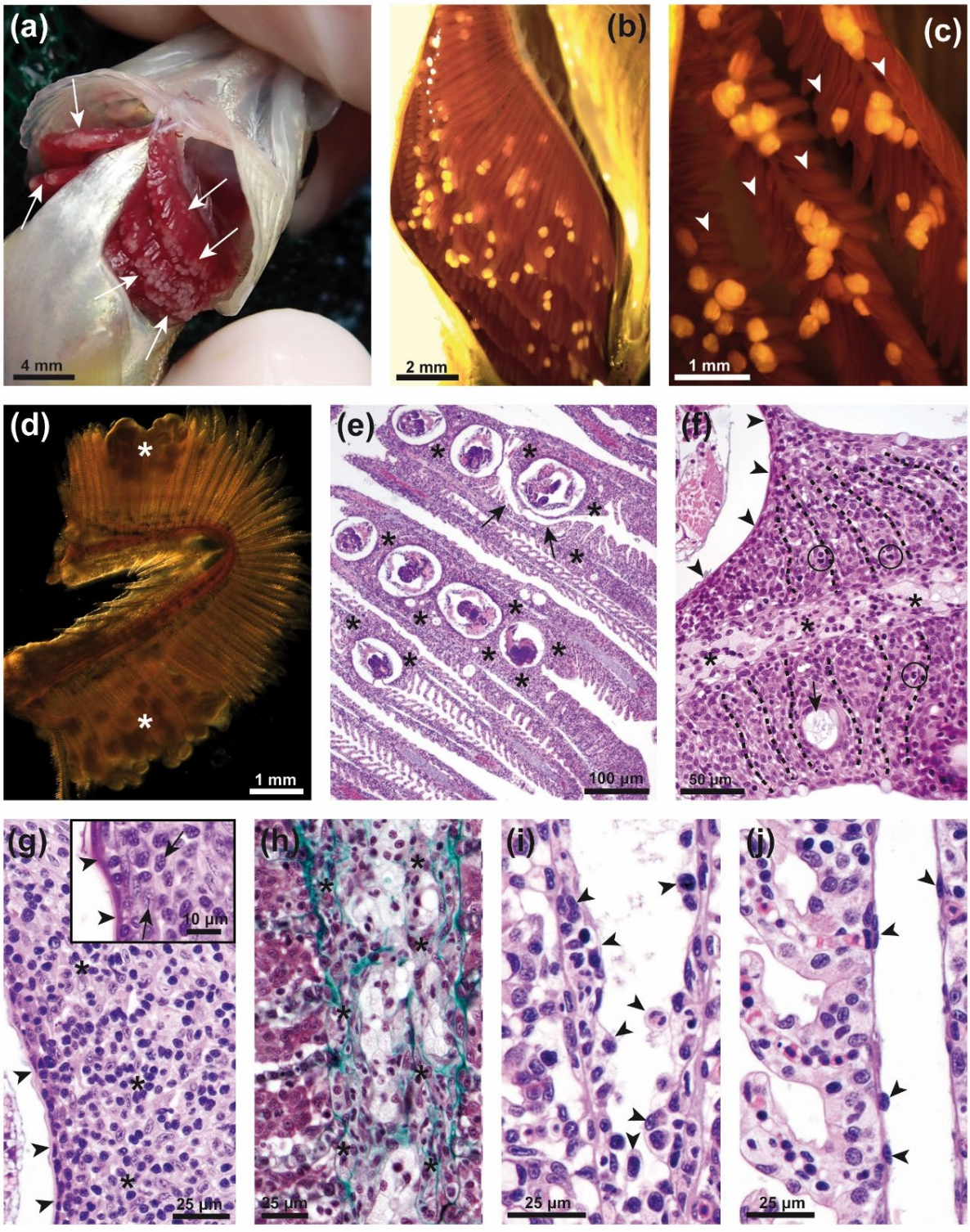
Figure 1. (a and b) Chronology of the mussel juvenile detachment (a) associated with the fish parasitosis (b) during the three stages of late glochidiosis of *M. margaritifera* delimited by dashed red lines. (c) No correlation between the parasitic loads and the fish body condition nor the opercular rate were detected in the correlation analysis. Individuals with parasitic loads in the upper quartile were represented in red. Confidence interval was 95%.

By naked eye visualization of infested fish, abundant, well-delimited, punctiform nodules were observed in the branchial tissue (Figure 2a). By stereomicroscopy, the immersed gills revealed a high number of ivory bean-shaped parasitic nodules located on the four holobranchs (Figure 2b), mostly at the trailing edges of the filaments (Figure 2c). Fish with less than 58 larvae/fish g exhibited a distinctive distribution of the larvae, clustered in bunches, which tended to gather at the dorsal and ventral regions of each holobranch (Figure 2d).

By light microscopy at low magnifications, the parasitic clusters were composed of several large and protruding nodules. Each nodule corresponded with a cyst of *M. margaritifera* larva, surrounded by a well-localized epithelial response in which adjacent lamellae, and sometimes the adjacent filaments, were fused obliterating the gill exchange surface (Figure 2e and f). Often, fused lamellae became elongated up to twice their length (Figure 2f and 3g). The parasitic cysts were completely covered by an intense epithelial hyperplasia and hypertrophy, the latter characterized by the high number of epithelial cells with large swollen cytoplasm, ovoid nucleus and small nucleoli (Figure 2g). Mitotic figures were frequently detected all over the cyst, although they were more abundant basally at the interlamellar epithelium of the parasitized regions (Figure 2f). The epithelial cells closest to the bivalve periostracum concentrically arranged and became thinner, elongated and intensely eosinophilic with H&E, being also PAS-positive (Figure 2g). Associated with the parasitosis, a lymphocytic inflammatory infiltrate was interspersed between the hyperplastic epithelium (Figure 2g). Moreover, a mixed inflammatory cell population, composed by macrophages and polymorphonuclear cells, was identified at the deeper layers of the filaments and related to the interlamellar system, particularly underneath the basal membrane of small ladder-like vessels (Figure 2h) and marginated over the endothelium of elongated sinus (Figure 2i).

No other lesions were recorded in other sampled organs from infested fish. On the other hand, control fish exhibited unfused lamellae, intraepithelial lymphocytes at the trailing edge of the filaments and scarce inflammatory cells associated with the interlamellar system (Figure 2j).

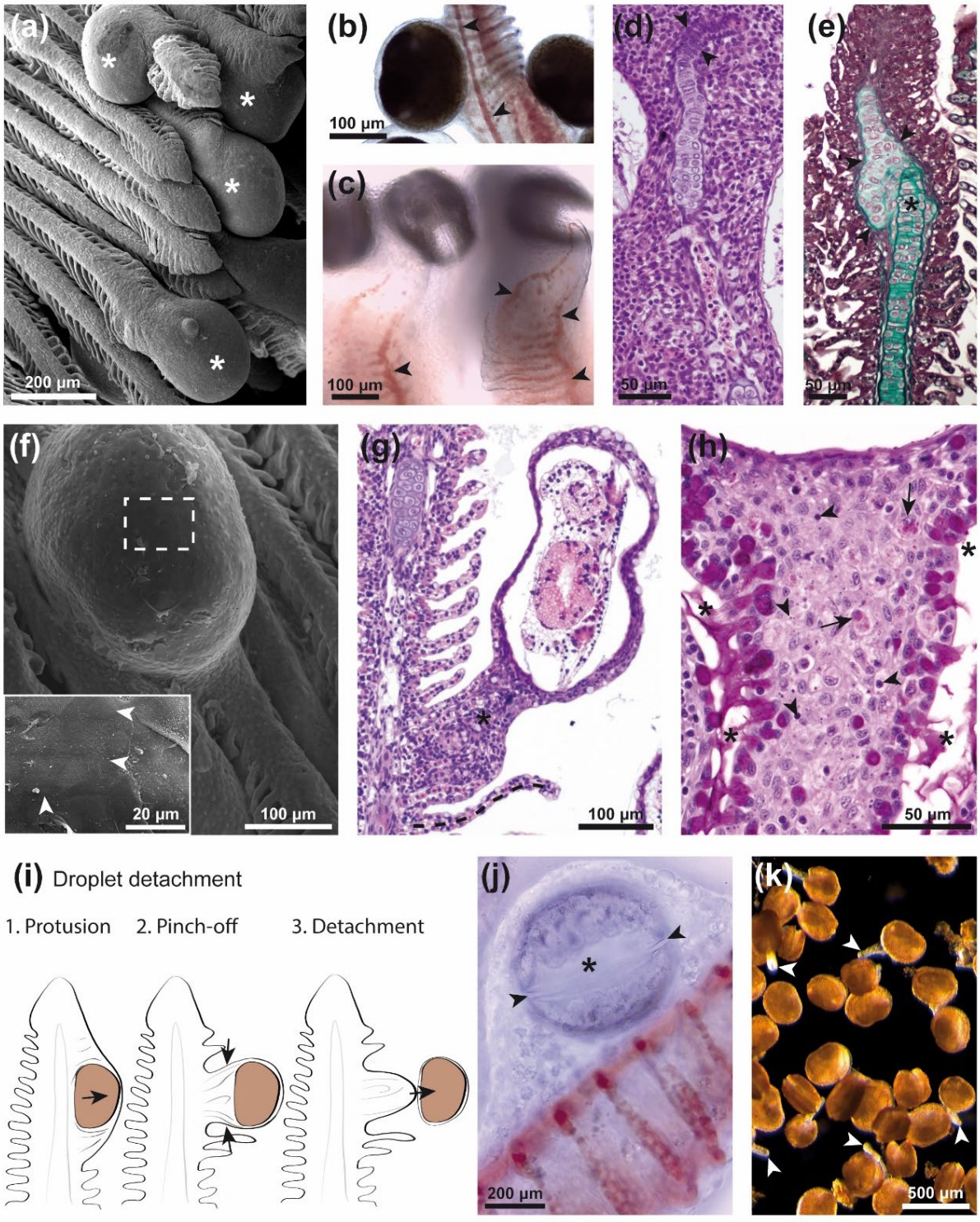
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Figure 2. Main morphopathological findings during the rising detachment stage of late *M. margaritifera* glochidiosis. (a) Gill macrophotography of an infested fish with punctiform lesions which corresponded with the encysted larvae (arrows). (b and c) Stereomicrophotographs showing the bean-shaped larvae located between each pair of hemibranchs indicated with arrowheads. (d) Distribution of parasites clustered in bunches at the dorsal and ventral regions of each holobranch (asterisks). (e) The localized epithelial response enclosing the parasitic cysts was accompanied by severe lamellar and filamental fusions (asterisks). Note the concave depressions on the filament surface contiguous to an adjacent larva (arrows). H&E stain. (f) Surrounding the outer surface of the parasitic cyst (arrowheads), the fused and elongated lamellae (dashed lines) were related to abundant mitotic figures (circles) and interlamellar cysts (arrow). Note the pleomorphic inflammatory infiltrate located at the interlamellar system (asterisks). H&E stain. (g) A high number of lymphocytes interspersed between epithelial cells, the latter became flattened and eosinophilic towards the larvae (arrowheads). H&E stain. Inset: The eosinophilic epithelial cells (arrows) beside the parasitic cysts (arrowheads) became thinner and more intensely stained under the PAS stain. (h) Interlamellar system with pleomorphic inflammatory cells (asterisks) underneath the intricate basement membrane highlighted with the Masson-Goldner trichrome stain. (i and j) Comparison between infested fish with marginated macrophages and polymorphonuclear cells over the interlamellar system endothelia (arrowheads, i) and control fish showing a normal lamellar morphology without leukocytes over the endothelial cells lining the interlamellar system (arrowheads, j). H&E stains.

The encysted larvae developed between 2100 and 2500 degree-days (sum of daily mean temperatures in ºC) and displayed a thin and refringent periostracum under fresh gill mounts, which allowed to observe the valves completely closed, the valve rims facing each other and the discernible mantle within the internal pallial and extrapallial cavities (Figure 3j). Once detached from the gills, the recently sloughed and viable juveniles exhibited mobile valves and a protractile foot protruding out of the valve limits (Figure 3k). Taking into account the motility of the valves, the mussel viability abruptly increased at day 209 PE (2220 degree-days) onwards from values below 63% to over 93% (Figure 1a). Some parasitic nodules bulged to a high extent on the gill surface (Figure 3a), molding the contiguous filaments observed as focal areas with concave shape (Figure 2e). Furthermore, fresh gill mounts highlighted the undisturbed arterioles underneath the parasitic cyst (Figure 3b) and exceptionally devious arterioles were related to the encysted larvae at the filamental tips (Figure 3c). In these cases, the growth plate of the filament was also deviated (Figure 3d) and the cartilage ray was thickened due to an irregular disposition of hyperplastic chondrocytes, observed by histology (Figure 3e). Under SEM, the nodular surface was covered by flattened and polygonal epithelial cells, overlaid by well-defined microridges (Figure 3f). However, at the most protruding regions, a localized epithelial degeneration occurred, characterized by the presence of faint microridges and cell boundaries (Inset, Figure 3f). Histologically, the contact area between these larger nodules and the filaments became reduced (50–100 μm width) creating a pedunculated, teardrop shaped structure supported by the hyperplastic epithelium of fused lamellae (Figure 3g). Occasionally, the encysted parasites were barely linked to the gill tissue by a very constricted and pinched-off peduncle which showed a superficial goblet cell hyperplasia (Figure 3h). Moreover, at deeper layers, the hyperplastic tissue was accompanied by moderate epithelial apoptosis, observed by pyknosis, cell shrinkage and the presence of apoptotic bodies (Figure 3h). Based on the overall morphopathological features described, larvae sloughed from the gill by a *droplet detachment* mechanism, illustrated in Figure 3i and simplified in three sequential steps: 1. protrusion over the surrounding limits, 2. pinch-off by reduction of the contact area between the gill and 3. detachment of the larvae without tissue disruption.



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Figure 3. Gill morphopathology of the most superficial parasitic cysts during the rising detachment stage of late *M. margaritifera* glochidiosis (a-h) and the *droplet detachment* (i) from encysted larvae into free-living juveniles (j and k). (a) Scanning electron microscopy (SEM) of a cluster of buoyant encysted larvae (asterisks). (b and c) Wet mount microphotographs comparing the unaltered arterioles (b, arrowheads) with exceptionally devious arterioles (c, arrowheads). (d and e) Deviation of the growth plate (arrowheads, d) and a well-localized cartilage hyperplasia (arrowheads, e) overgrowing over the cartilage ray (asterisk, e). H&E and Masson-Goldner trichrome stain, respectively. (f) SEM of a parasitic cyst completely covered by epithelial cells with the dashed area highlighting the most protruding area. Inset: Detail of the dashed area with poorly discernible microridges over the epithelial surface (arrowheads). (g) Histological microphotograph of the hyperplastic peduncles (asterisk) anchoring the larvae to the gill filaments. Note the presence of an elongate lamella (dashed line). H&E stain. (h) Constricted peduncle with a high number of goblet cells lined up the epithelial surface (asterisks) and high number of apoptotic (arrowheads) and phagocytic bodies (arrows) at deeper regions. PAS stain. (i) Schematic illustration of the three-steps droplet detachment of the mussel juveniles. (j) Fresh light microscopy of an encysted larvae with the valve rims facing each other (arrowheads) and their internal pallial cavity (asterisk). (k) Stereomicrophotograph of recently detached and viable juveniles with their protractile foot (arrowheads).

Adjacent to the parasitized areas, the proliferative lamellae were variably fused through their extension, ranging from complete and solid fusions to partially fused lamellae (Figure 4a-f). Solid lamellar fusions formed by hyperplastic epithelial and goblet cells, the latter mostly located at the external surface, obliterated the interlamellar spaces (Figure 4b). On the other hand, partially fused lamellae led to the occurrence of a high number of marked and well-delimited interlamellar cavities, barely visible by wet mount microscopy as translucent spaces (Figure 4a). These cavities were lined by epithelial and mucus-secreting cells, contained mucinous material and cell debris, and ranged from roundish and turgid interlamellar cysts of 10–70 μm diameter to big and elongated clefts up to 150 μm length, the last opened to the exterior (Figure 4c and d). By SEM, between the fused filaments and lamellae, narrow openings were also discernible on the surface of the filamental edges, lined by epithelial cells (Figure 4e and f).

3.2 The declining detachment stage (day 227–238 PE)

The parasitic loads rapidly decreased to virtually zero at day 238 PE (Figure 1b). At this stage, extensive regions of the holobranchs continued to exhibit partially fused and disordered lamellae by stereomicroscopy (Figure 5a). Histologically, half of the infested fish progressively reduced the severity and extension of their lesions displaying a less severe proliferative branchitis than that described during the rising detachment stage, with slightly hyperplastic and elongated lamellae, with their tips bent inwards (Figure 5b and c) and partially fused (Figure 5d and e). Such partially fused lamellae were accompanied by a high number of interlamellar cavities, mostly elongated clefts with abundant goblet cells arranged with their openings towards the epithelial surface (Figure 5e). On the other hand, solid lamellar fusions were less frequent in comparison to the previous stage, being restricted to well-localized areas that protruded over the filamental limits. In addition, the mixed inflammatory infiltrates related to the interlamellar system remained virtually unchanged (Figure 5c).

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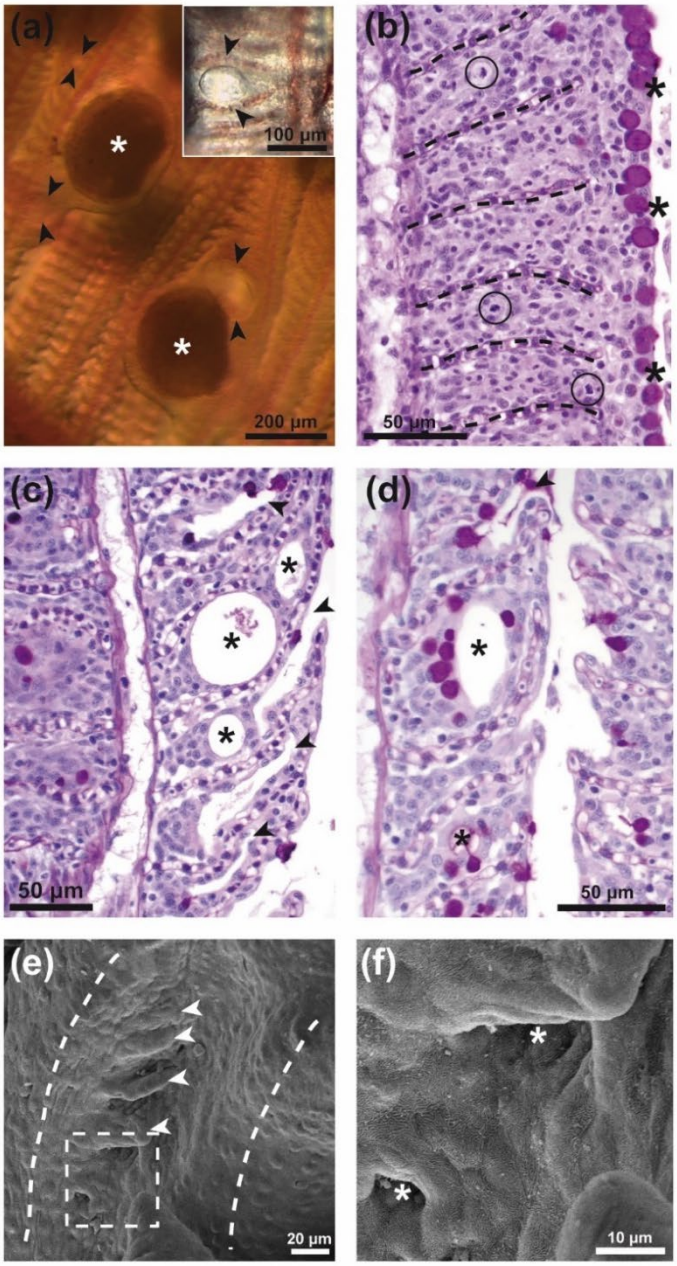


Figure 4. Fusions and interlamellar cavities adjacent to the encysted larvae during the rising detachment stage of late *M. margaritifera* glochidiosis. (a) The limits of the interlamellar cavities were barely visible by wet mount microscopy (arrowheads) close to the parasitic cysts (asterisks). Inset. Detail of an interlamellar cyst associated with two fused lamellae (arrowheads). (b) Solid lamellar fusion (dashed lines) associated with the presence of abundant interlamellar mitotic figures (encircled) and goblet cells at the surface (asterisks). PAS stain. (c and d). Partially fused lamellae characterized by the presence of interlamellar cysts (asterisks) and clefts (arrowheads) surrounded by mucous cells. PAS stains. (e) SEM of two fused filaments (dashed line) with small openings located between partially fused lamellae (arrowheads). (f) Detail of the dashed area highlighting two interlamellar openings (asterisks) lined up by epithelial cells.

Related to the hypertrophied and hyperplastic lamellar fusions, certain areas revealed marked intercellular spaces and epithelial apoptosis, the latter detected by the presence of abundant shrunken cells and apoptotic bodies (Figure 5f-h). Meanwhile the number of lymphocytes decreased markedly being only observed at the base of the affected lamellae (Figure 5f), abundant phagocytic cells were intimately associated with a localized epithelial apoptosis, identified by their large cytoplasm containing eosinophilic and PAS-positive vacuoles (Figure 5g and h). Moreover, at the outermost layers, profuse shedding and degenerating pavement cells were depicted by the loss of adherence with neighboring cells, pyknosis, and swollen and vacuolized cytoplasm (Figure 5g).

