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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 postexposure 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* L.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

1 INTRODUCTION

Freshwater mussels from the order Unionida are bivalves which play important and pervasive functions in the riverine ecosystems due their biofiltration capabilities and retention of matter and energy from the water to the benthos (Vaughn, 2017). 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, Haag & Roston, 2008; Denic, Taeubert & Geist, 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, McVicar & Waddell, 1988; Howerth & Keller, 2006; Ieshko, Geist, Murzina, Veselov, Lebedeva *et al.*, 2016; Nezlin, Cunjak, Zotin & Ziuganov, 1994; Treasurer & Turnbull, 2000). Those tissue changes, in the case of intense gill parasitosis, may lead to the impairment of fish welfare (Thomas, Taylor & Garcia de Leaniz, 2013), related to behavioral changes (Filipsson, Petersson, Höjesjö, Piccolo, Näslund *et al.*, 2018; Horký, Slavík & Douda, 2019), respiratory distress (Kaiser, 2005; Taeubert & Geist, 2013; Thomas *et al.*, 2013) and growth reduction (Ooue, Terui, Urabe & Nakamura, 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 specificity 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, Geist, Aldridge, Araujo *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, Thomas & Adams, 2018; Marwaha, Aase, Geist, Stoeckle, Kuehn *et al.*, 2019; Wacker, Larsen, Karlsson & Hindar, 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.

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 Ictiogenico 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 15°C 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 m³ 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 hr (Table S1).

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 postexposure (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 ($100 \times \text{weight}/\text{length}^3$) were calculated and standard deviation expressed ($\pm$). Complete necropsy of animals was performed and right holobranches and other organs (skin, thymus, digestive tract, heart, kidney and spleen) were immediately fixed in Bouin's fixative for 18 h.

Left holobranches 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 holobranches 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 holobranches were decalcified for 6h in a 10% ethylenediaminetetraacetic acid (EDTA) solution (Osteodec, Bio-optica®, Italy). Decalcified holobranches 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 CellS 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 Ictiogenico de O Veral" (Xunta de Galicia, Spain) 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).

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.

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

Days postexposure	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.

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).

Glochidia were superficially attached to the gills due 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 hr 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 hr PE. During this period, the clasped gill tissue inside the pallial cavity disappeared almost completely (Figure 1f).

At 24 hr 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.

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 conformed 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](#)).

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 hyperplastic 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.

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, Gumpinger & Csar, 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 holobranches as described in other *M. margaritifera* infestations (Clements *et al.*, 2018; Treasurer & Turnbull, 2000; Young & Williams, 1984).

Using light microscopy, the different orientation of holobranches in the paraffin blocks allows observation for the first time of 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, Austbø, Bjørgen, Skjødt, Hordvik *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, 1932; Scharsack, 1994).

During the first 12 hr 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, Collares-Pereira & Araujo, 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 hr 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 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* glochidia 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, Scheder, Gumpinger and Waringer (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 (Lovv, 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 immunitary 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 disappearance of the hyperplastic lesions and return to the normal gill architecture, since apoptosis was not a prominent feature in this study, other remodeling mechanisms should be considered such as the reduction of mitotic rate or the detachment of interlamellar cell mass (Nilsson, 2007).

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, Brijs, Näslund, Wengström, Adamsson *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, Purser & Al-Mousawi, 1987; Young & Williams, 1984). 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, Kovitvadi, Chatchavalvanich, Thongpan & Kovitvadi, 2011), could be regarded rather a different adaptation of the mantle margin of molluscs (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, 1932; 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 “*spaces*” 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, Camara and Ramos (2002) and Pekkarinen and Valovirta (1997). This artifact may occur during fixation in well-developed larvae due to the high osmolality 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; Spielberg, 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, Ieshko & Zotin, 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 immunitary 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.

Figures

Figure 1

Distribution of recently attached glochidia throughout the gills (a–d) and the branchial response during the first 6 hr postexposure (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).

Figure 2

Images of gill response and glochidial development during the first 3 days 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 phagocytosed 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.

Figure 3

Images of the intense early expulsion of immature glochidia at 10 and 14 days 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.

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 postexposure (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.

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SUPPORTING INFORMATION

Table S1. Water quality mean values at different interval days, grouped to represent the periods bewtweensampling times. Nitrogen compounds are total ammonia (N-NH^3), nitrite (NO^{-2}) and nitrate (NO^{-3}) are expressed in mg/L; temperature is expressed in Celsius degrees ($^{\circ}\text{C}$). Sample mean and standard error are provided.

Interval day	N-NH^3	NO^{-2}	NO^{-3}	$^{\circ}\text{C}$
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