Gill histopathology of Maria-da-toca *Hypleurochilus fissicornis* by metacercariae of *Bucephalus margaritae* (Digenea: Bucephalidae)

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Abstract Gills of Maria-da-toca *Hypleurochilus fissicornis* collected at Ponta do Sambaqui–Florianópolis island–Brazil, were analyzed to describe the histopathology caused by metacercaria of *Bucephalus margaritae*. Gills were submitted to the routine histological techniques for embedding in paraffin and permanent mounting in Balsam and stereoscopic analysis. Metacercariae showed a branchial infection site pattern for encystations. The branchial infection site pattern is half-basalward in the primary branchial filament with amplitude of the infection of 1–3 metacercaria. Cysts occurred within branchial abductor muscle and cartilaginous and osseous tissues of the gills. Each metacercariae had a contentional hyaline parasitic capsule and melanin-like pigmentation. The half-apical-ward region of the primary branchial filaments showed several dysplasia degrees, cartilage and osseous degeneration (pyknosis), thrombosis and immune exudated cells (mainly lymphocytes). Cytopathologies as thickening of

the epithelium lining of the secondary branchial filaments were a response of the branchial infection site pattern of the metacercaria. Interlamellar obliteration and fusion of the lamellae due to the hypertrophy and hyperplasia of the epithelial lining as well as chloride cells occurred. Pyknosis of pillar cells and epithelial lining cells from the secondary branchial filaments were also present. Bucephalosis in *H. fissicornis* gills is no-hemorrhagic and no-fatal branchitis, but could compromises the gill functions and could permits the secondary opportunistic infections.

Keywords Abnormal gill · Branchitis · Bucephalosis · Pattern of branchial infection

Introduction

Metacercaria constitute a typical quiescent parasitic larval stage and they are able to infect a high number of fish species as second intermediate host on the parasite life cycle. They cause severe damage to their host such as fibroblast proliferation (Ogawa et al. 2004), inflammatory response, breathing difficulties, necrosis and death (Eiras 1994). The intensity of parasite infection necessary to promote such alterations in one fish is variable according to the parasitic species and its quantity, host dimension and host health condition (Fergusson 1989; Roberts 2001).

Beach of Sambaqui in south of Brazil is a traditional fishing ground for locals and also the locality of mussel *Perna perna* (Linnaeus 1758) (Bivalvia: Mytilidae) culture. The mussel culture attracts the Blenniidae Maria-da-toca, *Hypleurochilus fissicornis* (Quoy and Gaimard 1824) that utilize the mussel for food, protection and spawning (Gerhardinger et al. 2006). This provide adequate condition for spreading and development of the *Bucephalus*

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margaritae Ozaki and Ishibashi 1934 (Digenea: Bucephalidae), commonly found encysted in the gill of the Blenniidae (Marchiori et al. 2010) and in the form of sporocysts and cercariae in the mantle cavity of *P. perna* (first intermediate host) (Silva et al. 2002; Marchiori et al. 2010). Open aquatic culture systems, as mussel ropes, have several uncontrolled variables, perturbations and interactions of diverse trophic levels because water column are in continual motion and changes (Lalli and Parsons 1997; Garrison 2010). In this way, understanding several aspects of the host-parasite interaction as well as helminth-etiological pathologies in different trophic levels could help to mitigate the parasitosis or its control in economic animal species.

This study describes the histopathology caused by metacercariae of *B. margaritae* in gills of *H. fissicornis*.

Materials and methods

Infected specimens ($n = 10$) of *H. fissicornis* were collected at Ponta do Sambaqui, Baía Norte, Florianópolis island, Brazil (27° 29'S–48° 33'W) from the ropes of a mussels farming's area of the experimental culture of *P. perna*. The gills were isolated from the opercular cavity under stereoscopy to metacercarial cysts counting.

In fact, this study is part of a larger work, where the authors identified and characterized the life cycle of the parasite species. The parasite identification was performed according to the morphological aspects of cercariae, metacercariae and adults sensu previously published work (Marchiori et al. 2010).