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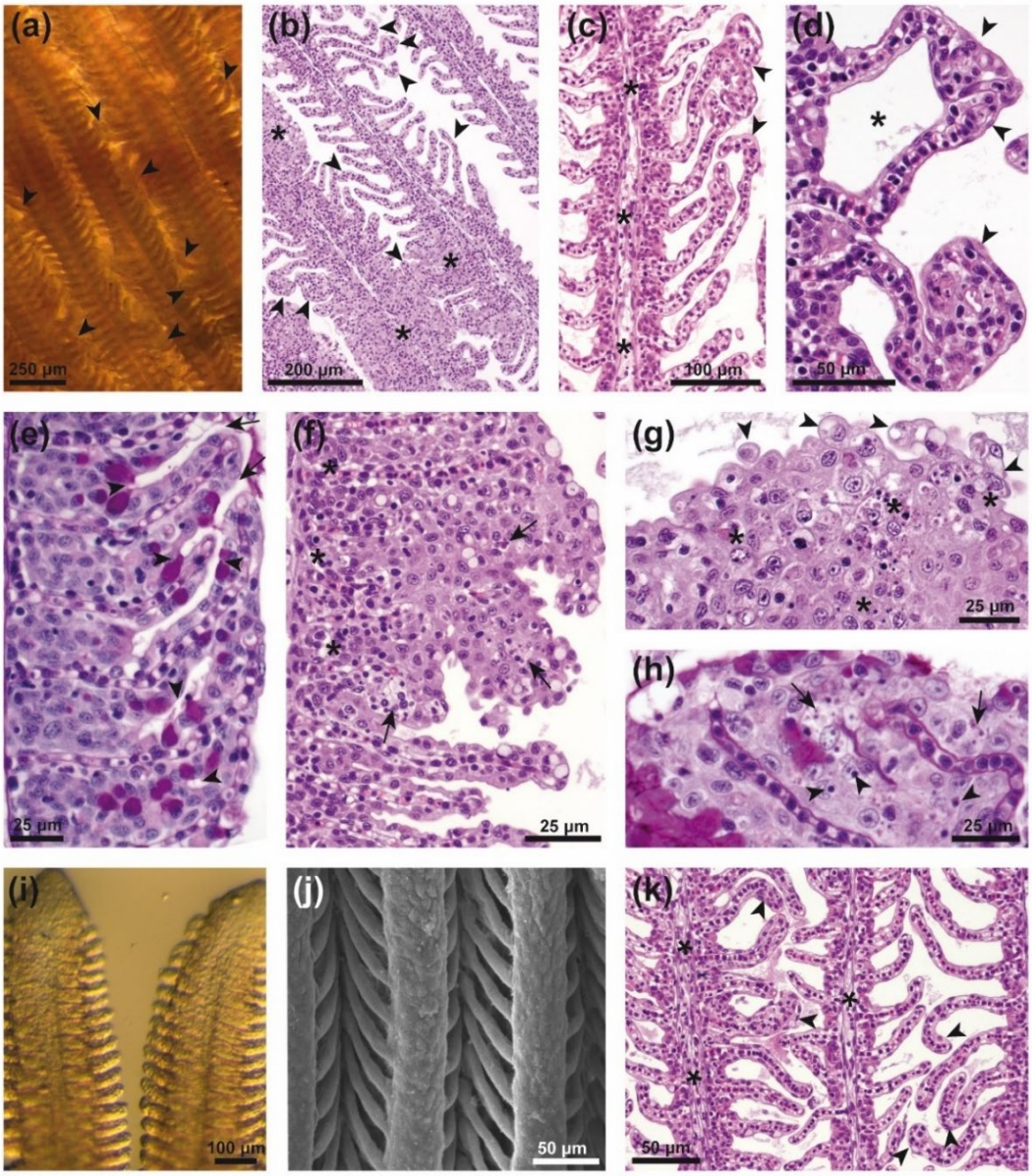


Figure 5. Gill morphopathology during the declining detachment stage (a–h) and the post-detachment stage of late *M. margaritifera* glochidiosis (i–k). (a) Stereomicroscopical image of disordered lamellae observed by tousled secondary filaments (arrowheads). (b) By histology, altered lamellae ranged from hyperplastic and fused lamellae (asterisks) to elongated and bent lamellae (arrowheads). H&E stain. (c) Detail of elongated lamellae with their tips bent inwards and slightly hyperplastic (arrowheads). Note the presence of a moderate number of pleomorphic inflammatory cells at the interlamellar system (asterisks). H&E stain. (d) Hyperplastic and fused lamellar tips (arrowheads) giving rise to an elongated interlamellar cavity (asterisk). H&E stain. (e) Detail of hyperplastic lamellae with most of the goblet cells arranged towards the interlamellar clefts (arrowheads) with openings towards the surface (arrows). PAS stain. (f) Partially fused lamellae showed areas of moderate lymphocytic (asterisks) and localized areas of epithelial degeneration (arrows). H&E stain. (g) Detailed microphotograph of abundant apoptotic bodies (asterisks) and vacuolized, swollen and sloughing epithelial cells at the outer surface (arrowheads). H&E stain. (h) Phagocytosis of apoptotic bodies (arrowheads) and small PAS-positive vacuoles (arrows) was associated with an area of lamellar fusion. PAS stain. (i and j) Normal structure of outer surface of the gill by stereomicroscopy (i) and scanning electron microscopy (j). (k) Histological photograph showing mild epithelial hyperplasia, elongations and lamellar deviation (arrowheads) together with a moderate number of mononuclear cells at the interlamellar system (asterisks). H&E stain.

3.3 The post-detachment stage (day 239–246 PE)

At this stage, parasitic cysts were absent (Figure 1b) and a quick and progressive regression of the previous lesions could be observed, leading to the observation of normal filamental and lamellar architecture in the infested fish group, resembling that of the control fish (Figure 5i-k). Only mild and sporadic deviation of the filamental tips were detected, accompanied by subtle histological changes, which consisted of mild lamellar elongations and bends, a rare mild hyperplasia of the lining epithelium and a moderate number of mononuclear cells remained restricted to the lumen of the interlamellar system (Figure 5k).

4. DISCUSSION

The gill morphological changes observed throughout late glochidiosis of *S. salar* were related to the larval detachment of *M. margaritifera* and the subsequent recovery process, providing valuable information to understand the mechanisms involved in the larval detachment and the gill response. Hence, this study entails an exhaustive example of how the morphopathological examination of fish gills during glochidiosis may serve as an essential monitoring tool to preserve fish welfare during the captive breeding programmes of endangered freshwater mussel.

During the late stages of this long-lasting parasitosis, no detrimental effects on the fish survival and body condition were observed, demonstrated by the absence of mortalities and the lack of relationship between the parasitic loads and the host fitness variables, such as the condition factor and the opercular movements. These evidences support previous observations during *M. margaritifera* glochidiosis in which sublethal parasitic loads up to 300 larvae/fish g do not have a detrimental effect on fish survival (Cunjak & McGladdery, 1991; Chowdhury et al., 2019; Taeubert & Geist, 2013) or fish body condition (Treasurer et al., 2006). However, recent studies measuring the individual
fish growth during a specific period described a delayed growth on fish parasitized by *Margaritifera laevis* and *M. margaritifera* (Chowdhury et al., 2019; Ooue et al., 2017). Regarding the opercular rates, no significant differences between control and exposed groups were observed and the correlation analysis between the parasitic load and the opercular rate did not show any relationship. Nevertheless, the high mean opercular rate observed in both fish groups, 2.7 times higher than the basal rates of Atlantic salmon fry, could be explained by the stressful procedure of hand-netting (Hawkins, Armstrong & Magurran, 2004a; Hawkins, Armstrong & Magurran, 2004b). This contrast with other authors in which *S. trutta* and *Micropterus salmoides* affected by glochidiosis showed—at resting conditions—a mild increase of the opercular rates around 1.2-1.5 times in comparison with control fish (Kaiser, 2005; Thomas et al., 2013).

During fish necropsy, stereomicroscopy revealed an extremely variable outcome of glochidiosis in *S. salar*, in which exclusively one out of four lately infested salmon remained highly parasitized with 102 larvae/fish g. These differences between fish of the same experiment suggest the involvement of an individual variability, rather than being restricted to the host species level (Taeubert et al., 2010; Wacker et al., 2019). Within the breeding programmes and the experimental designs, further procedures should increase the sample size

employing less-invasive methodologies to deal with the high parasitic load variabilities,. In this sense, the stereomicroscopical visualization of immersed gill tissue reveals as a potential diagnostic tool to determine *in vivo* the larval load. In comparison with a previous technique (Österling, 2011), the immersed technique enhances the stereomicroscopical visualization of parasites among filaments, avoids the introduction of instruments into the gill and exposes different holobranchs and gill regions. To implement this technique, future studies need to consider the shift on the parasites distribution over the gill from a random distribution during early glochidiosis (Castrillo et al., 2020) towards the focal distribution in dorsal and ventral gill areas observed in this study during the late glochidiosis. This aggregation could be influenced by the heterogeneous flow in the dorso-ventral axis (Strother, 2013) and the unidirectional water flow through the hemibranchs (Hughes, 1984), creating more sheltered regions to the water current as described in other gill ectoparasites (Kumar, Madhavi & Sailaja, 2017; Wootten, 1974).

The histopathological evaluation during the rising detachment stage (day 203–226 PE) highlighted the involvement of a proliferative branchitis surrounding the persistent larvae, similar to that described during early glochidiosis (Castrillo et al., 2020), regarded as a common and unspecific response against multiple pathogens of the salmonid gills (Tubbs, Wybourne & Lumsden, 2010). The hypertrophied and hyperplastic epithelial cells may establish long-lasting lamellar fusions playing an important role in the maintenance of the cyst covering and growth of the larvae. Such increase in size might exert pressure and tensional forces on the fused epithelium and could explain the elongation of the fused lamellae, already described during the *M. falcata* glochidiosis (Karna & Millemann, 1978).

In addition to the proliferative lesions, an inflammatory gill response was associated with the larvae, although it seems inefficient to prevent the larval encystment and development of *M. margaritifera*. In particular, the lymphocytic infiltrates observed in this study were similar to those observed around persistent glochidia at early stages (Castrillo et al., 2020). In addition, deeper inflammatory infiltrates, located at the interlamellar system, may also indicate a leukocytic migration through the sinusoidal vessels, described as a defensive mechanism against bacterial and parasitic gill diseases (Adams & Nowak, 2001; Grizzle & Kiryu, 1993). Due to this marked proliferative gill response, although inefficient to reject the mussel larvae, a tolerance defense strategy (Medzhitov, Schneider & Soares, 2012) should be further considered in those fish which remained highly parasitized at late stages of glochidiosis, as has been suggested in *M. margaritifera* (Barnhart et al., 2008; Marwaha et al., 2019). Other co-evolved parasitisms showed an increased tolerance response in its wild host, *S. trutta*, in contrast with the non-native host *O. mykiss* (Bailey, Strepparava, Wahli & Segner, 2019), however, the understanding of the fish immune response and the tolerance/resistance mechanisms in glochidiosis deserve further study.

Although the kinetics of larval detachment cannot be fully appreciated by the techniques employed, the evaluation of the fish gill lesions throughout the rising detachment stage allowed us to infer a disjoining sequence, termed as *droplet detachment*, due to the similarities with the protruding, pinch-off and detachment of a droplet fluid (Henderson, Pritchard & Smolka, 1997). Initially, the larval protrusion was observed by the apical displacement of the cyst and peduncle

formation, potentially aided by the ventilatory forces in the gill chamber for example, the water flow and the abduction and adduction of the holobranchs (Strother, 2013). Pedunculated cysts were covered by a thin layer of epithelial cells which suffered mild degeneration already described by Scharsack (1994), which could be attributed to the tensional forces due to the growing larvae and the water forces suffered at the outermost layers. Progressively, peduncles became constricted leading to the pinch-off of the larvae surrounded by a thin layer of epithelial cells, with no signs of "opening up" as previously described by SEM studies (Scharsack, 1994; Wächtler et al., 2001). This droplet detachment mechanism, in absence of tissue disruption, contrasted with the epithelial erosions involved in the early detachment of immature larvae in *S. salar* and *O. mykiss* (Castrillo et al., 2020; Scharsack, 1994), suggesting a different pathogenesis between the early rejection of immature larvae and the late detachment of viable juveniles.

Meanwhile, the goblet cell hyperplasia seemed to be a chronic response in glochidiosis (Treasurer & Turnbull, 2000), not detected at earlier stages (Thomas et al., 2013). This hyperplasia is particularly noticeable around constricted peduncles during the larval detachment and might contribute, by mucus secretion, to create a physical and biochemical barrier to preserve epithelial integrity at these detachment areas. In addition, the maintenance of the epithelial continuity and the goblet cell hyperplasia would prevent the occurrence of secondary infections (Dang, Pittman, Sonne, Hansson, Bach et al., 2020; Karna & Millemann, 1978) and the onset of an osmoregulatory compromise, the latter observed during the epithelial erosion caused by the detachment of mature trophonts of both marine and freshwater ciliated protozoa (Colorni & Burgess, 1997; Ewing & Kocan, 1987; Tumbol et al., 2001).

After the seven-months parasitism and the increase in water temperature, the larvae of *M. margaritifera* accomplished their metamorphosis, confirmed by collection of viable juveniles since day 209 PE onwards, around 2200 degree-days. Nevertheless, it remains unknown if temperature acts over the gill response, the juvenile development or both. The observation of the completely closed valves in the gills and the marked shift on the juvenile viability prior to the detachment peak suggest an active role of the parasite during the droplet detachment, where the larval movements of the foot inside the pallial cavity could provoke an internal motion as previously hypothesized (Kaiser, 2005; Waller & Mitchell, 1989). To determine the role of the larvae during the parasitism and the subsequent performance of the dropped-off juvenile mussels (Marwaha et al., 2017), further studies need to focus on the development and metamorphosis during this poorly known parasitic stage (Scharsack, 1994).

Once the inciting cause was removed by the larval droplet detachment, salmon gills recovered almost completely from the parasitism of *M. margaritifera* in around 19 days after the detachment peak. This study provides shreds of evidence of a quicker gill recovery than described in glochidiosis with *Lampsilis reeveiana* (Kaiser, 2005). In this case more severe lesions were attributed to a larval "excystment", whereas in *M. margaritifera* the non-erosive detachment preserved the basal membranes unaltered, essential for safeguarding the gill repair mechanisms (Ferreira Sales et al., 2017; Kudo & Kimura, 1983; Speare et al., 1999). In addition, the high temperature maintained during this trial is expected to increase the reparative

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mechanisms as occurring during wound healing (Jensen, Wahli, McGurk, Eriksen, Obach et al., 2015; Schmidt, 2013).

During the declining detachment stage, gill remodeling was the most important phase of the healing cascade observed in this study (Sveen, Karlsen & Ytteborg, 2020). In particular, apoptosis, phagocytosis and epithelial shedding mutually interplay to remove the cell excess, limit the detrimental effects of inflammation (AnvariFar, Amirkolaie, Miandare, Ouraji, Jalali et al., 2017) and preserve the branchial architecture during healing (Daoust & Ferguson, 1986). The presence of apoptotic hallmarks related to the more constricted peduncles and the remaining lesions after the detachment peak may indicate a regression of the hyperplastic tissue, as it was suggested during other gill proliferative diseases (Ferreira Sales et al., 2017; Speare et al., 1999) and reversible gill remodeling (Gilmour & Perry, 2018; Sollid, De Angelis, Gundersen & Nilsson, 2003). On the contrary, the absence of apoptosis during the parasitism could be explained as a beneficial mechanism both to the parasite by preserving the hyperplastic covering around the larvae (Bienvenu, Gonzalez-Rey & Picot, 2010) and also to the host by minimizing the gill surface area exposed to the external environment (Nilsson, 2007). In addition to this, the epithelial exfoliation could be involved in the removal of the outermost dying cells, as described also during the recovery of bacterial gill disease (Kudo & Kimura, 1983); providing evidence of how individualized cells slough rather than large intercellular masses (Nilsson, 2007). Due to the co-occurrence of mitosis, apoptosis and cell shedding, further quantitative studies are needed over time to provide insight about their mutual interplay during the mechanisms of gill remodeling.

During the reorganization of gill epithelium, the shift of the goblet cell distribution towards an interlamellar position points to the involvement of this cell type in the gill remodeling (Kudo & Kimura, 1983; Mueller, Sanchez, Bergman, McDonald, Rhem et al., 1991). In particular, the epithelial polarization, observed by the arrangement of goblet cells towards the abundant interlamellar cavities, may help to rearrange the intercellular junctions towards the typical basolateral position (Ronza, Villamarín, Méndez, Pardo, Bermúdez et al., 2019), and cleaving of fused lamellae, as occurring during the epithelial reshaping processes (Takeichi, 2014). Interlamellar cavities are regarded as advanced lamellar fusions during glochidiosis (Castrillo et al., 2020; Treasurer & Turnbull, 2000). In the absence of a mucociliary apparatus, these interlamellar cavities and the superficial openings might play an important role during the gill clearance and remodeling, allowing the hydraulic forces to act between lamellae, as it had been suggested during amoebic and bacterial gill diseases (Adams & Nowak, 2001; Speare, Ferguson, Beamish, Yager & Yamashiro, 1991b).

As a result, mild sequelae were observed at the end of this experimental parasitosis, in which the filamental tip deviation could slightly perturb the hydrodynamics through gills. Moreover, the mild presence of a mixed inflammatory infiltrates at the secondary arterio-venous system should be further studied in relation with the inflammation and healing of the gills, as this vasculature might be involved in the leucocyte mobilization by removing the interstitial fluid, waste products and cellular debris; supporting further similarities with the lymphoid system of higher vertebrates (Olson, 2002; Rummer et al., 2014).

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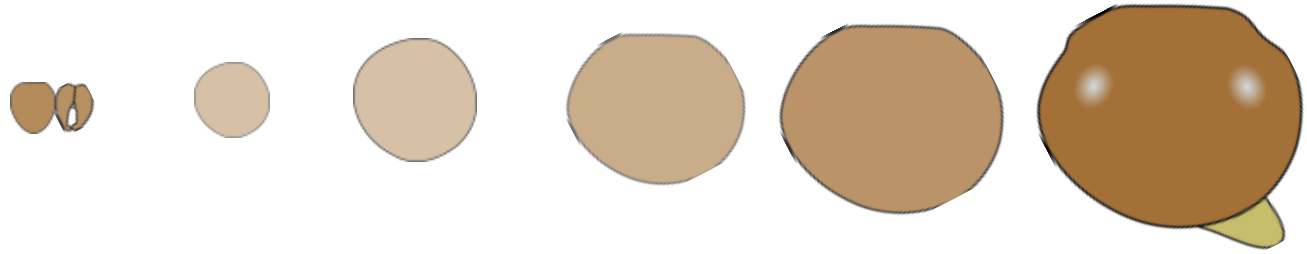
In summary, the multifocal proliferative branchitis, the non-erosive droplet detachment of the mussel juveniles and the quick and effective gill recovery suggests that experimental late glochidiosis did not severely harm fish health, supported by the absence of fish mortalities and clinical signs. This unique host-parasite interaction supposes a model to understand the role of the proliferative and remodeling epithelial responses during the defense and healing mechanisms of the gill. Moreover, the morphological techniques employed are revealed as a valuable tool during the conservation aquaculture of endangered freshwater mussels to optimize the artificial breeding programmes and evaluate the fish welfare.

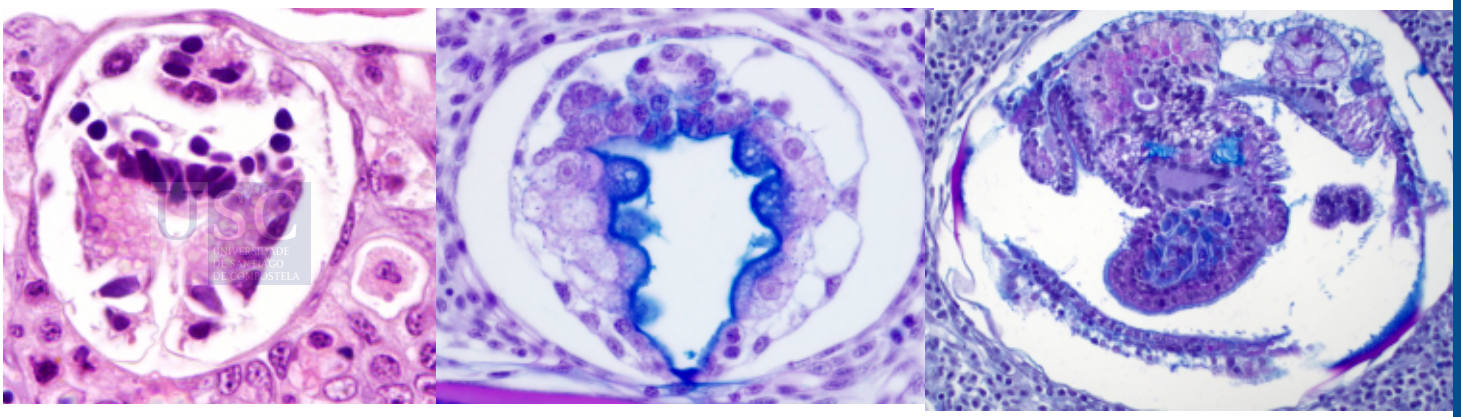
5. ACKNOWLEDGMENTS

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Study 3: Larval development of the Freshwater Pearl Mussel





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STUDY 3: LARVAL DEVELOPMENT OF THE FRESWATER PEARL MUSSEL

Manuscript I: The parasitic travel of *Margaritifera margaritifera* in Atlantic salmon gills: from glochidium to post-larva

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HIGHLIGHTS

- The knowledge of the larval morphogenesis of *M. margaritifera* is essential for understanding and monitoring this parasitism within the freshwater mussel culture.

- The intermediate larval stage, known as mushroom larva, show a distinct morphology among bivalves, where the mushroom body and the zip membrane might be involved in both isolation and nutrition from the fish.

- After metamorphosis, juveniles detach from the gills with a complete set of post-larval organs, and settle into the riverbed aided by the glandular byssal complex of the foot.

ABSTRACT

The larval development of the endangered freshwater mussel *Margaritifera margaritifera* (L.) represents one of the most striking parasitism among unionids, in which larva parasite the fish gills for several months. Despite the importance of this parasitic phase to successfully culture the freshwater mussel, the larval morphogenesis remains completely obscure. To describe the parasitic larval development and metamorphosis, Atlantic salmon (*Salmo salar* L.) were exposed to glochidia, sampled periodically to visualize the gills by stereomicroscopy and light microscopy and results were summarized throughout three developmental stages. Once attached to the fish gills, glochidia changed their morphology within the first days and acquired an intermediate stage termed mushroom larva due to the presence of the mushroom body and the zip membrane, both structures transitory and distinctive of this long-lasting parasitism. The zip membrane, located at the valve cleft, may play a unique role in the isolation and acquisition of non-particulate nutrients from the fish, while the prominent mushroom body of the mantle accumulated abundant intracytoplasmic lipid droplets. After 200 days, a successful metamorphosis was evidenced by the formation of a complete set of post-larval organs, pointing to the acquisition of different functionality, which will be essential for the settlement and deposit-feeding into the riverbed. Among the post-larval organs, the byssal complex of the post-larval foot was described for the first time at the end of the parasitic stage of unionids. In conclusion, this study provides an overview of the larval morphogenesis of *M. margaritifera*, from glochidium to post-larva, essential for understanding the parasitic interaction between the freshwater mussel larva and the fish host. Moreover, the morphological techniques and the hallmarks described might be applicable to optimize and monitor the larval developmental status during one of the most critical stages of the captive breeding programmes of endangered freshwater mussels.

Keywords: Freshwater Pearl Mussel, larval development, glochidium, mushroom body, byssal gland.

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1. INTRODUCTION

Freshwater mussels of the order Unionida are known as naiads, according to the mythological female nature deities which preside bodies of fresh water and protect the water quality and the freshwater ecosystem; hence they also provide a direct contribution to human and animal health which depend on these water sources (Strayer, 2017; Vaughn, 2018). These services are expected to occur in large mussel communities, where the semi-infaunal adults display important burrowing and biofiltration functions in the riverbeds (Boeker et al., 2016; Vaughn & Hakenkamp, 2001; Vaughn et al., 2008). Nevertheless, the freshwater mussels at the Northern Hemisphere are one of the largest and most threatened freshwater taxa worldwide (Lopes-Lima et al., 2018; Lopes-Lima, Froufe, Do, Ghamizi, Mock et al., 2017). In Europe, *Margaritifera margaritifera* (Linnaeus, 1758) is listed as *Critically Endangered* (Lopes-Lima et al., 2018; Lopes-Lima, Sousa, et al., 2017), where most of the populations lack recent recruitment due to the loss of the habitat required by the young mussels (Geist & Auerswald, 2007).

Before the juvenile stage, the *M. margaritifera* embryo is brooded within the marsupia of parent mussels and develops into glochidium. This unique larval stage is released into the water to encyst into the gill epithelia of its salmonid host, *Salmo salar* L. and *Salmo trutta* L. fry (Kat, 1984; Wächtler et al., 2001). After several months larva completes its metamorphosis and detaches from the host gills when temperature increases (Hruska, 1992). This embryological and larval development is regarded as a parasitic strategy for upstream dispersal and nutrition (Barnhart et al., 2008; Denic et al., 2015; Schwartz & Dimock, 2001), and contrasts with the pelagic veliger larva of other bivalves (Bändel, 1988; Carriker, 2001). Moreover, this interaction between the larva and the fish supposes one of the most striking parasitism among unionoideans, where the small glochidium of *M. margartifera* grows five folds from the initial size (Bauer, 1994; Denic et al., 2015; Howard & Anson, 1922; Scharsack, 1994; Scheder et al., 2011). Therefore, one of the eventual emergency conservation strategies relies on the captive culturing of the young mussels, in which the larva needs to successfully develop during this long-lasting parasitic larval stage on its suitable fish hosts (Gum et al., 2011).

Despite the importance of this parasitic phase for the subsequent survival of the juvenile mussels, very few studies have evaluated the larval development and the anatomy of freshwater mussel adults (McElwain & Bullard, 2014). For instance, this parasitic stage has been characterized in other unionids such as the margaritiferid *Pseudunio auricularius* during its parasitism in Siberian sturgeon (Araujo et al., 2002). Moreover, other species as *Hyriopsis bialatus* and *Utterbackia imbecillis* develop large glochidium with brief parasitic stages on the fish, thus, a supplemented tissue culture with fish serum could be successfully employed to describe the quick *in vitro* development of these larvae (Chumnanpuen et al., 2011; Fisher & Dimock, 2002). After metamorphosis and detachment from the gills, recent studies described the morphology of free-living young *M. margaritifera*, termed post-larval or juvenile stages (Araujo et al., 2018; Lavictoire et al., 2018; Schartum et al., 2016). However, up to our knowledge, there is only one study focused on the larval development of the threatened species *M. margaritifera*, by Scharsack (1994) in brown trout.

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In order to provide insight into the unique parasitic interaction between the *M. margaritifera* naiad and its suitable *S. salar* host, this study aims to thoroughly describe the larval development and metamorphosis of the parasitic larva *M. margaritifera* in the salmon gills by stereomicroscopy and light microscopy. This knowledge will contribute to a better understanding of the pathogenesis of this parasitism as well as to improve the management of parasitic and post-parasitic larvae during the freshwater mussel conservation programmes.

2. MATERIAL AND METHODS

Three independent experimental trials were performed between 2015 and 2018. Atlantic salmon fry (*S. salar*) were artificially infested with glochidia and sampled periodically for approximately seven months to evaluate the microscopical morphology of the encysted larva. These experimental trials were carried out in the framework of a conservation aquaculture program, held at Galicia (NW of the Iberian Peninsula) and focused on the propagation and captive breeding of this endangered freshwater mussel.

2.1 *In vivo* procedures in the salmon host

At the end of summer, salmon fry (age 0+) were submitted to a glochidial exposure (1; Figure 1a and b) following the methodology described by Castrillo et al. (2020). Briefly, wild gravid mussels of *M. margaritifera* (n = 2–4) were induced to oxygen and temperature stress to release and collect the glochidial spat. The released glochidia were evaluated in quantity and quality by light microscopy. Thereafter, fry were exposed by bath immersion to 1,000 glochidia/fish g for 30 min, and subsequently reared in open system tanks of 0.5 m^3 and a density below 1.1 fish/L.

Although a number of fish prematurely rejected the encysted larva, fish that remained infested after six months contained large larva encysted in the gill tissue, visible macroscopically by manual immobilization and abduction of the opercula (Castrillo, Varela-Dopico, Bermúdez, Ondina & Quiroga, 2021). This technique allowed us to the *in vivo* diagnosis and selection of the infested fish (2; Figure 1a and b) showing a variable prevalence betweem 8 and 35% (Figure 1a). Thereafter, infested fish (n = 40–78) were introduced in a recirculating system to synchronize the post-larval detachment (3) by progressively increasing the water temperature up to 17ºC (Figure 1a and b). Detached post-larva, also known as juveniles, were collected daily from the tank outlet by sieves and the start of the detachment of viable post-larva was determined at each trialwhen themajority of the detached individuals were viable (Figure 1a).

To reflect the temperature evolution throughout each trial, the water temperature was monitored every three hours with a temperature data logger (UX100, Onset HOBO®), represented in relation to each experimental procedure (Figure 1b). Moreover, to consider these different temperatures among trials, the elapsed accumulated temperature was calculated as the sum of the average daily water temperature between the maximal and minimal values, expressed in degree-days (Figure 1a).

2.2 Sampling procedure

Based on the proportion of infested fish in the tank, 6–10 exposed fish were sampled randomly at 40 min and 1, 3, 14, 21, 30, 60, 90, 120, 150, 180, 200 and 220 days post-exposure (PE; Figure 1b). Each fish was anesthetized with an overdose of Tricaine methane-sulfonate (MS-222, Sigma-Aldrich®) and a bicarbonate solution (1:2); and the euthanasia was confirmed by sectioning cranially the spinal cord. Holobranchs were excised and left gills were observed and photographed under a M125C stereomicroscope and a DM750 light microscope (Leica®). Additional squashes of small portions of the gill tissue without the supporting arch were visualized directly by light microscopy. Larvae morphologically oriented in a lateral view were photographed with the light microscope objective at 10x (n=5) to measure the larval shell length in the antero-posterior axis by ImageJ (Rueden et al., 2017).

(a)	trial	2015-16	2016-17	2017-18
Glochidial exposure of fish (1)	**date**	01/10/2015	22/09/2016	24/09/2017
	fish (*n*)	1000	1000	500
Selection of infested fish (2)	**days PE**	202	177	169
	degree-days	2110	1633	1560
	prevalence	8%	18%	35%
Start of the detachment of viable post-larva (3)	**days PE**	209	194	190
	degree-days	2202	1809	1907

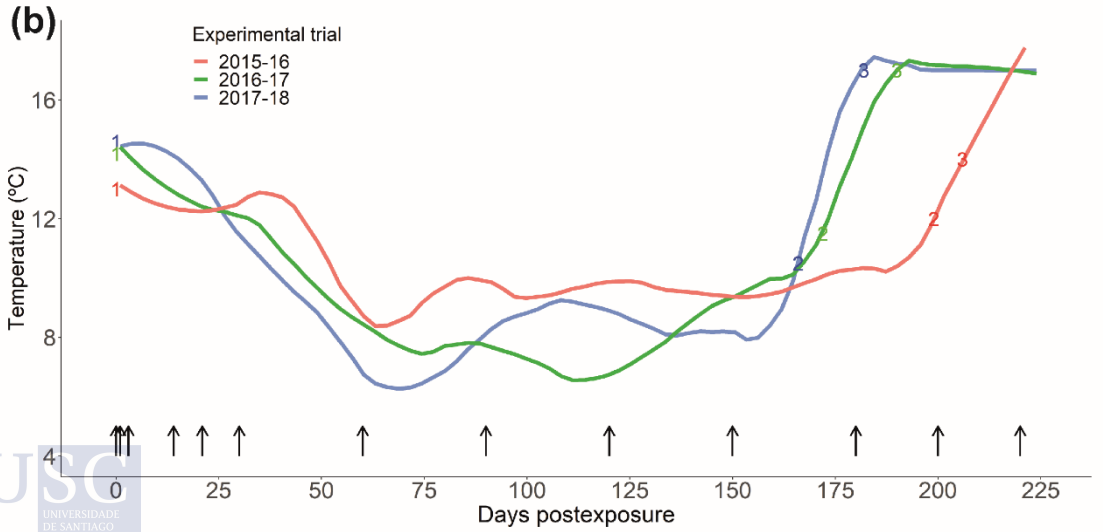


Figure 1. Summary of experimental trials performed in Atlantic salmon fry during the 2015-16, 2016-17 and 2017-18, divided in three procedures. (a) Summarizing table of the different yearly procedures. (b) Evolution of the smoothed daily water temperatures through each trial (represented by different colours) in relation to the different procedures (1–3) and the 13 samplings (arrows).

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Once the detachment from the fish gills was initiated, viable and unviable post-larvae were classified based on the presence or absence of pedal and valve movements, respectively, and photographed under the stereomicroscope to measure their length as previously described (n = 40, ± 0.1 μm). For the histological analysis of the detached post-larva, only the viable individuals were sampled to avoid potential post-mortem changes.

All procedures were carried out at the facilities of "Centro Ictiogénico de O Veral" (Xunta de Galicia) and followed the international (Directive 2010/63/EU, on the protection of animals used for scientific purposes), national (Law 6/2013 and RD 53/2013, on the protection of animals used for scientific experiments) and institutional regulations (USC Review Board).

2.3 Histology

Quickly after euthanasia, right holobranchs were fixed in Bouin´s, Dietrich´s and Davidson´s fluids to compare the effect of the different fixatives and minimize the shrinkage artifacts of larvae due to potentially high osmolarity (Castrillo et al., 2020). After fixation for 18 h, right holobranchs were decalcified for 6 hr in a 10% ethylenediaminetetraacetic acid solution (Osteodec, Bio-optica®) and processed for histopathology by routine methods. Sections were stained with hematoxylin and eosin (H&E) and additional periodic acid-Schiff (PAS), PAS-diastase, PAS-alcian blue (PAS-AB) and Masson-Goldner trichrome stains were performed. During the most representative samplings of the last trial, left holobranchs were fixed in 10% buffered formalin without the decalcification and stained with the calcium-specific stain alizarin red S; or sampled for cryosectioning and staining with H&E or the lipid-specific stain Sudan black B.

On the other hand, the sampled juveniles were fixed in Davidson´s fluid for 3 h, rinsed in 10% ethanol, stained with a light solution of eosin, wrapped in a lens paper inside the cassettes and embedded by pipetting them, before being dehydrated and processed as aforementioned.

All slides were observed and photographed using a BX51 light microscopy equipped with an EP50 digital camera and a polarized light (Olympus®).

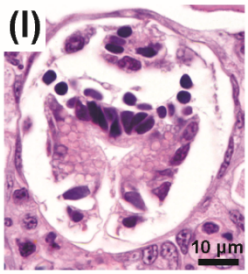
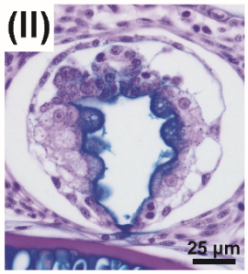
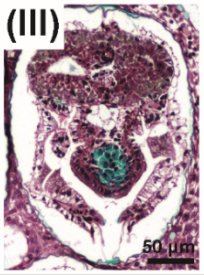
2.4 Statistical analysis

To estimate the relationship between the larval length and the accumulated temperature, the Kendall-Theil nonparametric linear regression and Spearman's correlation coefficient were calculated since data did not accomplish with the normality assumptions. On the other hand, the length of detached juveniles was compared between viable and unviable mussel with the parametric Student´s t-test for hypothesis testing. The significance level was 95% in all cases (p-value < .05). All the numeric data were analyzed by RStudio software (R Core Team, 2019).

3. RESULTS

During the encystment in the gills of Atlantic salmon, the parasitic larvae of *M. margaritifera* grew and displayed a progressive organogenesis which led to the metamorphosis and detachment of the viable post-larva. To summarize the larval development during this long-lasting parasitosis, the descriptions of each sampling point were grouped in three parasitic stages (I–III), based on the main morphological changes summarized in Table 1.

Table 1. Summary of the main morphological changes observed during the parasitic development of *M. margaritifera* larvae encysted in the Atlantic salmon gills, grouped in three parasitic stages (I-III) and represented each with a tranversal histological section.

Developmental stages	(I)	(II)	(III)
			
day postexposure (PE)	0-3	14-150	150-220
degree-days	0-37	130-1400	1400-2500
length (µm)	60-72	72-293	308-397
external morphology	walnut shape semi-transparent	roundish-bean shape semi-transparent	bean shape opaque
valve cleft	disintegrated gill tissue	zip membrane	zip membrane elongation
inner mantle epithelium	lost of the granular content	mushroom body	mushroom body regression
digestive apparatus	absent	discontinuous and immature digestive tract	mature digestive tract and primordium of the digestive gland
foot	absent	absent	ciliated and glandular foot
gills	absent	absent	three ctenidia
musculature	atrophy of the adductor muscle	absent	adductor and pedal musculature
other systems	absent	apparition of haemocytes	renopericardial, haemolymphatic and sensory systems

3.1 Parasitic stage I

This short stage comprised the first three days of the parasitism, between 40 min PE to 3 days PE. Between the day 0 and 1 PE, glochidia became encysted into the gill tissue and lost their typical morphology specialized for clasping to the fish mucosa (Table 1). Initially, glochidia were completely encircled by the gill tissue and showed a walnut shape, approximately 60 µm length and 70 µm height (Figure 2a). By histology, larvae were pinched to the gills by contraction of the larval adductor muscle clasping a small portion of host epithelium inside the pallial cavity. This pinched tissue became rapidly disintegrated in contact with the internal mantle epithelium, the latter characterized by the presence of eosinophilic, PAS positive and coarse intracytoplasmic granules (Figure 2b and c). Exceptionally, very few glochidia failed to encyst at day 1 PE, observed with the valves wide open inside the gill tissue (Inset, Figure 2a). At day 3 PE, larvae showed a negligible growth and the glochidial appearance was completely lost, confirmed by the absence of the clasped gill tissue, atrophy of the adductor muscle and loss of the granular appearance of the inner mantle (Figure 2d).

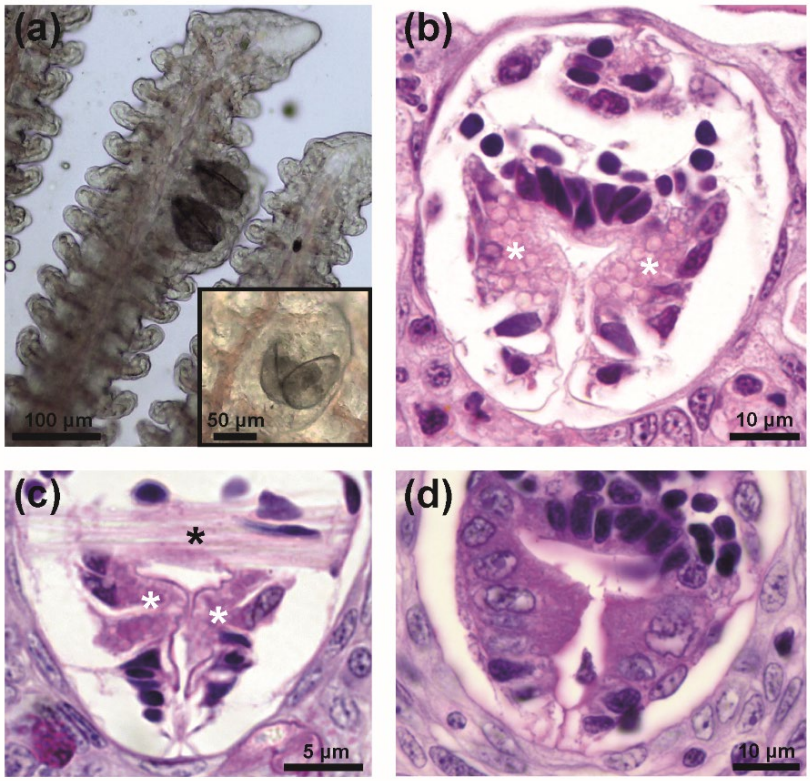


Figure 2. Stage I of the parasitic larva of *M. margaritifera* encysted in the Atlantic salmon gills during the day 0–3 post-exposure (PE). (a) Light microscopy of two recently encysted glochidia within the fresh gill tissue with closed valves and walnut shape. A few larvae failed to encyst, observed with their valves wide opened (Inset). (b–d) Histological sections of the closed bivalve, surrounded by the gill host tissue and showing at day 1 PE the inner mantle with coarse intracytoplasmic granules (white asterisks), eosinophilic (b) and PAS positive (c); becoming lost at day 3 PE (d). Anchored at both valves, the single larval adductor muscle (black asterisk, c) became atrophied and was absent at day 3 PE (d). H&E (b) and PAS stains (c and d).

3.2 Parasitic stage II

This stage, comprised between day 14 and 150 PE (around 130 and 1,400 degree-days), was characterized by a pronounced increase in length in most of the larvae (71.8 ± 3.2 up to 293 ± 2.4 µm), the formation of the zip membrane and the profuse development of the mantle, especially the mushroom body (Table 1).

In detail, larvae were externally characterized by the acquisition of bean-shaped valves, elongated in the antero-posterior axis since 500 degree-days PE (Figure 3a). Each valve was formed by a semi-transparent and single layer of thin and continuous periostracum (below 1 µm thick), in intimate contact with the surrounding host tissue (Figure 3a and b). Exceptionally, at the center of the hinge, the larval ligament was observed uncovered by periostracum (Figure 3c) and conformed internally by a basophilic U-shaped structure around 2 µm height (Figure 3d). The remaining valve cleft observed between the valve rims in the gill squash (Figure 3a), was filled by the zip membrane since 260 degree-days, identified as a membranous structure that isolated the pallial cavity from the surrounding host medium (Figure 3e–g). This membrane was composed of a homogeneous, acellular and basophilic material; stained magenta-blue under the PAS-AB stain (Figure 3f) and turkish-green under the trichrome stain (Inset, Figure 3g). Initially, the zip membrane arose as a thin and narrow structure which progressively became wider, increasing in width since 800 degree-days PE onwards (Figure 3g).

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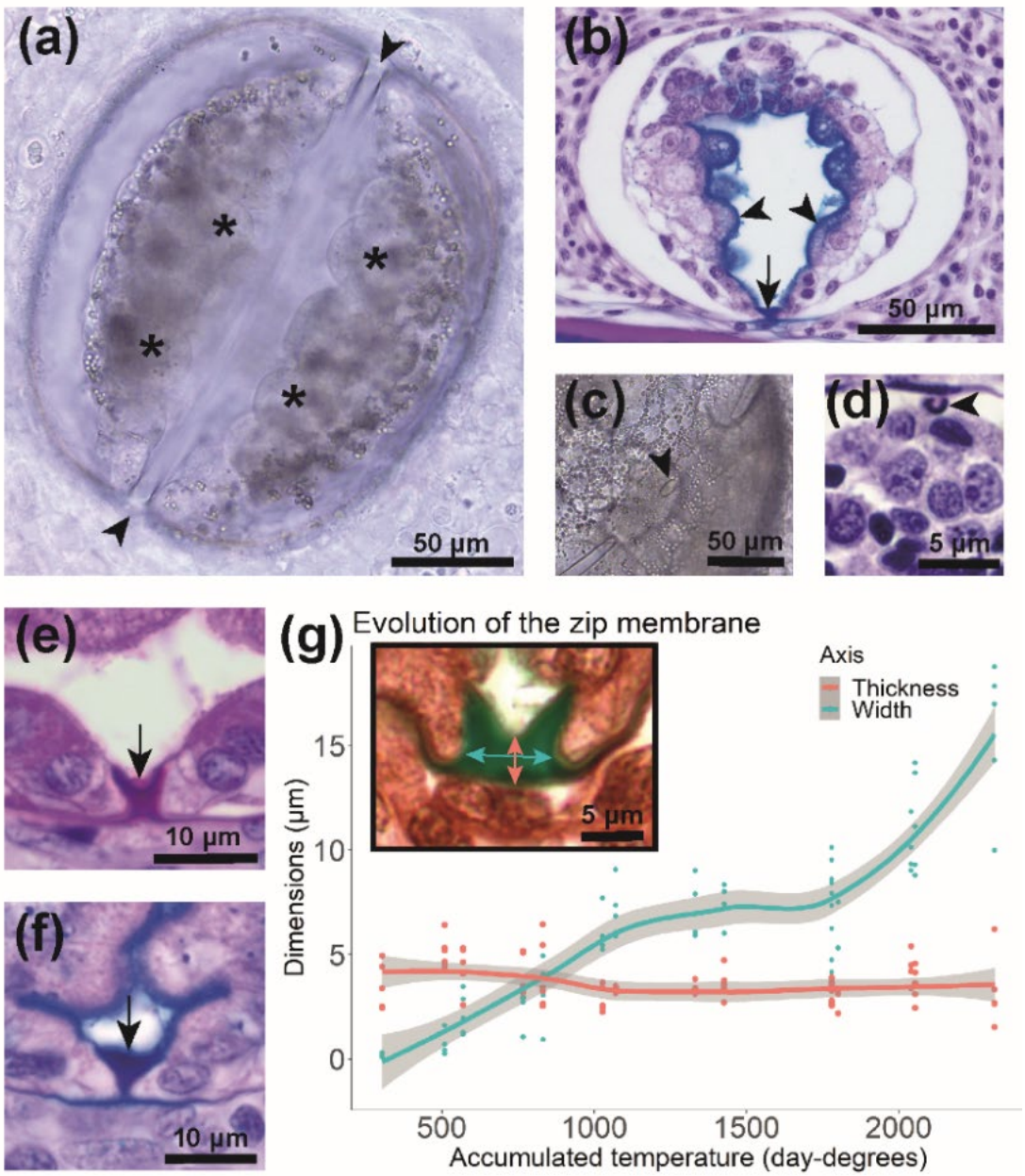


Figure 3. Stage II of encysted larva of *M. margaritifera* between the day 14 and 150 post-exposure (PE). (a) The gill squash highlights a pair of semi-transparent valves, the cleft between the valve rims (arrowheads) and the globose inner mantle cells (asterisks). (b) By light microscopy, the zip membrane (arrow) and the apical surface of the inner epithelium of the mantle was stained blue under the PAS-AB stain (arrowheads). (c and d) Microphotographs of the hinge region with a discontinuity on the periostracum (arrowhead, c), related to a basophilic U-shaped structure under the H&E stain (arrowhead, d). (e and f) Comparison of a histological section of the zip membrane (arrows) under PAS (e) and PAS-AB stain (f). (g) Evolution of the zip membrane dimensions (thickness and width) alongside the parasitism. Inset. Detail of the zip membrane highlighting the thickness as the distance among the periostracum of each valve (red arrow) and width as the distance among the external and outer surface of the zip membrane (blue arrow). Trichrome stain.