In the laboratory, the parasite larvae were collected from disrupted sporocysts of the mussels. Metacercariae and adults were fixed in 5 % formaldehyde. These fixed parasites were washed in distilled water to be stained in Gomori's Trichrome, clarified in creosote and mounted in Canadian balsam. Live cercariae were utilized to perform measurements, too.

The general aspects for the parasite identification are presented below:

The morphological measurements of the life cycle stages of the parasite were performed under camera lucida (in micra). The main measurements (and other features of the parasite) can be summarized as follows, according to: cercariae (body length and width; rinchus length and width; bulb shape; mouth shape and position; pharynx width), metacercariae (cysts length and width; body length and width; number of tentacles in the rinchus; number of projections in the tentacles; shape and dimensions of the projections in the tentacles; mouth position; tegument spines presence and they body surface distributional pattern) and adult (body length and width; tegument spines

presence and they body surface distributional pattern; rinchus shape; position of the tentacles projections; length and width of the tentacle projections; oral sucker position, length and diameter; pharynx width; body's vitellaria distribution and shape; intestine/vitellaria body distribution relationship; ovary shape and position; pre-pharynx presence or absence; pre-pharynx length and width; uterine loops/vitellaria distribution relationship; testicles shape, length, width, position; uterus shape and position).

To histological procedures were utilized five hosts specimens. The gills were fixed in Bouin's fluid (by 3 days) and softly decalcified in 5 % Formic acid in Formalin 10 % aqueous solution (by 3 days) to histological techniques for embedding in paraffin, microtomia thickness of 7 μ m, staining with Hematoxylin-Eosin and permanent mounting in Canadian balsam sensu Behmer et al. (1976).

Microphotographs were acquired using an "Olympus BH-2 microscope" (Japan) equipped with "Olympus Camera C-5060 digital camera" (Japan) associated.

Results

Branchial Infection Sites (BIS)

Metacercarial cyst were found on both left and right gill arches. The cysts could be observed as red points on gill surface similar to focal hemorrhages. Metacercaria (MTC) showed a BIS-pattern occurring in the branchial abductor muscle (BAM) from primary branchial filaments (PBF) and also within cartilaginous and osseous tissue of the branchial arches (BA) and branchial rays (BR) (Fig. 1A). MTC were delimited by an eosinophilic and thin contentional hyaline parasitic capsule (CPC) (Fig. 1B) of fibrous connective tissue formed by fusiform cells as fibroblasts. Histologically, the lesion feature comprised chondrocytes and osteocytes pyknosis, melanin-like pigment accumulation near to MTC and thrombosis of blood vessels. Grossly gill melanosis couldn't be noted.

The infection in the BR is half-basalward (Fig. 1A–C) and the amplitude was 1–3 MTC (Fig. 1B–D). Concerning to the BAM only one metacercariae was present within it.

Several degrees of half-apicalward BR displasia occurred above the BIS in the PBF. In some cases has occurred total degeneration of cartilaginous tissue on the cyst proximities. Telangiectasia and hemorrhagic processes were not observed. MTC showed an oriented pattern of distribution basal-upward one-after-another in PBF (Fig. 1D). Under light microscopy, apparently, each MTC has itself a CPC and chamber. Foreign-body-like giant cells associated with CPC were not observed in the lesions. Losses of PBF fragments as necrotic sequestration and secondary bacterial infections were not observed.

Fig. 1 Gill photomicrographs of *Hypleurochilus fissicornis* infected by metacercaria (MTC) of *Bucephalus margaritae*. Stain: H-E. **A** MTC (arrows) harbored in the branchial arc. Escal: 150 μ m. **B, C** Details of MTC half-basalward gill harboring and gill general response aspects. Escal: 70 and 90 μ m, respectively. **D** MTC (arrows) linear harboring pattern in the gill's PBF. Escal: 110 μ m. **E** Hypertrophy of the chloride cells (arrows). Escal: 60 μ m. **F** Gill bucephalosis general feature demonstrating apical lamellar obliteration (circle). Escal: 50 μ m. **G** Gill interlamellar space obliteration (arrow). Escal: 260 μ m. **BS** Blood vessel, **BA** Branchial arc, **PBF** Primary branchial arch, **BR** Branchial ray, **SBF** Secondary Branchial filament, **CPC** Contentional hyaline parasitic capsule, **EH** Epithelial hyperplasia and hypertrophy

Concerning to the BAM, there were local degenerations of muscular fibers near to the cyst with some degree of pyknosis. The MTC were harbored within the connective muscular capsule of the BAM.