Due to the translucent valves, the larval mantle could be observed composed of an inner and outer epithelium with abundant refringent vacuoles clustered concentrically, evidenced by visualization of the gill squashes (Figure 4a). The outer layer was composed of a simple squamous epithelium that progressively acquired a cuboidal morphology, with a peripherally

located nucleus and a vacuolized cytoplasm with extensions towards the inner epithelium (Figure 4a–f). The central zone of the inner epithelium was covered mostly by the mushroom body (Figure 4b). This structure was composed of abundant, tightly packed and highly globose cells with their cytoplasm extended apically, acquiring the characteristic mushroom-like appearance (Figure 4b). Both outer and inner epithelium contained abundant lipid droplets, intensely stained black-blue under the Sudan black B stain (Figure 4c). In detail, each cell contained a round and extensive nucleus (7-8 μm of diameter) with a marked nucleolus and abundant vacuoles up to 2 μm diameter dispersed all over the basal region of the cytoplasm (Figure 4d). Moreover, an abundant and finely granular intracytoplasmic material (blue-magenta under the PAS-AB, diastase-resistant and slightly green-blue under the trichrome stain) was observed at the mushroom body and being secreted towards the pallial cavity (Figure 4e and f). The apical membrane of these globose cells was lined with microvilli, forming a brush border epithelium (Figure 4f). At the marginal zone, the inner mantle epithelium between the mushroom body and the pallial edges was composed of basophilic cuboidal cells which progressively became more abundant at the end of this stage (Figure 4d).

At the visceral mass of the larvae, abundant immature cells were observed with a small nucleus and basophilic cytoplasm. At the central region, a discontinuous digestive tract was progressively evidenced since 300 degree-days as a cluster of cells concentrically arranged forming a small lumen around 1,000 degree-days (Figure 4g). The epithelial cells shaping this tubular structure showed a simple and columnar epithelium, lacking cilia and with nucleus at basal position (Figure 4g). Haemocytes were observed since 1,000 degree-days PE, with a PAS positive cytoplasm, loosely located at the dorsoposterior region without lining endothelium (Figure 4h).

In contrast to this pronounced larval development, a few larvae showed a delayed development between 300 and 1,000 degree-days PE, characterized on the gill squashes by the roundish shape, small size (approximately half the size of the bean-shaped larvae) and poorly discernible mantle structures (Figure 4i).

Tissue fixation led to the occurrence of shrinkage artifacts that hindered the evaluation of the internal morphology of the larvae by histology, especially at the end of this stage. An inwards displacement of larval structures and tissues was observed in the most marked cases as hyperstained and irregular cell masses without discernible cell boundaries (Figure 4j). By comparison of the different fixatives, Dietrich´s fluid avoided the cell shrinkage causing minimal histological distortion, despite a moderate inward tissue displacement that remained present during all the study. On the other hand, the fresh frozen sections did not exhibit this artefactual displacement (Figure 4k) and revealed an external empty cavity all over the outer surface of the zip membrane, well-delimited by flattened gill epithelial cells and completely isolated from the external medium (Figure 4l and m).

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3.3 Parasitic stage III

Between the day 150 and 220 PE (around 1,400–2,500 degree-days), the encysted larvae ranged from 308 ± 17 to 397 ± 27 µm length and suffered a metamorphosis, confirmed by the formation of most of the organs which constitute the plantigrade, or post-larva (Table1). The morphological differences between the pre-metamorphosed and the encysted post-metamorphosed larva or post-larva, could be chronologically established before and after the day 200 PE or 2,000 degree-days, almost coincident with the mean start of the detachment of viable post-larva (Figure 1a).

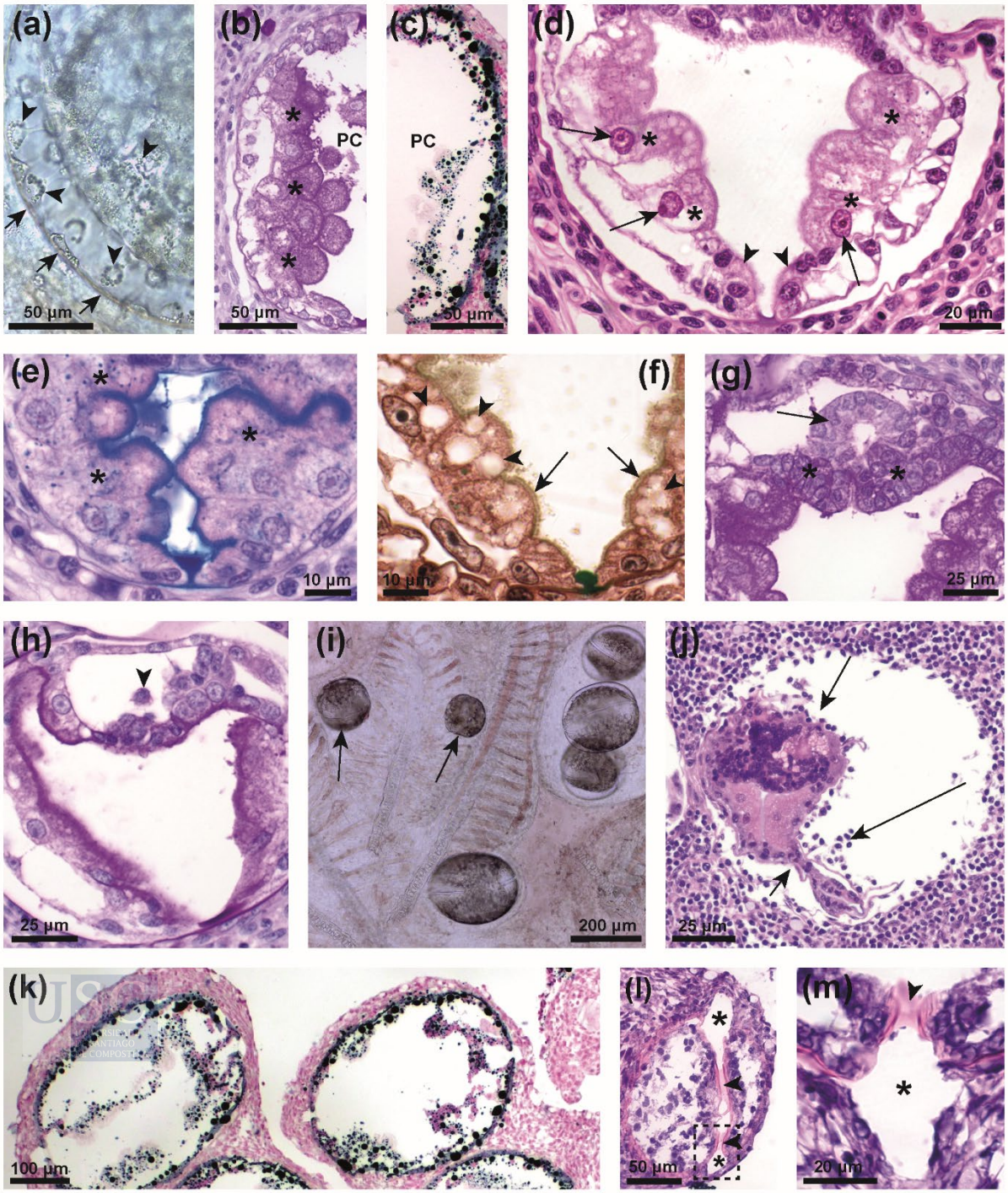


Figure 4. Stage II of the encysted *M. margaritifera* larva between the day 14 and 150 post-exposure. (a) Gill squash showing highly vacuolized mantle cells (arrowheads) which lied over the thin periostracum of the valves (arrows). (b) Light microscopy of the larval mantle displaying the outer mantle cells and the characteristic mushroom body at the inner mantle (asterisks), composed by globose cells showing a vacuolized and PAS positive cytoplasm which protruded towards the pallial cavity (PC). PAS stain. (c) Specular histological section of the mantle highlighting in black-blue the lipid vacuoles located both at the larval mantle surround the pallial cavity (PC). Sudan black B stain. (d) The larval mantle was composed by an outer and inner epithelium, the latter composed by the extensive mushroom body oriented towards the center of the pallial cavity (asterisks) and basophilic cuboidal cells at the pallial edges (arrowheads). Note the globose cells of the mushroom body with a round nucleus (arrows) with an evident single nucleolus. H&E stain. (e-f) Detail of the mushroom body containing a PAS positive intracytoplasmic granular material (asterisks, e) and abundant vacuoles (arrowheads, f). Note the brush-border at the apical region (arrows, f). PAS and trichrome stains, respectively. (g and h) Visceral region of the larva showing abundant immature cells (asterisks, g), a discontinuous digestive tract (arrow, g) and a haemocyte (arrowhead, h). PAS stains. (i) Gill squash evidencing two small larva at the upper left corner (arrows), with a roundish shape and poorly identified mantle structures, in contrast with larger larva (right and lower side). (j) Inner displacement (in the direction of arrows) of the larval internal structures together with the surrounding gill tissue of the fish, due to histological artifact during tissue processing. (k) Fresh frozen section without histological shrinkage, also evidencing abundant lipid vacuoles highlighted in black-blue. Sudan black B stain. (l and m) Detail of the well-delimited external cavity (asteriks) over the zip membrane (arrowheads), only visible in the frozen sections. H&E stains.

Between the day 150 and 200 PE (around 1400–1800 degree-days), the pre-metamorphosed larva was characterized by a partially developed digestive tract, foot and gills. Valves progressively darkened in contrast with the previous stage, hindering the visualization of the internal morphology on the gill squashes (Figure 5a). Regarding the mantle, the outer epithelial cells acquired a highly vacuolized cytoplasm with an intensely basophilic and PAS positive homogeneous material deposited as an amorphous thin layer over the inner side of the periostracum (Figure 5b–d). On the other side of the mantle, the inner cuboidal epithelium occupied a wider mantle region than the stage II and became as extensive as the mushroom body (Figure 5b).

At the visceral mass, the formation of the different portions of the digestive tract was accompanied by the apparition of a low number of haemocytes at the dorsoposterior region (Figure 5e). The digestive epithelium was hyperplastic and not completely differentiated, with poorly ciliated cells and abundant mitotic figures (Figure 5f and Inset). Moreover, since 1,600 degree-days PE, two bilateral evaginations arose antero-dorsally from the stomach, giving rise to a bilobed primordium of the digestive gland composed of highly granular epithelial cells (Figure 5g).

Towards the pallial cavity, the foot and the gills protruded ventrally and became hyperplastic showing an evaginated epithelium constituted by poorly organized and poorly differentiated cells with scant basophilic cytoplasm and abundant mitosis (Figure 5b, g and h). The primordium of the foot arose from the central region of the visceral mass (Figure 5g); while the gill buds (between one and two) began to evaginate at both sides of the posterior region (Figure 5h). All organs were infiltrated by abundant lipid droplets of variable sizes as demonstrated with the Sudan black stain (Figure 5i).

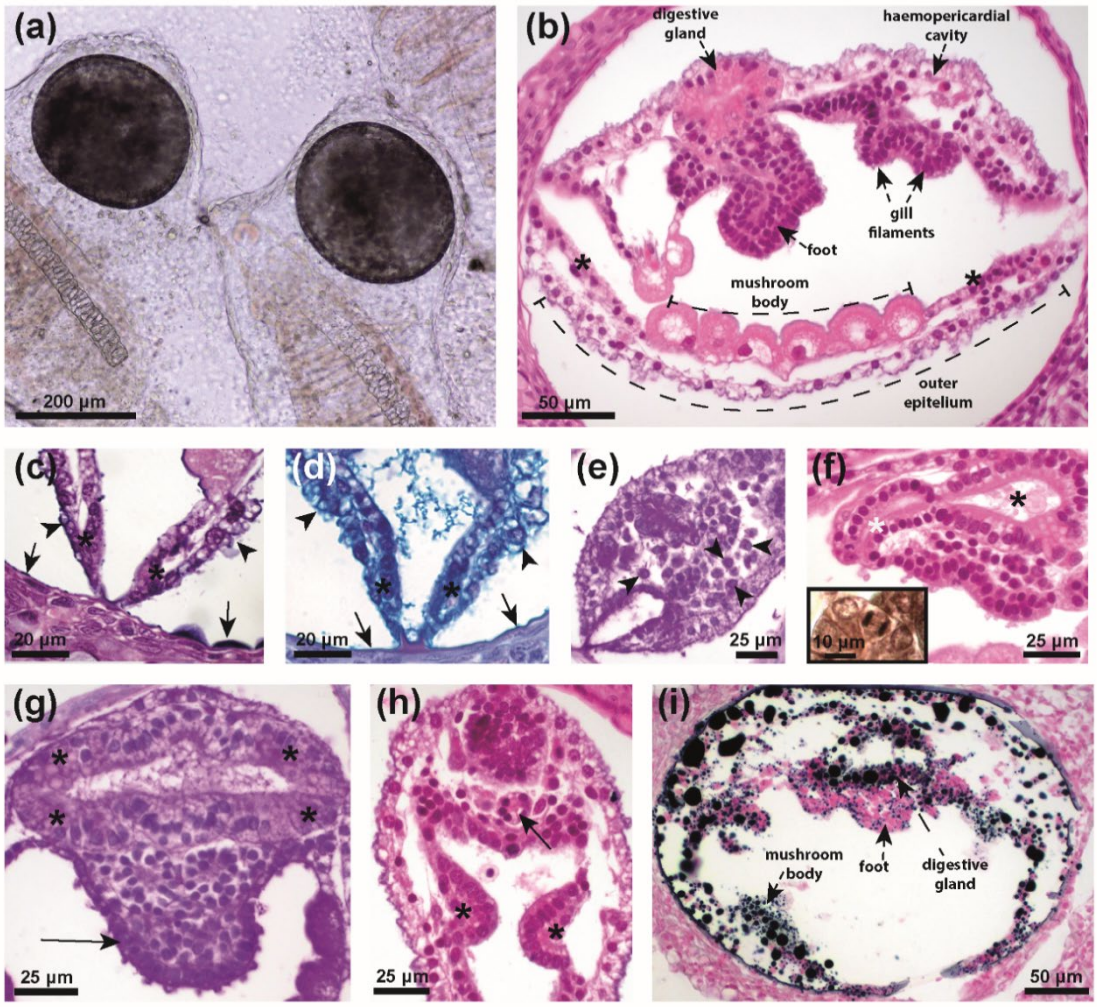


Figure 5. Stage III of the *M. margaritifera* pre-metamorphosed larva encysted in the salmon gills within the day 150 and 200 post-exposure. (a) Squash showing a pair of larvae with partially opaque valves, encysted in two gill filaments. (b) Low magnification of a histological section of an encysted larva showing the partially developed foot, gill filaments, digestive gland and haemopericardial cavity. The extensive inner cuboidal epithelium (asterisks) and the mushroom body can be distinguished in the inner part of the mantle, as well as the outer epithelium located towards the valves. H&E stain. (c and d). Detail of the mantle margin (asterisks) showing the basophilic and PAS positive amorphous material over the outer mantle epithelium (arrowheads) and below the periostracum (arrows). H&E and PAS-AB stains, respectively. (e) Dorsoposterior region of the larva containing certain haemocytes (arrowheads). PAS stain. (f). Anterior portion of the digestive tract showing a hyperplastic and poorly ciliated epithelium of the oesophagus (white asterisks) and stomach (black asterisk). H&E stain. Inset: Mitotic figure within the digestive lining epithelium. Trichrome stain. (g) Transversal histological section at the central region of the larva with the bilobed primordium of the digestive gland containing PAS positive granules (asterisks) as well as the primordium of the foot (arrow). PAS stain. (h) Transversal section at the posterior region of the larva where a pair of evaginated gill filaments (asterisks) and a portion of the intestine (arrow) were observed at the visceral mass. H&E stain (i) Frozen section showing abundant lipid vacuoles, highlighted black-blue all over the different larval tissues. Sudan black stain.

After metamorphosis, the post-larva was observed encysted in the gills between the day 200 and 220 PE (around 1,900–2,500 degree-days) and was characterized by the concurrence of post-larval organs with the larval structures described during the stage II (Figure 6 a–e). Valves were mostly opaque and black-brown all over their surface except the anterior and posterior adductor muscle insertions that appeared as partially translucid and roundish areas on

the valves (Figure 6a). The valve clefts were completely occluded by the zip membrane (Figure 7a–c), which was wider than previously (Figure 3h) and intensely magenta stained under the PAS-AB when compared to previous stages. Moreover, the pallial edges folded inwards so an outer and inner fold became discernible (Figure 7a–c). Each valve was compounded from an outermost layer of continuous periostracum with homogeneous thickness below 1 µm, birefringent under the polarized light (Figure 7d). Moreover, a fibrous ligament with similar staining properties to the periostracum was evidenced underneath the U-shaped ligament, protruding inwards as a semioval structure with approximately 22 µm length and 11 µm height (Figure 7e and f).

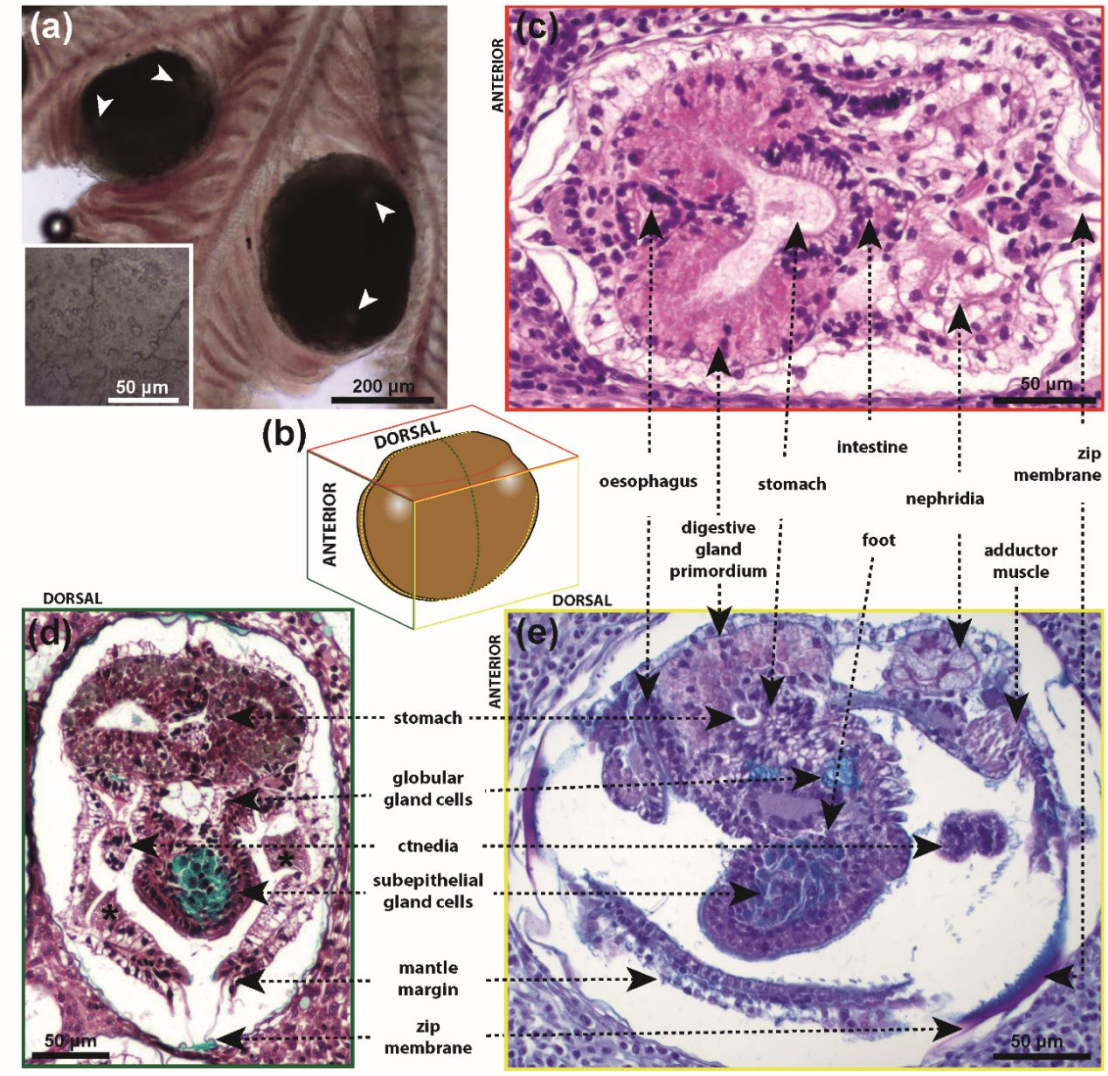
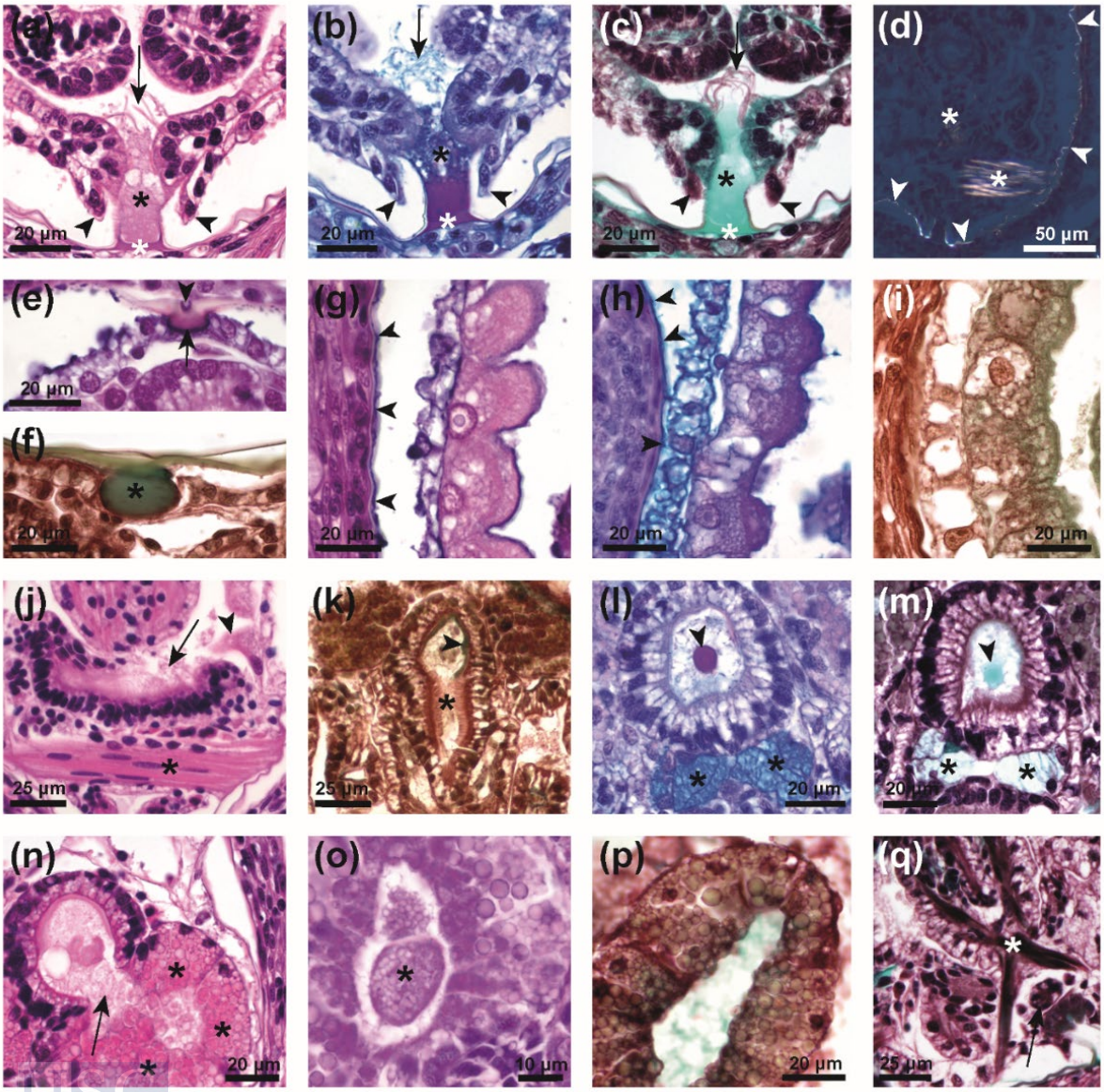


Figure 6. Tridimensional representation of the anatomy of the stage III of *M. margaritifera* post-larva encysted within the Atlantic salmon gills between the day 200 and 220 post-exposure. (a) Gill squash with two encysted post-larva showing almost entirely opaque valves, excepting the two insertions of the adductor muscles (white arrowheads). Inset: Higher magnification of the valve surface showing a black-brown colour. (b) Diagram of the post-larval shell, viewed from the left side, and showing the two adductor muscle insertions (white areas) in relation to three anatomical planes of section highlighted in red (dorsal), green (transversal) and yellow (longitudinal). (c-e) Representation of the main organs and structures in the different sections. (c) Dorsal histological section. H&E stain. (d) Transversal histological section. Trichrome stain. (e) Longitudinal histological section. PAS-AB stain.

A thin basophilic layer coated irregularly the inner face of the periostracum (Figure 7g). This material, stained blue by PAS-AB (Figure 7h), did not stain specifically under the alizarin red S nor the trichrome stain (Figure 7i). On the other side, the internal epithelium of the mantle resembled that of the adult mussel since the mushroom body at the central zone regressed in size, being replaced by a cuboidal epithelium (Figure 6d). At the marginal zone, the inner epithelium became columnar, bore long cirri and contained a blue and turkish-green intracytoplasmic material under the PAS-AB and the trichrome stains, respectively (Figure 7a–c). An abundant mucinous material was located between both mantle margins, becoming more eosinophil and deep-blue under the H&E and PAS-AB stains, respectively (Figure 7a and b).



The digestive tract showed a high differentiation of the different portions with an evident development of cuboidal epithelial cells with cilia (Figure 7j–m). The oral groove was observed as a broad and ciliated opening with poorly developed labial palps, observed as mild and non-plicated protrusions (Figure 7j). The oesophagus was plicated and ciliated, surrounded by the cerebroideal ganglion (Figure 6c and e). The stomach presented a dorsal region with round

Figure 7. Stage III of *M. margaritifera* post-larva encysted within the Atlantic salmon gills between the day 200 and 220 post-exposure. (a-c) Detail of zip membrane filling the valve cleft (white asterisks), as well as the outer margin (arrowheads) and inner margin of the mantle, the latter provided with long cirri (arrows). Note the lack of continuity of the valve periostracum with the zip membrane and the slightly different staining properties of the zip and the mucinous material located towards the pallial cavity (black asterisks). H&E (a), PAS-AB (b) and trichrome (c) stains. (d) Histological section highlighting the periostracum (arrowheads) and the muscle fibers (asterisks) under polarized light. Trichrome stain. (e) Transversal section of the hinge region showing the fibrous ligament (arrow) underneath the U-shaped ligament (arrowhead). H&E stain. (f) Longitudinal section of the fibrous ligament (asterisk) showing a semioval shape. Trichrome stain. (g-i) Detail of the outer mantle epithelium lying over the periostracum, with a basophilic material in between (arrowheads), stained under the H&E stain (g) and blue under the PAS-AB stain (h), but unstained with the trichrome stain (i). (j) Ciliated simple columnar epithelium of the oral groove (arrow), besides the anterior adductor muscle (asterisk). Note the proximity of one globose mantle cell of the mushroom body (arrowhead). (k) Stomach with the dorsal region covered by a thin gastric shield stained turkish-green with the trichrome stain (arrowhead), and a funnel-shaped style sac ventrally (asterisk). (l and m) The crystalline style (arrowheads) was visualized magenta and light-blue under the PAS-AB and trichrome stains, respectively. Ventrally to the stomach, the globular gland cells of the byssal complex (asterisks) were observed blue with the PAS-AB stain, but poorly light-blue stained with the trichrome stain. (n) Communication between the stomach and the primordium of the digestive gland (arrow), the latter containing a high number of eosinophilic granules (asterisks). H&E stain. (o and p) Detail of the digestive gland epithelium with abundant PAS-positive and yellowish granules under the PAS and trichrome stain, respectively. Note the vacuolized and finely granular PAS-postive material within the lumen (asterisk, o). (q) Detail of the intestine and rectum (arrow) anatomically related with the muscular chiasma formed by the retractor muscles (asterisk). Trichrome stain.A thin basophilic layer coated irregularly the inner face of the periostracum (Figure 7g). This material, stained blue by PAS-AB (Figure 7h), did not stain specifically under the alizarin red S nor the trichrome stain (Figure 7i). On the other side, the internal epithelium of the mantle resembled that of the adult mussel since the mushroom body at the central zone regressed in size, being replaced by a cuboidal epithelium (Figure 6d). At the marginal zone, the inner epithelium became columnar, bore long cirri and contained a blue and turkish-green intracytoplasmic material under the PAS-AB and the trichrome stains, respectively (Figure 7a–c). An abundant mucinous material was located between both mantle margins, becoming more eosinophil and deep-blue under the H&E and PAS-AB stains, respectively (Figure 7a and b).

shape and non-ciliated epithelium covered by a thin gastric shield. At the ventral region, the style sac was funnel-shaped, oriented posteroventrally, lined by a ciliated, pseudostratified columnar epithelium and contained the crystalline style (Figure 7k–n). Both crystalline style and gastric shield were acellular structures with diffuse limits that stained magenta or light-blue under the PAS-AB and the trichrome stains, respectively (Figure 7k–n). The lateral region of the stomach is communicated by two openings with the bilobed primordium of the digestive gland (Figure 6c), which contained refringent, eosinophilic granules of highly variable size up to 6 μm diameter (Figure 7n). These granules intensely stained PAS positive towards the lumen of the diverticula (Figure 7o) and were yellowish under the trichrome stain (Figure 7p). The intestine was lined by ciliated columnar epithelium and formed a short loop inside the visceral mass, but no typhlosole was observed (Figure 6c and 7q). The rectum passed through the renopericardial region, between the posterior pedal retractor muscles and opened to the pallial cavity at the anal region (Figure 7q).

Between the paired gill filaments, a ciliated foot extended from the visceral mass towards an antero-ventral position (Figure 6d and e). It was supported by abundant muscle fibers interposed in multiple directions all over the foot and by two well-developed retractor muscles which created a muscular chiasma dorsally at the medial line (Figure 7q and Figure 8a). Two different types of glandular tissue could be observed into the foot, conforming the byssal complex. At the proximal region of the foot, between the pedal ganglia and the stomach, large

globular and vacuolized gland cells with PAS positive cytoplasm not stained by H&E nor the trichrome stain (Figure 7m) were observed in a clustered fashion (Figure 8a). This gland tissue extended towards the posterior region of the foot and opened into a diverticulated and ciliated byssal duct which continued ventrally and opened into the byssal groove, a narrow longitudinal invagination at the posteroventral midline of this organ (Figure 8b). No byssus thread was observed at the lumen. At the apical region of the foot, a large number or unicellular glands were densely packed underneath the lining epithelium. These gland cells contained abundant small intracytoplasmic granular material which stained PAS positive and turkish-green under the trichrome stain; this material was also observed inside the cytoplasm and over the surface of the epithelial cells (Figure 8a–d). Superficially, the foot was lined by a ciliated, simple columnar epithelium (Figure 8a–d).

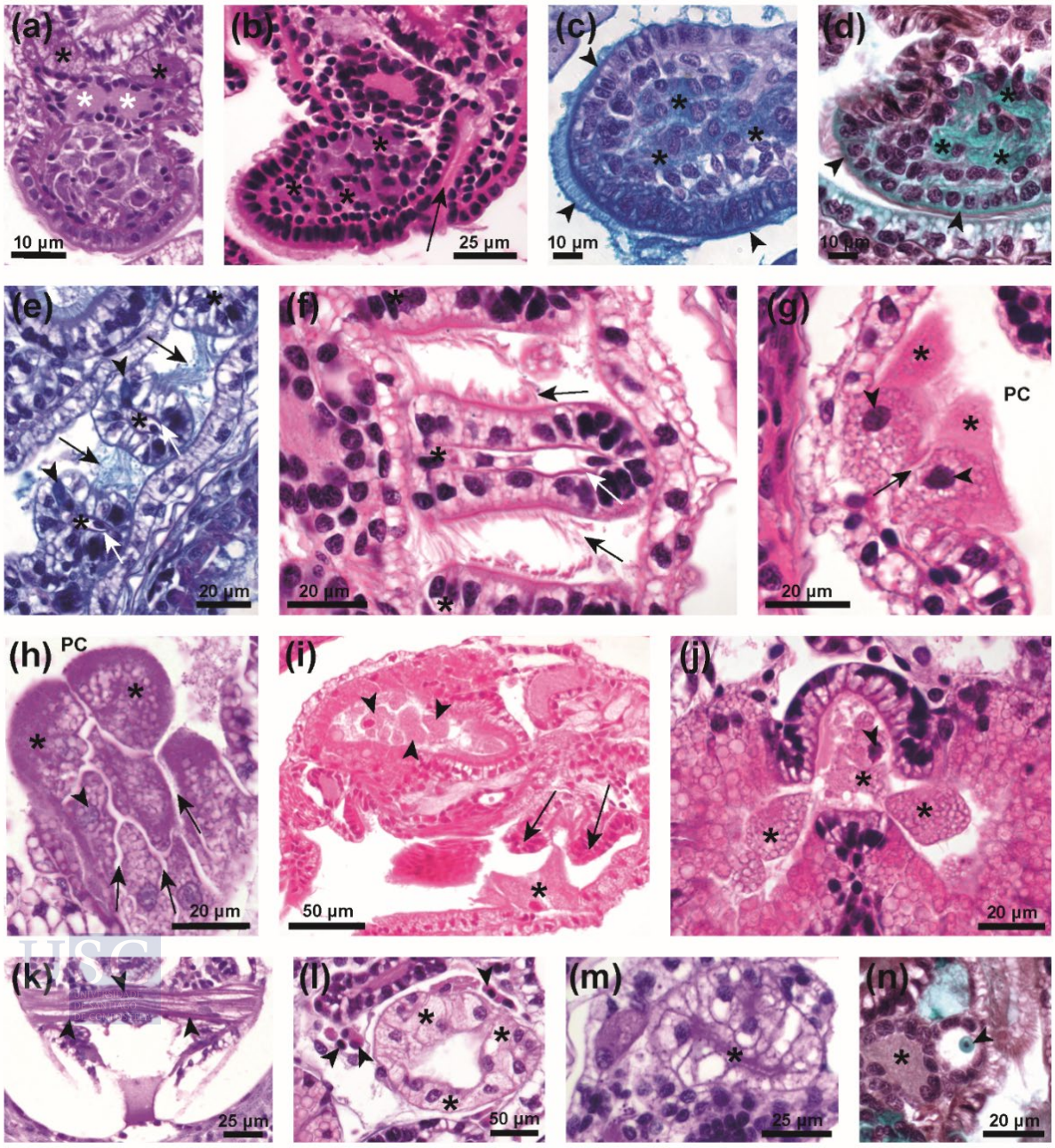


Figure 8. Stage III of the *M. margaritifera* post-larva encysted within the Atlantic salmon gills between the day 200 and 220 post-exposure. (a) Transversal sections of the foot showing PAS-positive globular gland cells of the byssal complex (black asterisks), in a dorsoposterior location in respect to the bilobed pedal ganglia (white asterisks). PAS stain. (b) Longitudinal section of the foot, exhibiting the byssal duct (arrow) at the posteroventral midline and highly packed subepithelial gland cells of the byssal complex (asterisks) at the apical region. H&E stain. (c and d) Detail of the granular content of the subepithelial gland cells (asterisks) at the apical region of the foot, highlighted blue under the PAS-AB stain and turkish-green under the trichrome stain. Note the presence of similar tinctorial features at the cytoplasm of the highly ciliated epithelium and over the surface of the foot (arrowheads). (e and f) Three ctenidia (asterisks) positioned between the mantle and the visceral mass, supported by chitinous rods (white arrows) and lined by a ciliated epithelium (black arrows) and a few goblet cells (arrowheads). PAS-AB and H&E stains, respectively. (g and h) Detail of the mushroom body with marked intercellular spaces (arrows), shrunken nucleus (arrowheads) and vacuolized and PAS positive cytoplasmic portions partially sloughed (asterisks) towards the pallial cavity (PC). H&E and PAS stains, respectively. (i) Low magnification showing the remnants of the mushroom body (asterisk) molded by the adjacent ctenidia (arrows) and abundant cellular material (arrowheads) within the lumen of the stomach and the digestive gland primordium. H&E stain. (j) High magnification of the central stomach and the lobulated digestive gland primordium containing cellular fragments with shrunk nucleus (arrowhead) and abundant vacuoles (asterisks). H&E stain. (k) Detail of the posterior adductor muscle conformed by PAS positive myofibers (arrowheads). PAS stain. (l) Renopericardial cavity containing few haemocytes (arrowheads) and the nephridium with a highly vacuolized epithelium (asterisks). H&E stain. (m) Nephridium displayed an apical cytoplasmic portion with a finely granular PAS positive material oriented towards the lumen (asterisk). PAS stain. (n) Statocyst near the pedal ganglia (asterisk) lined by a cuboidal simple epithelium containing a turkish-green statolith (arrowhead) with the trichrome stain.

Anchored to the posterior region of the visceral mass, three pairs of ctenidia occupied each lateral region of the pallial cavity (Figure 8e and f), being oriented towards an antero-ventral direction (Figure 6d and e). Each ctenidium was highly vascularized, supported by thin chitinous rods and lined by a simple columnar epithelium with few goblet cells (Figure 8e). The lateral epithelial cells bore abundant long cilia which progressively increased in number during this stage and contacted the cilia of the adjacent filament (Figure 8f).

The globose cells which comprised the remaining mushroom body were scarce and presented wide intercellular spaces, a shrunken nucleus and highly vacuolized cytoplasm with abundant PAS positive material at the apical border (Figure 8g and h). Moreover, abundant cellular portions were observed partially sloughed at the pallial cavity (Figure 8h) and the remaining epithelial surface of the cytoplasm displayed concave depressions being molded by the adjacent ciliated ctenidial and pedal epithelia (Figure 8i). Remnants of cells similar to those aforementioned were observed inside the lumen of the stomach and the primordium of the digestive gland, characterized by a shrunk nucleus, abundant vacuoles and finely PAS positive granular material within the cytoplasm (Figure 7o, 8i and j).

At the dorsal region, the anterior and posterior adductor muscles measured around 20 μm of diameter and were intermingled by abundant bundles of myofibers (Figure 8k). The renopericardial cavity was located at the posterodorsal region of the visceral mass, containing a paired and well-developed nephridia, with excretory cells arranged towards a lumen and showing a highly vacuolized cytoplasm with PAS positive substance at the apical border (Figure 8 l and m), and a high number of haemolymphatic cells, as haemocytes and granulocytes (Figure 8l). In addition, the complete development of the nervous system was confirmed by the presence of the cerebroideal, visceral and pedal ganglia (Figure 8a). Besides the bilobed pedal ganglia, a paired statocyst could be identified (Figure 8n).

During the three parasitic stages, larvae grew a 670% in length, from 59 ± 2.6 to 397 ± 23 µm, and the larval length was positively correlated to the degree-days (Spearman's coefficient .97), displaying a significant linear regression ($y = 67.9 + 0.149x$, p-value < .05; Figure 9a).

After detachment from the gill, the post-larva showed a variable size in which the viable individuals were significantly longer (361 µm) than the unviable ones (314 µm) (p-value < .001; Figure 9b). Externally, valves showed ivory, smooth and continuous periostracum lacking any remnant of the previous surrounding gill tissue of the host (Figure 9c). The internal morphology of the viable post-larva was similar to that described during the previous stage III (Table 1), but lacking the zip membrane, so that the pallial cavity communicated with the medium through the valve cleft, where the foot occasionally protruded towards (Figure 9d). Dorsally, these valves remained anchored by the semioval fibrous ligament (Inset, Figure 9d).

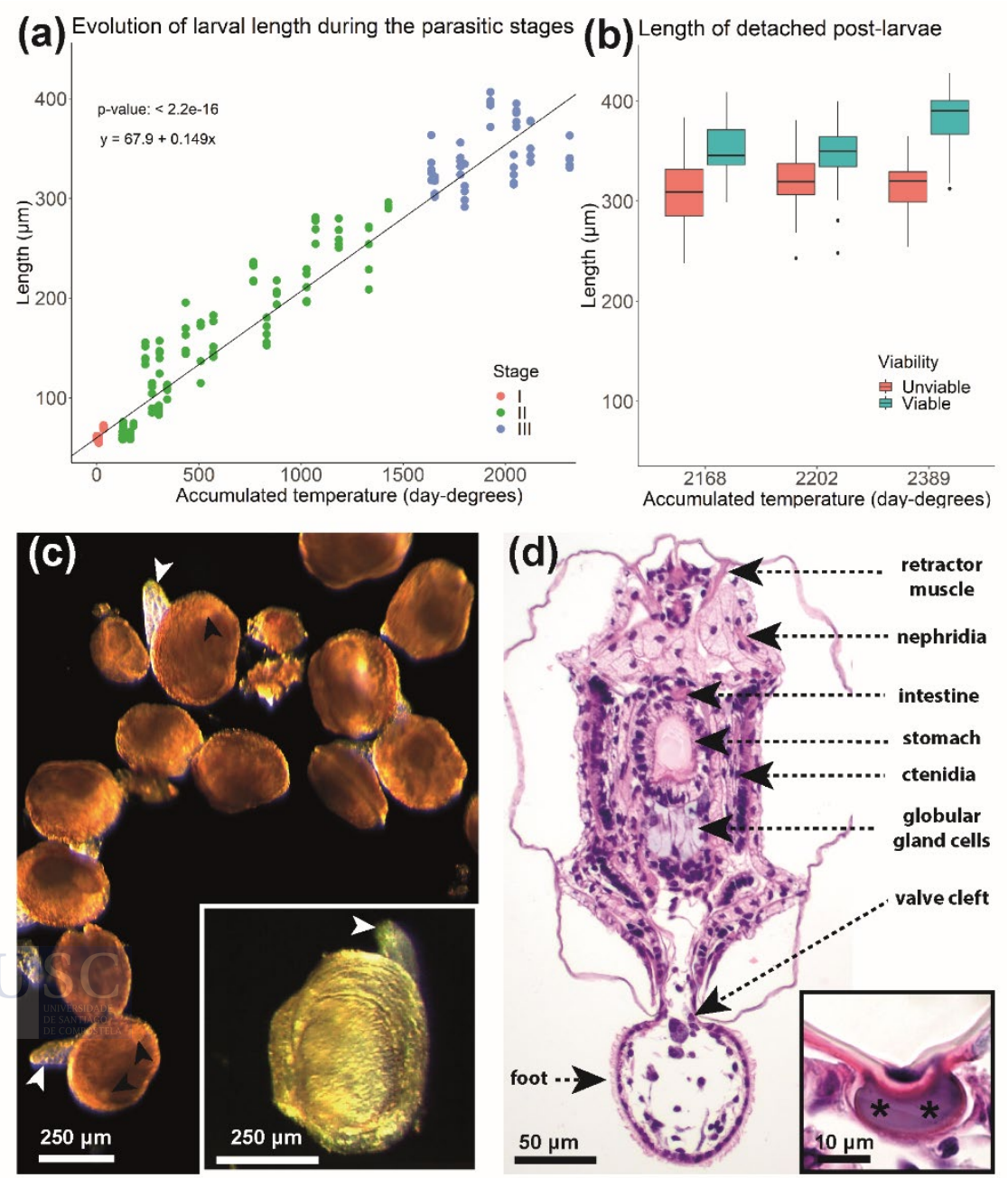


Figure 9. (a) Evolution of larval length during the three parasitic stages (I-III), highly correlated with the accumulated temperature. (b) The length of the viable detached post-larva showed a significant bigger size than the unviable detached post-larvae (***; *p*-value < .001). (c) Stereomicrophotograph of the viable post-larva, recently detached from the fish gills with ivorish valves and protruding foot through the valve cleft (white arroheads). In some specimens, the insertion of both adductor muscles (black arrowheads) could be observed. Inset: Detail of the external surface of the continuous periostracum without any remnant of the gill tissue. (d) Histological image at low magnification of a detached post-larva, showing a similar morphology to the encysted larvae except for the foot which protruded towards the valve cleft. Inset. Detail of the semioval fibrous ligament at the hinge region (asterisks).

4. DISCUSSION

This study provides an exhaustive description of the larval development and organogenesis of the freshwater mussel *M. margaritifera* in the gills of Atlantic salmon (*S. salar*). This knowledge is essential to establish the sequential growth stages of the larva and also to understand the host-parasite interaction, which has been scarcely addressed.

During this 7-month parasitism, larva overwintered in the fish gills and grew over six-folds in length; up to 308–397 μm, similarly to previous studies in *M. margaritifera* (Araujo et al., 2018; Bauer, 1987a; Eybe et al., 2015; Marwaha et al., 2017; Schartum et al., 2016; Young & Williams, 1984b). This slow but marked development contrast with other larger glochidia species with around 200–400 μm length, which showed a negligible growth during its much shorter parasitism (Bauer, 1994; Wächtler et al., 2001). Despite this different development rate among species, the organogenesis took place in a similar continuous fashion, divided from two to six sequential stages, as depicted in margaritiferids (Araujo et al., 2002; Scharsack, 1994) and other unionids (Bändel, 1988; Chumnanpuen et al., 2011).