Cytopathologies were present in two ways: 1—mainly in the half-apical region of the PBF when MTC were harbored in the half-basal of BR causing alterations in the secondary branchial filaments (SBF) or 2—in *toto* PBF when MTC were installed more basally within BA. In these

areas it was observed hypertrophy and hyperplasia of the epithelial lining and chloride cells (Fig. 1E). The abnormal chloride cells showed light eosinophilia. The thickening of the SBF epithelium lining due to the hyperplasia and hypertrophy was present and resulted in interlamellar obliteration and fusion of lamellae (Fig. 1F–G). Pyknosis of pillar cells and lining epithelial cells were present. Immune cell exudations, mainly lymphocytes, were present in abnormal PBFs.

Discussion

Gills are complex and comprise a very large body surface area to the homeostasis of the fishes. The large gill surface area shows a potential for rapid and clinically ionic and protein change in acute diffuse injury with pathological importance. This organ responds to the injury by several ways, as encapsulation, depending on the type and severity of the causative agent and also the length time of exposure (Evans et al. 2005).

Encapsulation of infective stages of metazoan parasites is a common pattern observed in several studies in different host-parasite levels of interaction as a response to the injury (Fergusson 1989; Faliex 1991; Laruelle et al. 2002). Simões et al. (2006) describe to *Ascocotyle (Phagicola) pindoramensis* (Travassos 1928) (Digene: Heterophyidae) under ultrastructural examination and report the presence of tegumental specializations. In addition, these authors record that these tegumental specializations of the MTC (as scale-like tegumental spines) may promote irritation or erosion on host's tissues. Faliex (1991) analyzed the effects of *Labratema minimus* (Stossich 1887) (Bucephalidae) MTC in the liver of *Liza ramada* (Risso 1827) and *Atherina boyeri* Risso 1810 (Teleostei) and observed different organizational features of CPC structure. These latter authors also discussed about the possibilities of the consume of the host tissues remains by the parasites and such data are supported by two aspects: (1) the presence of lipid inclusion bodies and granular material into MTC's cystic cavity and, (2) the positive metacercarial growth observed. Concerning to the mussel *P. perna* (first intermediate host) the findings of Silva et al. (2002) showed no signs of parasitic encapsulations or immune cell exudation processes by the host. This CPC-absence aspect could be related to the simpler cercaria revetment, differently from a more complex MTC tegumental structure, reinforcing the irritative power of metacercariae tegument ornamentation on host tissues.

Encysted MTC of several Digenea species stimulate the local melanocytes in fishes and they are grossly visible due to the dermal melanosis (Ogawa et al. 2004). In the present study, the melanosis, properly so called, was not observed as “black spots” and the macroscopic aspect of the lesion resembles telangiectasia points as observed by Shoiabi et al. (2010). Evans et al. (2005) record that melanin pigment accumulation is a normal aspect of fish gills.

Etiological agents generally show a cell and tissue specificity pattern (Thomson 1983) and gill lesions tend to be diffuse (Evans et al. 2005). Thomson (1983) and Carlton and McGavin (1998) write that parasitic larval phases show chemotaxis by cellular, tissular or systemic host sites for infection, from a specific way. The occurrence of *B. margaritae* MTC within gill connective tissues-type of *H.*

fissicornis corroborate records concerning to the affinity of this larval stage to the connective tissues from other body regions such as dermal skin layer (Fergusson 1989) and caudal fin rays (Ogawa et al. 2004). Olson and Pierce (1997) reported that the affinity to the gill cartilage is characteristic of Heterophyidae. The infectivity of *B. margaritae* to the gills of *H. fissicornis* seems to occur when the gill's skeletal tissues are cartilages and not yet totally calcified. In Vertebrata the ossification process shows a transitional and gradual mosaic-pattern from cartilage to bone as they age (Hall 2005). This may propitiate infections early in host, compounding an adequate way to the penetration of the MTC, as observed in several studies.