The stage I could be regarded as a short and transitional period in which the typical morphology of the small glochidia vanished. During the first three days of parasitism, the pinched epithelial cells of the gill became rapidly disintegrated by the enzymes contained in the coarse granules of the inner mantle (Castrillo et al., 2020; Lefevre & Curtis, 1910; Pekkarinen & Valovirta, 1996). Subsequently, during the long and intermediate stage II, comprised between the day 14 and 150 PE, the encysted larva was enclosed by the valves and the zip membrane in between the valve cleft, providing an interface between the outer gill tissue of the fish and the inner larval tissues.

Valves were articulated at the dorsal region by a U-shaped ligament which might counteract the larval adductor muscle of the glochidia, supporting previous descriptions of the hinge ligament in other unionid described by scanning electron microscopy (Chumnanpuen et al., 2011; Schwartz & Dimock, 2001; Uthaiwan, Chatchavalvanich, Noparatnaraporn & Machado, 2001). Once the larval adductor muscle was lost, a fibrous ligament became evident at the terminus of the parasitism and resembled the primitive and provisional ligament structure of *M. margaritifera* which is also vanished six months after detachment from the fish gills (Pennec & Jüngbluth, 1983). These different ligament formations suggest a discontinuous ligament ontogeny as described in several Palaheterodont taxa (Bändel, 1988; Sartori & Ball, 2009).

Each valve was comprised of a single and extremely thin layer of periostracum with no signs of calcification under the alizarin red S stain, similar to previous electron microscopy

descriptions of the *M. margaritifera* glochidium (Nezlin et al., 1994; Pekkarinen & Valovirta, 1996). This contrast with the much thicker shell of many other glochidia within the family Unionidae, which contained an inner and porous calcareous layer underneath the thin periostracum, referred to as cuticle or pellicle (Bändel, 1988; Chumnanpuen et al., 2011; Lasee, 1991; Lima et al., 2006; Rand & Wiles, 1982; Schwartz & Dimock, 2001; Uthaiwan et al., 2001). To structurally support the thin valves of *M. margaritifera* larva, fluid-filled reservoirs described in the flanges could tighten the clasping valves of the glochidia (Pekkarinen & Valovirta, 1996) and, similarly, the fluid enclosed within the encysted larva might also maintain the structure of the larval valves. Nevertheless, the progressive increase in the valve opacity and the accumulation of an acidic material between the periostracum and the outer mantle might indicate the onset of the shell biomineralization towards the end of the parasitic phase (stage III), as has been described during the early shell secretion by the outer mantle epithelium in the post-larval stages of bivalves (Addadi, Joester, Nudelman & Weiner, 2006; Checa, 2018).

At the valve cleft, the zip membrane, recently described as a C-shaped proteinaceous barrier located over the valve rims (Castrillo et al., 2020), delimited completely the pallial cavity from the outer gill microenvironment during most of the *M. margaritifera* parasitism. This membrane might isolate the larva and avoid the exit of the disintegrated clasped tissue or enzymes from the pallial cavity through the valve cleft, limiting the triggering of the host immune response. However, before the formation of the zip membrane, the material aforementioned is susceptible to elicit an early immune response as described during the first 14 days of glochidiosis, related to the early detachment of immature larva (Castrillo et al., 2020). In this sense, despite a specific immune response against the glochidia of *Lampsilis reeveiana* was described in *Micropterous salmoides*, there is no information about the antigenicity of the different parasite structures (Dodd et al., 2006). Further studies need to elucidate the antigenic role of the different larval structures during these critical early stages, which could bet in touch—prior to the formation of the zip membrane— with surrounding gill tissue. Moreover, the external surface of the larva itself, with the scleroproteinaceous periostracum and zip membrane, should not be dismissed.

Considering the extreme thinness of the larval shell, the formation of the zip membrane, and the absence of a functional digestive apparatus during most of the parasitism, non-particulate nutrients of the gill interstitial fluid might diffuse through these external structures and serve as the main source of nutrition to the *M. margaritifera* larva, as evidenced by stable isotope analysis (Denic et al., 2015). This source of nourishment from non-particulate organic matter has been already described in the veliger larva of the family Ostreidae through the velum (Manahan & Crisp, 1982).

In intimate contact with the valves, the mantle of the encysted larva developed a different morphology characterized by the extensive mushroom body; a tissue described exclusively during the parasitic stage of *M. margaritifera* (Scharsack, 1994) and other few unionids (Arey, 1932c; Blystad, 1923; Chumnanpuen et al., 2011). For the first time, the accumulation of lipid droplets during the larval development of *M. margaritifera* was demonstrated by the observation of abundant lipid vacuoles in the larval mantle. These results complement previous descriptions in *U. imbecillis* larva, in which the mushroom body accumulated lipid droplets and

glycogen granules, observed by transmission electron microscopy (Fisher & Dimock, 2002); although no glycogen material was discernible with the PAS-diastase stain in our study. This abundant lipid content could be regarded as energy storage essential for the metamorphosis into post-larva, similar to that described during the early development of marine bivalves (Gallager, Mann & Sasaki, 1986). Based on the fact that the lipid droplets appeared only while the larva remained encysted in the fish, Fisher and Dimock (2002) hypothesized that the mushroom body might take part in the uptake of nutrients through the microvilli located in their apical surface, in a similar way to the epithelium of the digestive gland of adult bivalves (Owen, 1974). Such microvillar appearance of the mushroom body was also evidenced in *M. margaritifera* by electron microscopy (Scharsack, 1994). Based on this parasitic nutrition, the quantification of the lipid content could serve as a potential indicator of the larval status during the parasitism as described during the development of *Unio crassus* and *Anodonta anatina* juveniles (Douda, 2015). On the other hand, the gill cryosection revealed its potential, not only for the preservation of lipids, but also for the evaluation of the larval structures by reducing the histological fixation artifacts. To deal with the apparition of these artifacts, an thorough selection of the best and most representative images was required.

Due to this morphology and functionality, different from the glochidium, we propose to name “mushroom larva” to the larval form described during the parasitic stage II, grounded in the first descriptions of a similar intermediate “mushroom body stage” coined during the parasitism of unionids (Blystad, 1923). Nevertheless, the marked vacuolization of the mantle of anodontine glochidia, prior to the encystment into the fish (Pekkarinen, 1996; Wood, 1974), may allude to a previous embryonic nourishment during the matrotrophic brooding in those large glochidia with short parasitic periods (Schwartz & Dimock, 2001). Besides nutrition, the larval mantle of unionids has been also proposed as a multifunctional organ involved also in respiratory, osmoregulatory and excretory functions (Castilho, Machado, Reis & Sá, 1989; Rand & Wiles, 1982; Schwartz & Dimock, 2001). Some of the most relevant organs involved in the aforementioned functions, i.e., the ctenidia and nephridia, were morphologically absent or non-functional during most of the parasitism; thus, it seems plausible that the extensive larval mantle might counteract the hyperosmotic fish tissue (Nezlin et al., 1994; Pekkarinen & Valovirta, 1996). Further studies are needed to evaluate the permeability of the zip membrane and the periostracum to the nutrients, gases, salts and waste products, together with the potential role that the mantle cells might play in the exchange of these substances as it has been proposed by Robertson and Coney (1979).

Between the day 150 and 220 PE, the stage III was associated with the larval metamorphosis, characterized histologically by an intense and progressive organogenesis, leading to the detachment of viable post-larva since approximately the day 200 PE (1,900–2,200 degree-days), similar to previous studies in which the onset of post-larval detachment started around 1,700–1,800 day-degrees (Castrillo et al., 2021; Eybe et al., 2015; Marwaha et al., 2017; Taeubert et al., 2013). This detachment was successfully synchronized by the high cumulative temperatures in combination with the increase of water temperature over 15ºC, both considered as important trigger factors of larval metamorphosis (Hruska, 1992; Marwaha et al., 2017; Scheder et al., 2014). On the other hand, it was reported that extremely cold water (1–

3ºC) might also slow down this development rate up to a diapause stage (Murzina et al., 2017; Scheder et al., 2014); although this was not observed in this study, since the larval growth was linear (approximately 0.15 µm/day) and temperatures were over 6ºC.

The morphological features described after the day 200 PE—exclusively in the metamorphosed post-larva—could be employed to monitor the larval metamorphosis in the fish gills during the breeding programmes of *M. margaritifera*. Among those features, the indirect observation of the anterior and posterior adductor muscles through the valves might serve as a promising tool to easily distinguish the larval metamorphosis by wet mount of fish gills; being comparable with the formation of the pigmented larva eye spot which precedes the metamorphosis of *Ostrea edulis* (Waller, 1981) or the visualization of the adductor muscles reported in the detached juvenile unionid of *Hyriopsis myersiana* (Kovitvadhi, Kovitvadhi, Sawangwong & Machado, 2007). On the other hand, the presence of globose cells of the mushroom body contrast with previous studies which described a completely absent mushroom body after 10 months of parasitism (Scharsack, 1994). These mushroom body remnants in the recently detached post-larva might suggest a premature detachment and could be considered as a potential indicator of an incomplete metamorphosis, in which the artificial increase in water temperature might synchronize, but also precipitate the detachment of the outermost larvae (Castrillo et al., 2021).

The appearance of the post-larval organs since day 200 PE indicated a completely different morphology which is essential for the settlement into the benthos and the acquisition of a deposit-feeding lifestyle, as has been described for the *M. margaritifera* juveniles (Araujo et al., 2018; Lavictoire et al., 2018; Schartum et al., 2016). The post-larva anatomy, acquired at the end of the parasitism, is surprisingly similar to that described during the plantigrade stages in many different species of marine bivalves, which is crucial for the settlement and nourishment in the benthos (Baker & Mann, 1997; Bower & Meyer, 1990; Carriker, 2001).

The post-larval foot was internally characterized by different glandular tissues described for the first time in *M. margaritifera*, resembling some of the glandular tissues which conform the byssal complex of the recently detached juveniles of the freshwater mussel *Lampsilis cardium* (Lasee, 1991) but also other marine bivalves (Gruffydd, Lane & Beaumont, 1975). The subepithelial gland cells located at the tip of the foot were morphologically similar to the gland type A of *Ostrea edulis* (Cranfield, 1973), which exhibit similarities to the tip attachment glands of scallops, where a mucinous merocrine substance is secreted through the epithelium by ducts and is involved in the pedal attachment after metamorphosis (Bower & Meyer, 1990; Gruffydd et al., 1975). Similar cells, termed as “violet cells”, were similarly characterized in the foot of other adult freshwater mussels, although the role of these cells has not been elucidated yet (McElwain & Bullard, 2014). In addition to the subepithelial gland cells, globular gland cells extending over the pedal ganglion towards the dorso-posterior region of the foot were morphologically similar to the gland type D4 of *Ostrea edulis* (Cranfield, 1973). Its location and anatomical relation to the byssal duct suggests that this gland tissue corresponded with the primary byssus gland described in scallops (Bower & Meyer, 1990; Gruffydd et al., 1975). This duct opened into the byssal groove, a longitudinal invagination described in the foot of *M. margaritifera* juveniles (Araujo et al., 2018). Nevertheless, the formation of the

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byssal thread was not confirmed until the formation of a macroscopical byssal thread in more advanced juvenile stages of *M. margaritifera* (Lavictoire et al., 2018), *Lampsilis radiata* and *Elliptio complanata* (Smith, 1998). In absence of a byssal thread after metamorphosis and detachment from the fish gill, the pedal attachment has been proposed in several juvenile unionids as an essential mechanism for the settlement and burrowing by action of the adhesive mucus (Bradley, 2011; French & Ackerman, 2014) and the well-developed muscle system of the foot. Despite the potential role of the byssal complex in the settlement of juvenile unionids, the post-larval foot remain poorly studied and require further characterization of the different gland tissues and secreted products.

Moreover, these mucinous and/or collagenous secretions observed over the tip of the foot may be involved in the physical and immune protection, but also in the agglutination of feeding particles. Considering that deposit-feeding occurs in many autobranch juveniles before the gills are capable of suspension-feeding, the highly protractile and ciliated foot could create a feeding current that transports the fine particulate algae and bacteria from the river sediment to the labial palps (Yeager et al., 1994). Once the food is ingested, the ciliated stomach displayed a similar morphology to the typical type IV stomach of freshwater bivalves, being capable of sorting, triturating and digesting food particles. In communication with the stomach, the bilobed primordium of the digestive gland presented a similar granular appearance to *M. margaritifera* and *P. auricularius* larva at the end of the parasitism (Araujo et al., 2002; Scharsack, 1994), but contrast with the ductal system of the bivalve´s digestive gland, lined by digestive and basophilic cells (Owen, 1974). This different morphology might point towards different post-larval nutrition during and/or just after the parasitism, in which the abundant granules might correspond with a proteinaceous material involved in the enzymatic digestion and extracellular digestion of the digestive gland.

Within the lumen of the stomach and digestive gland primordium, a large number of vacuolized cell fragments were present, similarly to that described at the end of the parasitic stage in other freshwater mussels (Arey, 1932c; Chumnanpuen et al., 2011; Scharsack, 1994) and resembled sloughed cell fragments from the mushroom body to the pallial cavity. This abundant material inside the digestive lumen when the valves remained closed by the zip membrane leads to the speculation that the mushroom body might be detached and ingested, similar to the ingestion of the large and yolky test cells during the metamorphosis of the primitive encapsulated pericalymma larva of protobranch bivalves (Okusu, 2002; Zardus & Morse, 1998). To support this hypothesis, further studies are needed to corroborate this ingestion and reuse of the mushroom body and discard other potential physiological processes of the digestive gland related to the rhythmic feeding of bivalves (Owen, 1974).

The observation of the ctenidia during the stage III of *M. margaritifera* development provides the first histological evidence of the early gill formation during metamorphosis of this species, being complementary of the previous scanning electron microscopy description by Scharsack (1994). The presence of gill filaments, endowed with lateral cilia and the haemolymphatic vessels underneath, suggests that gills were able to generate a ciliary water current and haemolymphatic circulation, essential for respiration. On the other hand, the unreflected and partially ciliated ctenidia, only located at the inner demibranch, resemble the

first post-larval stages which are not completely effective for suspension-feeding until the first year post-detachment (Lavictoire et al., 2018; Schartum et al., 2016).

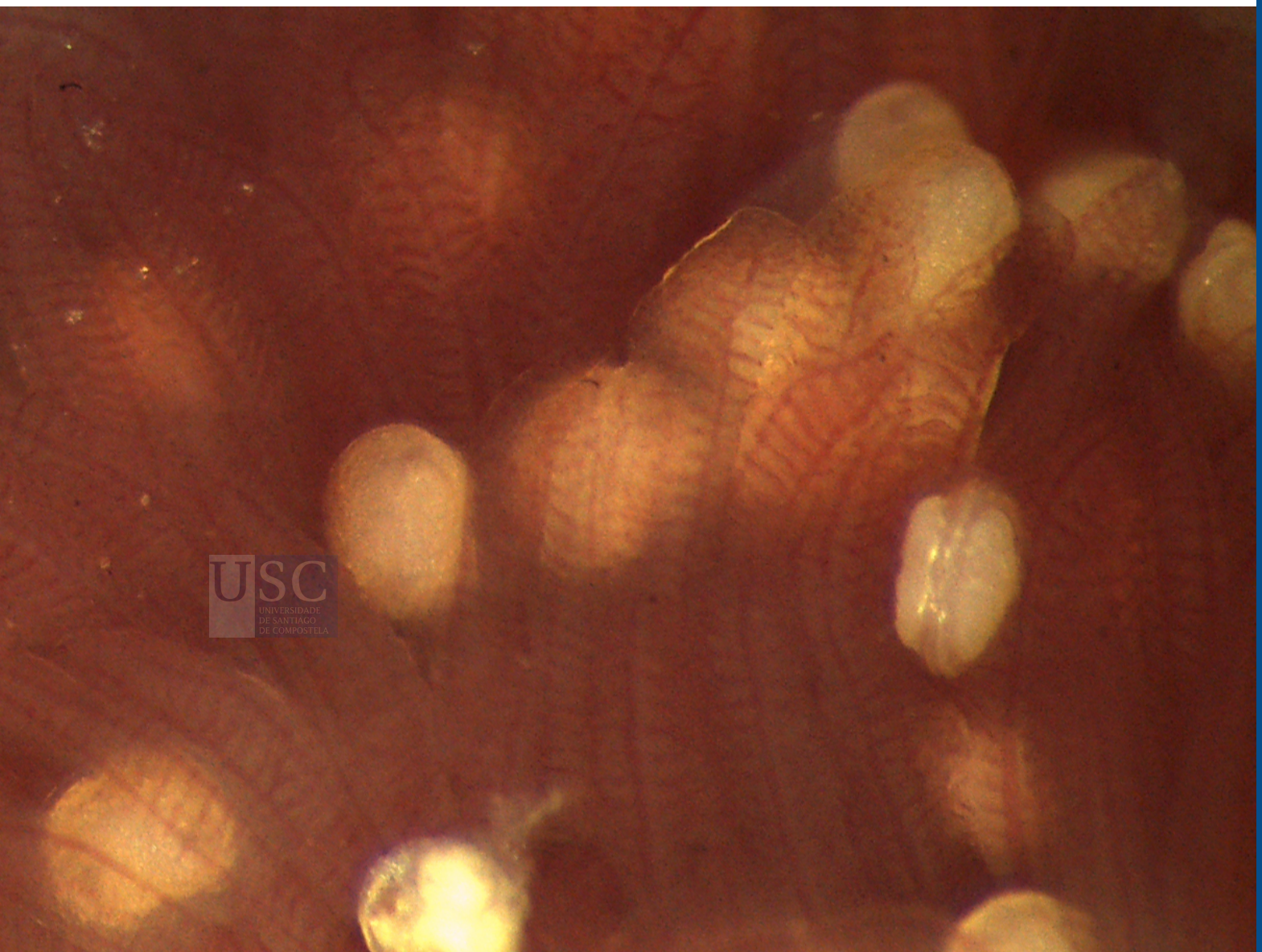
Based on the continuous larval development of *M. margaritifera* described in three parasitic stages (I–III), an intense morphogenesis from glochidia to post-larva was observed, highlighting an intermediate larval stage with unique morphological features involved in the parasitic interaction between the bivalve larva and the fish host. In conclusion, this study provides an overview of the larval morphogenesis of *M. margaritifera*, from glochidium to post-larva, essential for understanding the parasitic interaction between the freshwater mussel larva and the fish host. The intermediate stage—termed as "mushroom larva"—was characterized by a distinct morphology among bivalves in which the mushroom body and the zip membrane might be involved in both isolation and nutrition from the host fish. After metamorphosis and detachment from the gills, these unique larval structures are lost and the complete set of post-larval organs and structures allowed to continue with their free-living stage in the riverbed. Moreover, the morphological techniques and the hallmarks described in this sequential larval development (i.e., the lipid content and the presence of the adductor muscle at metamorphosis) could be directly employed to optimize and monitor the larval developmental status during the most critical stages within the conservation programmes of this endangered bivalve.

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General discussion


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GENERAL DISCUSSION

This Ph.D. Thesis has arisen to deepen into the parasitic interaction between the FPM and the Atlantic salmon, to understand and provide solutions for two of the most important concerns within the FPM culture: the impact of glochidiosis on fish health and the optimization of the parasitic stage of the larvae. To overview most of the results at a glance, the main morphopathological findings during glochidiosis (Study 1 and 2) were chronologically integrated with the larval morphogenesis (Study 3), as briefly illustrated in the Figure 1. A drop-down graphical abstract was also included in the Annex 2.

The first two studies (1 and 2) aimed to characterize the lesions suffered by the salmon and to study the pathogenic mechanisms during early and late stages of glochidiosis, by applying routinary techniques used in ictiopathology (top and center of Figure 1). On the other hand, the Study 3 takes advantage that the evaluation of the parasitized gills also provided novel information about the larval morphogenesis of *M. margaritifera* which has been scarcely addressed, stablishing three developmental stages (glochidium, mushroom larva and post-larva) (bottom of Figure 1).

Therefore, based on the chronology of the gill responses and the larval development, four main events may be established: the encystment of the glochidium into the gill tissue (day 0–3 PE), the premature rejection of immature larva (day 3–14 PE), the development of the mushroom larva (day 14–150 PE), and the metamorphosis and droplet detachment of post-larva (day 150–225 PE).

5.1 The encystment of the glochidium into the gills

To initiate its parasitic travel in the Atlantic salmon, glochidia attached to the gills by the closure of the valves due to the contraction of the larval adductor muscle. Just after pinching the filaments, the gill epithelium became hyperplastic engulfing the glochidia within the first 6 hours (Study 1). Meanwhile, the adductor muscle—which was previously essential for the attachment to the gills—was absent after 3 days PE, indicating that valves lost their capacity to be actively adduced, maintained closed by the epithelial response. This attachment and encystment mechanism support that epithelial hyperplasia may play an important role in the parasitic cyst formation, similar to the epithelial hyperplasia surrounding completely the *I. multifiliis* trophonts, a fish ectoparasite of the gills and skin (Dickerson & Findly, 2014; Ventura & Paperna, 1985).