Some Heterophyidae and Bucephalidae species immediately harbor and encyst in the gill while others search the proximities of calliperest gill blood vessels where they produce a multilayered CPC (Armitage 2000). This aspect may be related to the low irrigation and nutritive diffusion of this kind of connective tissue and its relative isolation of the immune system (Thomson 1983; Gartner and Hiatt 1999). The MTC nutritive and gaseous exchange implications could explain the half basal-ward pattern observed in this study to the BAM and PBF, concerning to the gill blood vessels main tributaries. In fact, if MTC settlement occurred in apical portions of the gills perhaps it would be easier to lose this portion due to the fragility of an abnormal organ (necrotic and edematous) as observed in MTC-positive PBF structure. Ecologically it is important to the parasite complete its life cycle properly in one appropriated host site (Thomson 1983) and the loss of a gill fragment with the cyst could be inadequate to the parasite life cycle. Furthermore, the breaking of gill fragments could permit a secondary nature infection because the skin comprises the first barrier against pathogens (Gartner and Hiatt 1999; Roberts 2001).

The PCP could represent a plus mechanism of protection against the host immune system as described for several pathological agents, such as virus, bacteria and metazoans (Roberts 2001). A significant immunological response to the MTC depends on the PCP presence (Fergusson 1989). The latter author reported that when PCP is present there is little immune response, what could explain the absence of foreign-body-like giant cells and the low quantities of granulocytes in this study. These features were very closer to the observation of Shoiabi et al. (2010). Furthermore, PCP mechanisms, no-losses of PBF fragments as necrotic sequestration and no-severe inflammatory processes could represent a way to increase the parasite time of permanence in host environment, i.e., host body. This could be a parasite natural selection mechanism ward to low virulence, as cited by Ganusov (2003). This low helminth virulence could permit the parasite to reach its adequate definitive host papa-terra *Menticirrhus*

americanus (Linnaeus 1758) (Sciaenidae) (Marchiori et al. 2010) to complete its life cycle, when different hosts are needed for the complete parasite life cycle, as Bull (1994) explained.

As seen in this study, Evans et al. (2005) and Shoiabi et al. (2010) observed that gill chronic responses to the injury includes hyperplasia of the chloride cells and, in more severe cases, includes marked epithelial hyperplasia with fusion of adjacent lamellae and obliteration of the interlamellar spaces. Edema, hypertrophy or hyperplasia of the gill epithelium could result in difficulties to gaseous and metabolites exchange with the water due to the higher distances between host blood supply and environment (Evans et al. 2005). As also observed by Faliex (1991), Laruelle et al. (2002) and Ogawa et al. (2004) necrotic features were present in this study and they may represent a response of circulatory disturbs caused by cysts formation. Cysts could modify, through ischemia, the pressure on the PBF afferent and efferent branchial artery and SBF arterioles. Circulatory disturbances are recorded as typical of migrant parasitic larvae (Thomson 1983; Carlton and McGavin 1998) and those caused by bucephalosis, in this study, was not sufficient to promote gill necrotic sequestration. When chronic gill tissue degeneration was present there was strong inflammatory cell influx, mainly lymphocyte cells, to the affected area and lower quantities of granulocytes. The lymphocytes are the most commonly cell type on the fish blood (Roberts 2001) what could explain the strong presence of this blood cell in the inflammatory process observed in the present study. The gill's bucephalosis itself doesn't seem to cause death processes in *H. fissicornis*. However, other anatomical and physiological complications derived from other infected organs may result in fish mortality.

Conclusions

The bucephalosis in the gills of *H. fissicornis* is no-fatal; it constitute a no-hemorrhagic branchitis without necrotic sequestration and melanosis; it follows a mosaic-pattern of gill's ossification permitting metacercariae harboring and encystations; it may compromise the gill functions and could permit secondary opportunistic infections.

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