As a result of the glochidial attachment, it is plausible that the first cue triggering this proliferative response would be the physical pressure of the valves against the gill epithelium. In addition, the external surface of the valve flanges, lined by a thin periostracum, could act as an antigenic structure activating the quick epithelial hyperplasia. However, other antigenic substances within the pallial cavity are also susceptible to become externalized by passively flowing through the valve cleft, which remains permeable since the zip membrane has not been formed yet. Among these putative internal antigens, the pinched gill tissue within the pallial cavity—which would suffer necrosis due to physical compression of the valves and reduction

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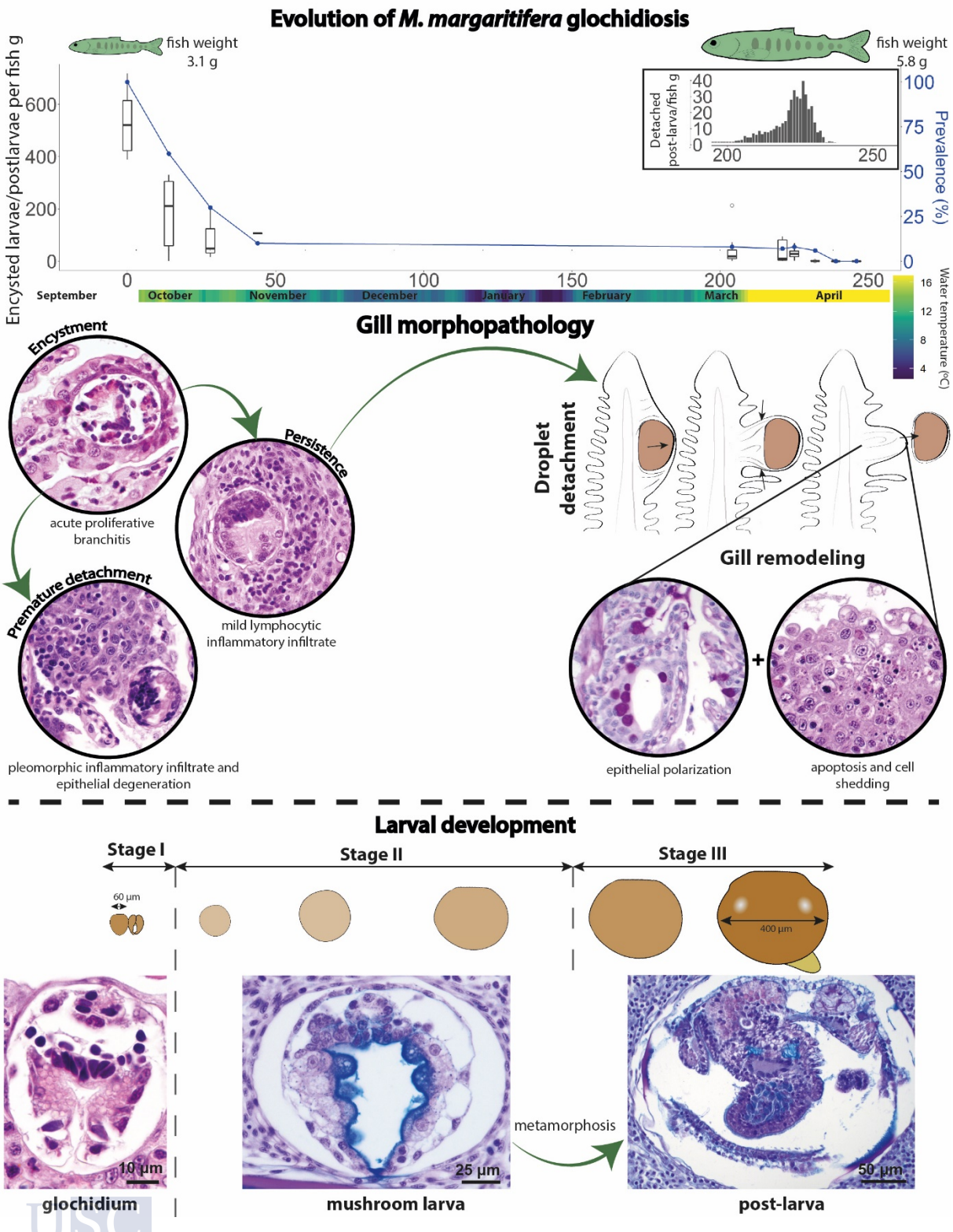


Figure 1. Summarizing diagram of the main results obtained alongside *M. margaritifera* glochidiosis, represented in the X-axis from September (day 0 post-exposure) to April (day 250 post-exposure). The evolution of the parasitosis, the morphopathological changes of the Atlantic salmon gills, and the larval development are integrated from the top to the bottom of the illustration.

of blood flow—might elicit an additional inflammatory response. Moreover, the enzymatic content secreted by the glochidial mantle may be considered capable to activate the immune response of fish, in the same way that enzymatic secretions of other ectoparasites impose a great antigenic capacity to salmon (Fast, Burka, Johnson & Ross, 2003).

These results show how the glochidial attachment is effective to trigger a proliferative response of the epithelium essential to encyst into the fish gills. Nevertheless, there are reasons to think that additional antigenic structures within the pallial cavity might become exteriorized and exert a potential antigenic capacity in the surrounding gill microenvironment.

5.2 The premature rejection of immature larva

Despite the initial glochidial encystment, some of the larvae suffered an early rejection during the first 14 days of glochidiosis, failing to successfully develop into post-larvae (Study 1). It is remarkable that the use of routinary histological techniques was scarcely used during *M. margaritifera* glochidiosis, and only a number of studies have employed similar techniques to evaluate the morphopathological changes associated with other glochidiosis. This approach allowed to characterize both larval morphology and gill lesions, relating for the first time the detachment mechanism of *M. margaritifera* larvae to an erosive branchitis with a pleomorphic inflammatory response. During this premature rejection, the epithelium surrounding the detaching larvae showed degenerative and edematous changes (Study 1), which suggests an epithelial damage with the potential loss of the intercellular junctions, leading to the final disruption and erosion of the outer surface of parasitic cysts. This erosive rejection observed in the suitable Atlantic salmon during the first two weeks of *M. margaritifera* glochidiosis was similarly described in preliminary studies in unsuitable host species in different freshwater mussels (Reis et al., 2014; Rogers-Lowery & Dimock, 2006; Scharsack, 1994; Waller & Mitchell, 1989). However, this process seems to occur in a more delayed manner in the suitable than non-suitable hosts, as the non-suitable *O. mykiss* rejected completely all *M. margaritifera* larvae in the first 24 h (Scharsack, 1994).

The erosion of the gill epithelium might exacerbate the underlying inflammatory responses due to the presence of necrotic debris and the invasion of other opportunistic pathogens because of the rupture of the epithelial lining (Study 1). Despite most of the larvae were rejected completely without any other morphological alteration, a few rejected larva showed a discontinuous and fragmented periostracum during this stage. This rupture of the outer barrier and externalization of internal structures of the larvae may contribute to elicit a more intense inflammatory response at this level, as it has been described in the gills after the rupture of other parasitic structures known as xenomas (Lovy et al., 2007).

These sequential events during early glochidiosis point towards an idiosyncratic individual response where the immune response of fish is involved, as previously hypothesized by Chowdhury et al. (2018) and Thomas et al. (2013). In this sense, the proliferative and inflammatory response during the first two weeks indicates the activation of a quick innate immune response at a mucosal level acting as the first defensive mechanism against parasitic diseases (Alvarez-Pellitero, 2008; Forlenza, Scharsack, Kachamakova, Taverne-Thiele, Rombout et al., 2008). Nevertheless, the involvement of the innate immunity is completely

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uncertain in *M. margaritifera* glochidiosis and only Chowdhury et al. (2021) pointed to the role of these mechanisms. On the other hand, Dodd et al. (2006) evidenced the presence of systemic specific antibodies (presumably IgM) in primed *M. salmoides* to *L. reeveiana* glochidium, and related this acquired immunitary response to a less successful larval development. The involvement of these immunoglobulins in an effective rejection of the larvae should be interpreted cautiously because these specific responses were demonstrated only after 100 days of exposure, and our study revealed that the early rejection occurs more quickly, within the first two weeks. Nevertheless, the role of IgM and other mucosal immunoglobulins, as IgT and IgD, should not be dismissed in glochidiosis due to their proven importance in the recognition of bacterial microbiota and parasites such as *I. multifiliis* (Perdiguero, Martín-Martín, Benedicenti, Díaz-Rosales, Morel et al., 2019; Xu, Takizawa, Parra, Gómez, Von Gersdorff Jørgensen et al., 2016).

5.3 The development of the mushroom larva

Despite all larvae were rejected by more than 50% of the fish, other larvae remained encysted—continuing their parasitic travel in the fish gills—and described as persistent larva (Study 1). However, the exhaustive morphological evaluation during the Study 3 revealed that those persistent larvae displayed a completely different morphology between the day 14 to 150 PE, characterized by the presence of a prominent mushroom body occupying most of the mantle. Up to our knowledge, there are only a few studies describing the mushroom body during the parasitic stage of freshwater mussels, where it was conformed by highly globose and vacuolized cells similar to those previously described in *M. margaritifera* (Scharsack, 1994), and other few unionids (Arey, 1932c; Blystad, 1923; Chumnanpuen et al., 2011; Fisher & Dimock, 2002). Due to the prominence of the mushroom body and its potential multifunctional role during the parasitic stage of *M. margaritifera*, this distinctive and intermediate parasitic stage was proposed to be termed as mushroom larva (Study 3).

One of the most striking features of *M. margaritifera* larvae is the marked growth within the gills; however, its mechanism of nutrition in the fish tissue remains completely a mystery. The first, and, up to our knowledge, unique hypothesis proposed is that the large amount of gill tissue clasped by the glochidia could be digested and employed as a nutritive resource (Arey, 1932c; Lefevre & Curtis, 1910). This theory makes sense in those mussel species with large glochidia which do not grow during the parasitic stage, but not in *M. margaritifera* where the larva increases in size and carbon content up to five-folds (Denic et al., 2015), being the small clasped fish tissue negligent as the exclusive food source. The zip membrane was characterized for the first time as a membranous, C-shape and proteinaceous barrier located all over the valve cleft, which delimits the pallial cavity from the exterior (Study 1 and 3). This finding provides an important progress to understand the potential exchange routes between the mushroom larva and the fish gills. The continuity of valve or the zip membrane demonstrates that nutrients, salts, waste products and gases should be transferred in a non-particulate manner, discarding the uptake of particulate nutrients as fish tissues or cells. Thus, the exchange of non-particulate material through the zip membrane or the thin valves stands out as the most plausible hypothesis to explain the exchange of substances during the *M. margaritifera* parasitism (Study 3). For a

better understating of the parasitic nutrition and the exchange of other substances, further permeability analysis of both the valves and the zip membrane would be necessary, as already investigated in other cyst-forming parasites (Lemgruber, Lupetti, Martins-Duarte, De Souza & Vommaro, 2011). Moreover, based on these relevant implications of the zip membrane, additional histological studies employing trichrome and PAS stains are needed in other freshwater mussels to confirm or discard the presence of a similar membranous structure at the valve cleft.

As larva developed, the proliferative gill response evolved since the day 14 PE towards less severe degenerative lesions and inflammatory infiltrates, with the predominance of lymphocytes; suggesting a chronification of the gill lesions (Study 1). The shift in the composition of the inflammatory infiltrates (pleomorphic vs. lymphocytic) concurred with the formation of the zip membrane of the mushroom larva. Considering the antigenic structures proposed previously, it would be inferred that this chronification may be caused by the cessation of the release of substances from the pallial cavity due to the presence of the zip membrane. As a result, the zip membrane not only limits the entry of particulate nutrients, but also it isolates the larva from the potential exit of material from the pallial cavity towards the exterior. Consequently, this isolation from the surrounding tissue may reduce the activation of the fish immune system and modulate the inflammatory response.

Due to the long-lasting parasitism, the lesions suffered by fish between the day 44 PE (end of the Study 1) and the day 203 PE (the onset of the Study 2) were not included, in order to focus on the critical early and late stages of glochidiosis. Nevertheless, the morphopathological pattern of the gills observed during the larval evaluation in the Study 3 (at day 60, 90, 120, 150, 180 PE; data not shown) suggests a chronification of the gill lesions resembling that above described, with similar proliferative lesions accompanied by the presence of interlamellar cysts, and filamental and lamellar fusions.

5.4 The metamorphosis and droplet detachment of post-larva

The morphogenesis of *M. margaritifera*, described in the Study 3, indicates the completion of metamorphosis into post-larva after the day 200 PE, where the encysted larva was composed by a complete set of post-larval organs. These results reported in Atlantic salmon complement previous studies, describing a similar and complete metamorphosis in the brown trout (Scharsack, 1994).

The simultaneous evaluation of the gill lesions, together with the larval development, allowed to describe for the first time (Study 2) the detachment mechanism of encysted post-larvae from the fish gills. This process was termed as droplet detachment due to the similarities with the protruding, pinch-off and detachment of a droplet fluid; where a characteristic pedunculated structure temporarily held together the fish tissue and the parasitic cyst. This post-larval detachment, which occurred at late glochidiosis, contrasts with the erosive rejection of immature larvae described during the first 14 days PE. Besides, the morphology of the post-larva might provide some clues to understand why this droplet detachment occurred only during late glochidiosis. Among the post-larval organs, the foot has been proposed to protrude among the valves, pressing and rupturing the surrounding fish tissue (Arey, 1932b; Kaiser, 2005;

Waller & Mitchell, 1989). Nevertheless, in the Study 3 the nearly detaching post-larvae always remained encysted in the gills with the valves closed by the zip membrane and the foot did not protrude out of the pallial cavity, suggesting that the valves have limited mobility and the foot does not rupture the cyst wall before the detachment. However, the well-developed musculature of the foot, inserted into the dorsal region of the valves, might move the foot inside the pallial cavity and exert physical forces into the overall cyst. These contractions may lead to oscillatory-like movements of the entire parasitic cyst which could favor the final pinch-off of the larvae during the droplet detachment. Hence, the post-larva of *M. margaritifera* may play an active role in the droplet detachment from the fish gills, but maintaining their valves closed by the zip membrane.

5.5 Impact of glochidiosis on fish health

The morphopathological evaluation of the gill lesions helps to understand how *M. margaritifera* glochidiosis may impair the gill multifunctionality. In this sense, the gas exchange might be reduced throughout the parasitism because of the proliferative lesions, due to the reduction of the surface area of the water-blood barrier and the increase of the diffusion distance (Study 1 and 2). This respiratory impairment might explain the reduced swimming capacity during the first day PE (Taeubert & Geist, 2013), and the delayed time to recover the respiratory movements to basal rates in brown trout after 5–6 months PE (Thomas et al., 2013). On the contrary, in our work, no detrimental effects on respiration were reported during late glochidiosis (Study 2), but the stressful procedure of hand-netting could mask more subtle effects, only observed under resting conditions. Besides the proliferative lesions, the evolution of morphopathological changes during glochidiosis suggests a complex host-parasite interaction with two different gill responses, each one related to a different larval outcome and impact on fish health:

- The early rejection of immature larvae (Study 1) was related to a rapid development of intercellular edema (spongiosis) surrounding the cysts, which might contribute, jointly with the hyperplastic response, to the increase of the diffusion distance and the decline of the gas exchange. Moreover, the epithelial degeneration and erosions might increase the epithelial permeability, compromising the osmoregulatory function.
- The persistence of mushroom larvae was associated with a proliferative response with no edematous lesions, erosions or tissue disruption around the persistent larvae (Study 1). After metamorphosis, post-larvae were detached by a droplet detachment mechanism which maintained the continuity of the lining epithelium (Study 2). The preservation of the epithelial continuity would help to prevent the occurrence of secondary infections (Dang et al., 2020; Karna & Millemann, 1978).

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However, the impact on fish health may not be restricted only to the presence of the parasitic larvae, but also it can be affected by the potential sequelae that could remain present after the end of parasitism, when the larvae or post-larvae detached. In this sense, after the erosive rejection of the glochidia, a complete gill recovery was reached in 30 days (Study 1). A similar proliferative response surrounding the larva (Study 1) may be involved in the re-

epithelization of the erosive lesions, and hence considered the first phase of the wound healing process during early glochidiosis, already proposed by Arey (1932a) and Reis et al. (2014). Simultaneously, the pleomorphic inflammatory infiltrates described during the Study 1 may play a double role; on one hand, they clear the damaged tissue and debris, but on the other hand, they might cause additional damage to the epithelium by releasing enzymes and free radicals (Daoust & Ferguson, 1986). From 21 days PE onwards, the epithelial remodeling was the most relevant and ultimate phase of gill healing, based on the progressive regression of the proliferative lesions related to the interlamellar cysts and lamellar synechia (Study 1).

Besides, the morphopathological description after the droplet detachment indicates that the gill lesions associated with the late stages of *M. margaritifera* glochidiosis were transitory and recovered almost completely in 19 days (Study 2). This quick recovery contrast with other gill processes that recovered in 4–6 weeks after the removal of the inciting cause (Daoust & Ferguson, 1986; Ferreira Sales et al., 2017; Kudo & Kimura, 1983). The droplet detachment of post-larvae would favor the gill healing process since there is no need of re-epithelization, because the continuity of the lining epithelium remains. This fact would minimize the inflammatory response and preserve the integrity of the underneath basal membrane, the latter considered as a determinant factor during gill healing (Daoust & Ferguson, 1986). After the post-larval detachment, an epithelial remodeling resembling that described during early glochidiosis was achieved by the mutual interplay of apoptosis, polarization and cell shedding described between the day 227 and 246 PE (Study 2). Otherwise, the presence of pedunculated structures at day 203 PE (Study 2) could be interpreted as an anticipated epithelial remodeling of the adjacent tissue beside the peduncles, before the post-larvae pinch-off from the gills.

Finally, the comparison of the different healing processes between early and late glochidiosis might indicate that the gill healing cascade after the erosive rejection of the premature larva consists of more healing phases (re-epithelialization, inflammation and remodeling) than the droplet detachment (remodeling). In any case, the absence of granulation tissue during these recovery processes contrasts with the classical wound healing cascade in fish skin (Sveen et al., 2020), supporting the fact that fibrosis is a rare response in the gills (Mallatt, 1985; Speare et al., 1991a). Despite these different healing processes during early and late glochidiosis, our studies suggest a safe margin of one month from the larval detachment to allow fish to recover from glochidiosis. Up to now, no other histopathological studies described the gill healing after glochidiosis, with only some preliminary studies after the parasitism by *M. margaritifera* larvae in Atlantic salmon (Treasurer & Turnbull, 2000), and by *L. reeveiana* larvae in largemouth bass (Kaiser, 2005). The recovery of fish in only one month after the detachment constitutes an important finding of this work, supporting that fish affected by glochidiosis could be set free into a suitable habitat or even reused in other experimental procedures, according to the current legislation regarding the protection of animals used for scientific purposes (Directive 2010/63/EU, 2010; Real Decreto 53/2013, 2013).

The comparison of the two gill responses and recovery mechanisms during the complex *M. margaritifera* glochidiosis provides the first evidence to suggest that fish that remained parasitized until the post-larval detachment suffer less severe gill damage, preserving the epithelial continuity, than fish rejecting prematurely the immature larvae. This fact may be

attributed to the inflammatory response against pathogens, which could be considered a double-edged sword to fish health (Medzhitov et al., 2012). On one hand, the initial proliferative gill response involved in the glochidial encystment is regarded as a gill defense mechanism to isolate and protect the gill from further damage (Ferguson & Speare, 2006; Mallatt, 1985). On the other hand, the quick and intense inflammatory response during the early larval rejection might cause detrimental lesions of the gill epithelia, such as the degenerative and erosive changes, already reported in other parasitizations which colonize fish mucosae (Holzer, Piazzon, Barrett, Bartholomew & Sitjà-Bobadilla, 2021; Losada, Bermudez, Failde & Quiroga, 2012). This opens the hypothesis if the early rejection of larvae might cause a more detrimental impact than the permanence and metamorphosis into post-larva, dissociating the disease severity from the pathogen burden, as proposed by Rogers and Dimock (2003) in *U. imbecillis* glochidiosis.

This perspective has been linked to glochidiosis by Marwaha et al. (2021) and Methling et al. (2019) because of the existence of a tolerance strategy, a host defense strategy aimed to maintain the host health without decreasing the pathogen burden (Ayres & Schneider, 2008). In contrast with tolerance, resistance has been described in *Unio tumidiformis* glochidiosis as the opposite host defense mechanism where the non-suitable fish species prematurely rejected all the larvae (Reis et al., 2014). In *M. margaritifera* glochidiosis, tolerance may occur since the fish health is preserved by maintaining the continuity of the gill epithelium, while the mushroom larvae persist and metamorphose into post-larvae. Moreover, tolerance might be also supported by the activation of mechanisms to compensate an impaired respiratory capacity, e.g., by the increase in the haematocrit levels (Filipsson et al., 2017; Marwaha et al., 2019) or the redirection of blood flow to non-affected gill regions (Booth, 1979). Resistance may be associated with the premature larval rejection by an erosive branchitis. As a result, both tolerance and resistance strategies may overlap during glochidiosis to preserve fish health, but with antagonist impacts to the parasite, as resistance does affect the parasite, meanwhile tolerance exerts a neutral or even positive effect on the parasite fitness (Kutzer & Armitage, 2016; McCarville & Ayres, 2018; Medzhitov et al., 2012). To demonstrate these tolerance/resistance strategies, further studies need to characterize the role of immune responses involved in the high host specifity of FPM. These opposite mechanisms have already been highlighted in other salmonid disease caused by the myxozoan *Tetracapsuloides bryosalmonae* in Europe, where opposite immune responses have been described between the native brown trout and the foreign rainbow trout (Bailey et al., 2019).

5.6 The monitorization of the larval development and metamorphosis of *M. margaritifera*

The exhaustive description of the larval development of *M. margaritifera* provides an excellent instrument to study the larval morphogenesis of *M. margaritifera*, from glochidium to post-larva, essential for understanding the parasitic interaction between the PFM larva and the fish host. On the other hand, the complex parasitism, with low larval survivals and impaired developments, evidence the need to improve the existent tools (i.e., the quantification of the

larval growth and survival) and develop new ones for monitoring the growing larva within the fish gills.

During the parasitic stage, the larval growth has been frequently calculated by measuring the maximal diameter of the larvae through microscopical visualization of the fresh gills (Bauer, 1987a; Taeubert et al., 2010). However, the larval elongation, described around 500 degree-days or the day 40 PE, can lead to wrong measurements during the evaluation of the larval growth, when using this method. In fact, the confusion between the glochidial length (60 µm) and height (70 µm), described by Bauer (1992), and Pekkarinen and Valovirta (1996), would explain the marked growth over six-folds in length described in the Study 1, in contrast with the commonly reported five-folds growth of *M. margaritifera* (Denic et al., 2015). To deal with that, the semi-transparent valves allow to determine the orientation of the larva within the gill tissue by the morphological evaluation of the position of the valve cleft, the hinge, the pallial cavity, the mushroom body and even the insertion of the adductor muscles. Hence, the larva oriented in an oblique position could be initially discarded, only measuring the height and/or length of properly oriented larvae or post-larvae within the fish gills. Thus, the determination of the larval orientation would improve the measurement accuracy during the evaluation of the larval growth, and it also would favor the use of more accurate terminology, i.e., the length or height. On the other hand, the measurement of the larval size by histology would lead to marked underestimations of the larval length, since larvae were measured independently of the plane of section (Ieshko et al., 2016; Murzina et al., 2017). Therefore, further morphometric studies are required to identify potential anatomical landmarks which would enable the monitorization of the larval growth by histology. Among the larval structures, the zip membrane outstands as a potential anatomical landmark due to its position at the valve rims, shape and histochemistry properties, which may facilitate the visualization and determination of the larval size in the histological sections.

To evaluate the larval survival hitherto is compulsory to do a lethal sampling, performing a necropsy of fish at different moments of the parasitism to quantify the parasitic loads. However, it is known that the early larval rejection provokes a reduction of the prevalence of the parasitism and the fish parasitic loads, increasing the parasitic load variability (Study 1). To deal with the high error during the estimation of the parasitic loads after day 14 PE (Study 1), it would be necessary a minimum sample size of around 30 individuals, based on sample size estimations calculated in previous trials. This number of fish would be excessive to periodically sample within the *M. margaritifera* culture. Taking into account the results obtained with the stereomicroscopical visualization of immersed gill tissue (Study 2), this technique reveals as a potential diagnostic tool to determine *in vivo* the larval load. Hence, gill stereomicroscopy would reduce the number of fish employed during each infestation and optimize the efforts, avoiding the laborious method of counting all the encysted larva on each holobranch. Once this technique would be standardized and proven that do not cause any detrimental effect to both larva and fish, it may enable to sample a bigger number of fish or even evaluate the parasitic loads in the same individual at different moments of the parasitism. In this sense, preliminary data during both early and late stages of glochidiosis have indicated a good sensitivity and

specificity of this *in vivo* technique, in comparison with the traditional gill visualization after the necropsy (Al-Soufi, 2021).

Regardless of the technique employed to visualize the gills, parasitic loads are commonly expressed in larvae per host fish (Bauer, 1987a; Young & Williams, 1984b) or standardized, more recently, by the fish weight and expressed in larvae/fish g (Marwaha et al., 2019; Taeubert & Geist, 2013). These markers of the parasitic loads are highly dependent on the prevalence of the parasitism and the fish weight; thus, to allow additional comparisons between different populations and studies, further studies need to provide more detailed information about the terminology and methodologies employed in the calculation of the parasitic loads. As similarly recommended in ecology and parasitology, we propose to homogenize the terminology to express the parasitic loads during glochidiosis, for instance, by using abundance (larvae/fish), intensity (larvae/infested fish) and density (larvae/infested fish g) (Margolis, Esch, Holmes, Kuris & Schad, 1982).

Larval growth and survival have been used as general indicators of larval fitness. However, it is required the development of more specific biomarkers to evaluate the larval fitness during the parasitic stage. The lipid content has been already proposed as an indicator of the *Unio crassus* and *Anodonta anatina* juvenile fitness just after the parasitic stage, in which the lipid content might have critical consequences on the vitality and survival of juveniles during the early post-parasitic stage (Douda, 2015). The accumulation of lipid vacuoles within the larval mantle of *M. margaritifera* during the Stage II (Study 3) evidences that lipids may constitute a potential biomarker of the larval energy storage and even to predict the post-larval fitness just after the parasitism. Because lipids are easily dissolved by the organic solvents routinely employed in histopathology, the use of fresh frozen tissue stands out as a complementary histological technique that may aid the detection of larval lipids, highly overlooked in freshwater mussels. The non-use of fixatives would also skip the common artifactual shrinkage of the larva, that was minimized in our work using less hyperosmotic fluids as Dietrich´s fluid (Study 3). Moreover, cryosections avoided the collapse of certain structures and cavities, as the pallial cavity and the external cavity described over the zip membrane in the Study 3.

Towards the end of the parasitic stage, an additional and important concern is to identify the completion of the metamorphosis and to determine the optimal moment to initiate the synchronization and/or collection of juvenile naiads. During the Study 3, viable post-larvae with a size of 308–397 µm length detached from the fish gills after 200–220 days (1900–2500 degree-days), synchronized by the artificial increase in the water temperature. A similar detachment of viable mussels was reported with 300–480 µm length after 150–330 days (equivalent to 1700–3400 degree-days; see Table 2 in Section 1.4). These highly variable time periods and size ranges demonstrate the difficulty to establish a rule of thumb to determine the metamorphosis based on the time and/or post-larval length. Thus, the sequential description of larval development (Stages I–III) and the simultaneous collection of viable post-larvae from approximately the day 200 PE (2000 degree-days) allowed to detect later certain morphological traits indicative of the end of the metamorphosis. Some of these morphological changes consist in the presence of the anterior and posterior adductor muscles, the highly ciliated epithelium of the foot, the glandular tissue of the byssal complex and the ciliation of the lateral epithelial cells

of the ctenidia. Among them, the indirect visualization of the adductor muscle by wet mount of the gills is proposed as a potential marker to easily distinguish the larval metamorphosis and initiate the synchronization of the juvenile collection at the hatchery. Although the adductor muscle was detected as soon as 90 days PE (Scharsack, 1994), the presence of this structure could not be evidenced until 200 PE in the Study 3, when an evident group of myofibers of 20 µm of diameter was identified histologically. In contrast with the presence of post-larval features, the detection of some remnants of the mushroom body after 225 days PE might be also considered as additional indicators, suggestive of an uncomplete metamorphosis (as indicated in the Study 3), since this structure was not observed during the longest parasitism of 300 days (Scharsack, 1994).

These proposed tools and methodologies are susceptible to be integrated within the common procedures to monitor and provide a better overview of the larval and post-larval fitness during the FPM culture. In this sense, the morphological assessment of the parasitized fish gills may help to simultaneously evaluate the growth of larval length, the larval survival and the accumulation of lipids, together with the identification of potential morphological flagships of metamorphosis. This approach may aid to detect the reduced larval survival during the early glochidiosis (Study 1) and the delayed growth of the persistent mushroom larvae (Study 2), therefore contributing to solve the main constrains of the parasitic stage during the *M. margaritifera* culture (Bauer, 1987a; Jung et al., 2013; Marwaha et al., 2019; Taeubert et al., 2010; Young & Williams, 1984a, 1984b).

5.7 Further directions

The findings highlighted in this Ph.D. Thesis could be integrated with the existent bibliography to create a handbook for managing the parasitic stage during *M. margaritifera* culture and design further conservation measures. In this sense, the approach of studying this host-parasite interaction as a disease might serve to apply strategies commonly used in Veterinary Medicine, which could be complemented with the traditional perspective employed in the Conservation of threatened unionid mussels (Österling, Larsen & Arvidsson, 2020). Thus, the development of useful, viable and feasible tools is required to monitor both larval development and fish health. As fish welfare is susceptible to be affected during glochidiosis, further studies should develop less invasive techniques which would ensure that animals are bred, cared for and used following the three principles of replacement, reduction and refinement for animal experimentation (Directive 2010/63/EU, 2010). In this sense, the proposed *in vivo* stereomicroscopical visualization of immersed gills opens new strategies to monitor the parasitic loads; but also the biopsy of a parasitized portion of fish gills may contribute to it, based on previous studies describing this technique as a non-detrimental tool to diagnose other gill diseases (Nowak & Lucas, 1997). This biopsied tissue would be visualized by wet mount microscopy and histology to monitor the gill responses during glochidiosis, the growth and development of larva, and even the overall gill status, to discard the presence of other pathogens which would worsen the morphopathological pattern. Therefore, the findings of this Ph.D. Thesis enable a better understanding of the pathogenesis of glochidiosis, and eventually they

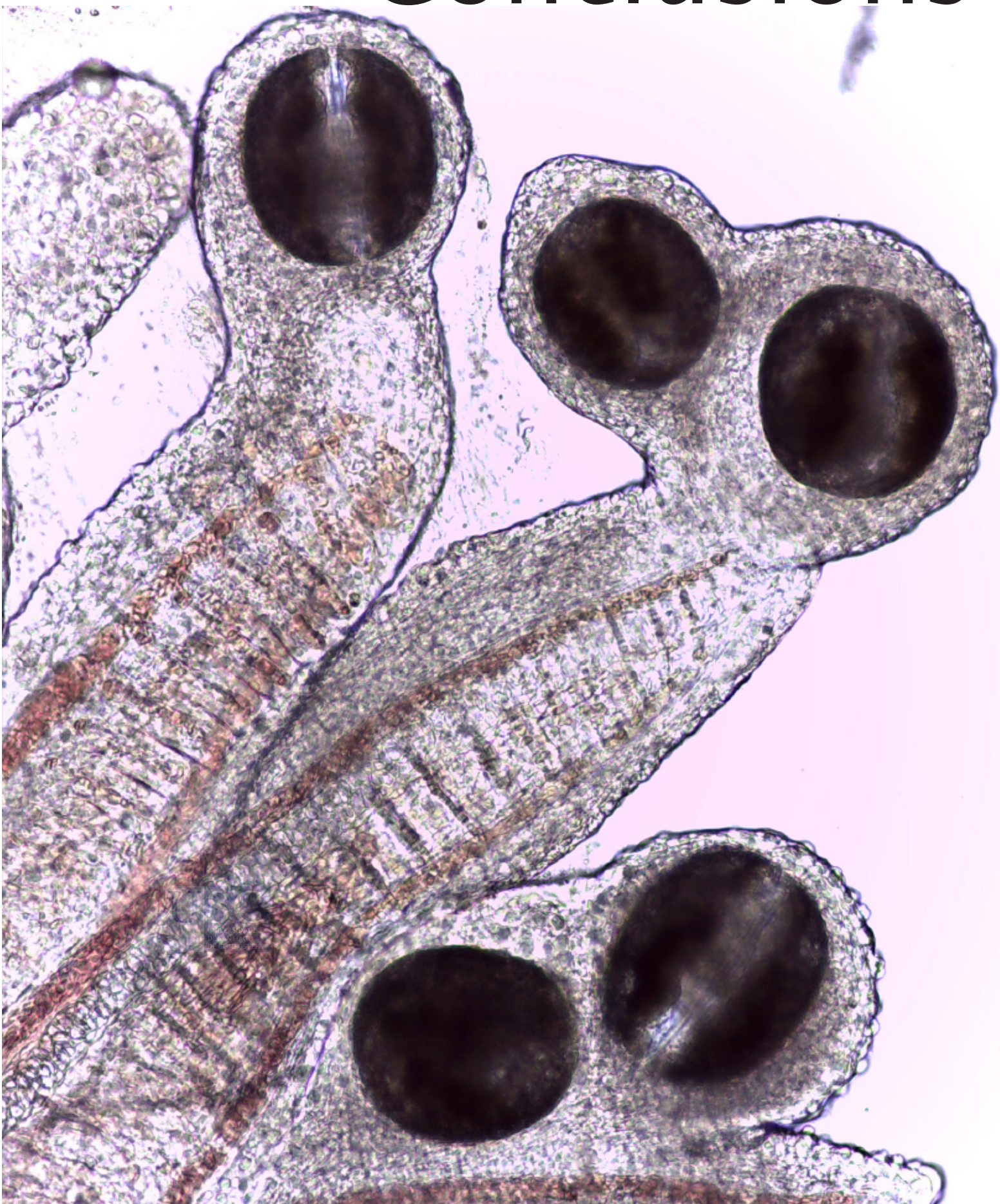
would allow troubleshooting during the culturing procedures and provide further recommendations.

To better understand the pathogenesis of glochidiosis, it is necessary to elucidate the causal relationships among the fish responses and the larval outcomes. The gill morphopathological changes described during the Study 1 and 2 suggest that the premature rejection or the permanence and development of the mushroom larvae in gills are driven by the underlying fish immunity. Therefore, the evaluation of the immune response of fish might serve to better understand the defense mechanism against the parasitic larva of *M. margaritifera* and subsequent gill healing mechanisms. In this sense, the combination of transcriptomics, mucus proteomics and immunohistochemistry characterization of immune cells might serve to better understand the early rejection of immature larvae, the gill re-epithelization after this erosive rejection, and the epithelial remodeling after the droplet detachment. The high host suitability of FPM provides an ideal frame to compare the fish response between the intraspecific suitabilities within brown trout and Atlantic salmon populations and even with the non-suitable species as rainbow trout. As a result of the important role of fish immunity, further strategies should focus on the immunomodulation of glochidiosis through the search for genetic markers of tolerance/resistance and therapeutic strategies, the latter proposed Dubansky, Whitaker and Galvez (2011) based on the evidence that the immunosuppression *Lepomis macrochirus* with cortisol injections improves the larval survival of *U. imbecillis*. Despite our studies pointed towards the involvement of the non-specific innate immunity, additional research should determine the potential antigenic structures of the glochidium which might trigger the early immune response, where the role of the immunoglobulins at a local level could not be discarded, requiring further evaluation.

The ultimate goal of science is to increase knowledge for the common benefit of society. Therefore, it would be logical to engage the general public in the world of science to increase public support and awareness, which might finally lead to more informed choices. In the research area of Conservation of freshwater mussels, scientific outreach takes a fundamental role because conservation activities are exclusively supported by governments and public institutions. Hence, new formulas are required to involve citizen collaboration in the protection of endangered naiads, as occurring with the sponsorship of more symbolic species, e.g., marine mammals. One essential task of the different stakeholders—including researchers—is to outreach this knowledge in the simplest and most accessible manner to society. Then, citizens will truly appreciate the drinking tap water, farmers will protect their creeks and rivers, and politicians will understand the importance of supporting the research and the conservation of freshwater mussels. Thus, additional efforts should be oriented to make society understand why the FPM are real jewels in the aquatic ecosystems.

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Conclusions



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CONCLUSIONS

1. The Atlantic salmon encysts the glochidium of *M. margaritifera* by a proliferative branchitis; however, during the first 14 days of glochidiosis certain individuals suffer an erosive and pleomorphic branchitis which prematurely rejects the immature larva, possibly by the activation of a quick and effective immune response.

2. A distinctive intermediate parasitic stage, involved in the larval nutrition and isolation from the fish immune response, was characterized by the presence of the zip membrane and the extensive mushroom body of the mantle, and hence proposed to be named as mushroom larva.

3. The *M. margaritifera* larvae which remained encysted in the salmon gills showed a marked growth and morphogenesis from glochidium to post-larva. After metamorphosis, *M. margaritifera* post-larva acquires a complete set of organs that are implicated in the droplet detachment and settlement into the riverbed.

4. Salmon that remained parasitized until the post-larval detachment suffered a less severe histopathological gill damage than fish rejecting prematurely the immature larvae; by reducing the inflammatory response, preserving the epithelial continuity and favoring the gill healing. Thus, two different gill responses during *M. margaritifera* glochidiosis were characterized, each related to different larval outcomes and impacts on fish health.

5. The recovery of Atlantic salmon fry from *M. margaritifera* glochidiosis was complete one month after the parasitism, relying on a quick and effective epithelial remodeling that is achieved by the mutual interplay of apoptosis, polarization and cell shedding.

6. The combination of stereomicroscopy, wet mount microscopy and histology of the fish gills exhibit direct applicability to monitor the fish health and the larval and post-larval fitness during the FPM culture.

7. New tools and indications are proposed to optimize the culturing and experimental procedures involving the parasitic stage of the FPM on fish, allowing to evaluate the growth, survival and lipid content of the larva, and metamorphosis into post-larva.

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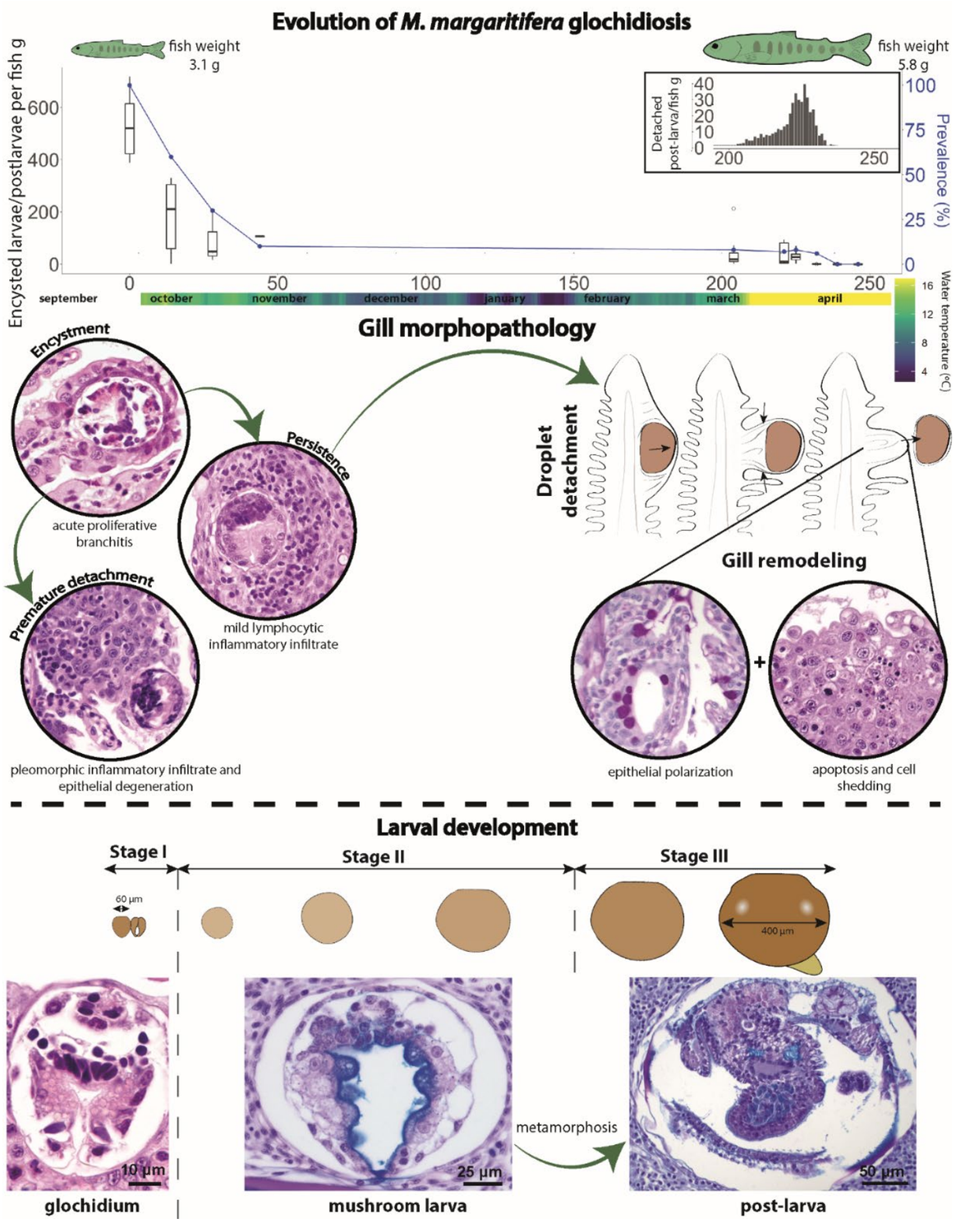
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2. GRAPHICAL ABSTRACT

Summarizing diagram of the main results obtained alongside *M. margaritifera* glochidiosis, represented in the X-axis from September (day 0 post-exposure) to April (day 250 post-exposure). The evolution of the parasitosis, the morphopathological changes of the of the Atlantic salmon gills, and the larval development are integrated from the top to the bottom of the illustration.



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RESOLUCIÓN DE AUTORIZACIÓN DE PROXECTOS DE EXPERIMENTACIÓN ANIMAL

Expediente núm.: 02/17/LU-002
Interesado: Roberto Bermúdez Pose
Procedemento: RESOLUCIÓN DE AUTORIZACIÓN DE PROXECTOS DE EXPERIMENTACIÓN ANIMAL
Data de inicio: 1 de Noviembre de 2017
Forma de inicio: solicitude do interesado

ANTECEDENTES

O interesado, como responsable do proxecto **Recuperando el mejillón de río. Estudio de los factores críticos** presentou con data 29 de xuño de 2017 (rexistro de entrada **72839/RX1687427)**, solicitude para a realización do proxecto de experimentación animal cuxos datos detállanse a continuación:

- Denominación do proxecto: **Recuperando el mejillón de río. Estudio de los factores críticos**
- Nome do centro usuario: (AE-LU-002).
- Persoa responsable do proxecto: **Roberto Bermúdez Pose**
- Establecemento onde se realizarán os procedementos do proxecto (ou lugar xeográfico no caso de traballos de campo): Facultad de veterinaria(AE-LU-002) y Centro Ictioxénico del Veral.
- Clasificación do proxecto : **Tipo II.**

CONSIDERACIÓNS LEGAIS E TÉCNICAS

I. Real decreto 53/2013, de 1 de febreiro (B.O.E. nº 34, do 8 de febreiro), polo que se establecen as normas básicas aplicables para a protección dos animais utilizados en experimentación e outros fins científicos, incluíndo a docencia, establece no seu artigo 33 as condicións de autorizacións dos proxectos con animais de experimentación.

II. Artigo 89 da Lei 30/1992, de 26 de novembro, do réxime xurídico das Administracións Públicas e do procedemento administrativo común (B.O.E. núm. 285, 27 de novembro de 1992), modificada pola Lei 4/1999, de 14 de xaneiro, establece que a resolución que poña fin o procedemento decidirá todas as cuestións expostas polos interesados e aquelas outras derivadas do mesmo.

XUNTA DE GALICIA
DELEGACIÓN TERRITORIAL
DE LUGO
Xefatura Territorial da Consellería
do Medio Rural

Ronda da Muralla, 70
LUGO

galicia

Esta xefatura territorial é competente para ditar resolución de conformidade co artigo 11 do Decreto 245/2009 de 3/ de abril, polo que se regulan as delegacións territoriais da Xunta de Galicia e o Decreto 166/2015, de 13 de novembro, polo que establece a estrutura orgánica da Consellería do Medio Rural.

Revisada por parte do Servizo Provincial de Gandería de Lugo a documentación achegada na solicitude e e visto o informe **favorable** da avaliación do proxecto, de data 20 de octubre de 2017 emitido polo órgano habilitado Comité de Ética da USC, este Departamento Territorial resolve, **AUTORIZAR** o proxecto solicitado.

A autorización deste proxecto terá unha **duración de 5 ANOS**, transcorridos os cales, deberá renovar a autorización do mesmo.

A autorización é unicamente válida nas condicións que figuran no expediente. Ante calquera cambio significativo no proxecto que poida ter efectos negativos sobre o benestar dos animais, deberá solicitar a confirmación da autorización ao Servizo Provincial de Gandería.

Esta autorización poderá ser suspendida no caso de que o proxecto non se leve a cabo de acordo coas condicións de autorización e retirala previo expediente tramitado ao que se lle dará audiencia.

Contra a presente resolución, que non pon fin á vía administrativa, poderá interpor **recurso de alzada** ante o conselleiro de Medio Rural da Xunta de Galicia no prazo **dun mes** contado a partir da recepción da notificación da presente resolución, conforme coa Lei 30/1992, do 26 de novembro, (B.O.E. núm. 285, 27 de novembro de 1992), de Réxime Xurídico das Administracións Públicas e do Procedemento Administrativo Común na súa redacción dada pola Lei 4/1999, do 13 de xaneiro.

Lugo, 15 de Novembro de 2017
A xefa territorial da consellería do medio rural de Lugo

XEFATURA TERRITORIAL LUGO

Mª Olga Iglesias Fontal

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA



This Ph.D. Thesis arose with the aim to understand and provide solutions for culturing the endangered Freshwater Pearl Mussel during its parasitic stage in the Atlantic salmon. To optimize the production of young mussels and preserve fish health, the morphopathological pattern of glochidiosis was characterized during the early and late stages of glochidiosis and related to the larval development and metamorphosis of *Margaritifera margaritifera*, from glochidium to post-larva. The results here enclosed contribute to a better understanding of the pathogenesis of glochidiosis that may aid to manage the parasitic stage during the culture of freshwater mussels.