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ORIGINAL PAPER

Ultrastructure of *Didymocystis semiglobularis* (Didymozoidae, Digenea) cysts in the gills of Pacific bluefin tuna (*Thunnus orientalis*)

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Abstract Tuna are active pelagic fish with an extraordinary migratory activity, well known for their unique physiology reflected in high metabolic rates. However, knowledge of microbial and environmental diseases is still limited. We have analyzed the ultrastructure of the digenean trematode *Didymocystis semiglobularis* isolated from the gill arch of Pacific bluefin tuna (*Thunnus orientalis*) by transmission electron microscopy (TEM). The parasite is demarcated from the rest of host tissue in a sac of host origin, composed of active fibroblast and scattered bundles of collagen tissue, with no macrophage accumulations. TEM micrographs reported in this study reveal a wide multilayered tissue isolating the host from the parasite capsule and more internally complex and compact layers dividing the parasite capsule from the body itself, which encapsulates eggs at different developmental stages. Since the size and shape of the parasite would imply host tissue activation at the site of infection, no histopathological changes were observed in the architecture of the tuna superficial layer. No degeneration or necrosis was observed in the upper layer of the host tissue.

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Introduction

Pacific bluefin tuna (*Thunnus orientalis*) are highly migratory pelagic fish renown for their high aerobic capacity and migrations across ocean basins. Bluefin tuna also represent the most valuable finfish aquaculture product currently recognized, whom production accounts for more than half of the total world capacity, concentrated in the Mediterranean Sea and with increasing penning efforts in Mexican waters (FAO Fishery statistics 2002; Miyake et al. 2003). Bluefin tuna shows remarkable physiological amenabilities including high metabolic rates, high cardiac outputs, and high capacity to conserve metabolic heat (Carey and Teal 1969; Block and Stevens 2001; Blank et al. 2004). Their flexible physiological tolerance enables them to experience large ambient temperature changes when diving below the thermocline or encountering cold surface waters at high latitudes (Block et al. 2001).

The long-distance migrations and exposure to a wide range of ambient water temperatures facilitate infections from several parasitic groups. Parasite communities of wild and reared bluefin tuna display remarkable diversity of species, among which the highest levels of prevalence and abundance are achieved by members of Didymozooidea superfamily (Trematoda, Digenea Poche 1907) (Munday et al. 2003; Mladineo and Tudor 2004). According to Nikolaeva (1985), family Didymozoidae numbers 212 species placed in 81 genera and 23 subfamilies, parasitizing generally marine fish, with only four species described from freshwater. Their distribution is wide, occurring in tropical and subtropical world oceans, while 23 species have been isolated from Mediterranean fish. Didymozoids are gonochorists or hermaphrodites, mostly encysted in pairs, with a wide and species-specific distribution in host tissues. In bluefin tuna, they are found to inhabit different

niches, parasitizing gills, cartilaginous, mucous, and connective tissue in nose, mouth, and ocular and gill cavity, as well as digestive organs, kidneys, gonads, skin, and fins (Yamaguti 1958, 1970). The diversity and broadness of their predilection sites reflect in the capability to elicit various degrees of pathologies in reared fish (Mladineo 2007). Little is known about their life cycle, except the existence of many paratenic hosts (small pelagic fish and cephalopods) that propagate the metacercariae to tuna (Lester and Newman 1986). Although it is generally accepted that after the ingestion in a final host, larval stages are transported by the blood stream from the digestive system to various tissues, both mechanisms of recognition at the site of infection and the stimuli that induce two individuals to aggregate at the same site enabling their encystment in a single cyst are still unknown.

In general, most digeneans are parasites of the digestive tract where they feed on mucus, epithelial cells, and occasionally blood. There are few reports of significant pathogenesis and perhaps none of mortalities associated with such infections. This is probably because worms are small relative to the size of their host. They are mobile so they do not produce permanent damage at a single site and their diet is innocuous. The digeneans that do cause significant problems for their hosts as sexual adults are mainly those that occur in nongut sites. For example, blood flukes (Sanguinicolidae) occurring in blood vessels of fish interfere with blood flow, induce erosions of blood vessels, and, since their eggs do not have a direct route for their exit to the water, pass across tissues which causes major inflammatory responses (see Cribb 2005).

In order to better understand the host tissue reaction to abundant tissue digeneans in a highly valuable host, we studied the morphology of *Didymocystis semiglobularis* cysts embedded in the cartilaginous base of gill arch between gill rakers in the Pacific bluefin tuna (*T. orientalis*).

Materials and methods

Animals

Pacific bluefin tuna (*T. orientalis*) were captured by hook and line off San Diego, CA, USA, held on board of the fishing vessel in wells filled with flowing seawater, and transported by truck to the Tuna Research and Conservation Center in Pacific Grove, CA, USA. The tuna were held in two 109-m^3 circular tanks containing seawater.

Didymozoid cysts were measured and ruptured with fine needles under the stereomicroscope. Individuals were fixed under coverslip pressure, stained in Borax carmine, and mounted in Canada balsam. Identification was done following Ishii (1935) and Yamaguti (1958, 1970).

Samples of *D. semiglobularis* cysts were removed from the cartilaginous base of gill arch between the gill rakers in the Pacific bluefin tuna and fixed by immersion with 3% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2, kept at room temperature for a brief period of time, and stored at 4°C. The whole cyst was then postfixed in 2% OsO_4 in 0.1M sodium cacodylate for 1h at room temperature, stained *en bloc* with saturated uranyl acetate in cold acetate buffer, and embedded in Epon. Ultrathin sections (70–90nm) were stained with saturated uranyl acetate solution in 50% ethanol and lead solution. Sections were observed using a Philips 410 microscope (Philips Electron Optics, Mahwak, NJ, USA). Images were recorded either on film or using a Hamamatsu C4742-95 digital imaging system (Advanced Microscopy Techniques, Chazy, NY, USA).

Results

Host tissue

The outer host tissue layer, in contact with the exterior side of the cyst presumably touching the gill arch, is composed of an undulated layer formed by a thick epithelium (Fig. 1a) ranging from 10 to 40μm wide. Within this layer, excretory cells (Fig. 1b, c) show a wide variety of cytoplasmic organelles such as large kidney-shaped nuclei (N), small mitochondria (M), and particularly extensive rough endoplasmic reticulum (rER; Fig. 1c). Together with those cellular elements, long, narrow, and large nucleated structural cells are visible (Fig. 1a, c, between arrows).

Cells belonging to this external layer are tightly packed between the external plasma membrane and a double basal lamina (Fig. 1d, double arrow) in direct contact with a thick (~80μm) internal layer of connective tissue where dense bundles of collagen fibers run in different orientations (Fig. 1d, asterisk).

The inner host layer seems to be in close contact with the outer parasite cyst layer. It is formed of a multilayered connective tissue with collagen fibers (Fig. 2, asterisks) and noncellular membranes where an intensive pinocytotic activity exists. Between collagen bundles, few fibroblasts are scattered, showing large centrally located and granulated nuclei (Fig. 2a) together with large lysosomes within a thin rim of cytoplasm (Fig. 2b, c). Inside the cytosol, large vacuoles are also visible (Fig. 2b, double arrow) while rare cases of phagocytosis intrusions can be observed (Fig. 2c, arrowhead and inset). The discontinuous layer of connective tissue is composed of elongated fibroblasts with large oval nuclei, apparently fused with a layer of cellular elements of variable shape and size. A viscous matrix fills the space between the individual cells and between the next membranous layer (Fig. 2a). Deeper, beneath the fibroblast-

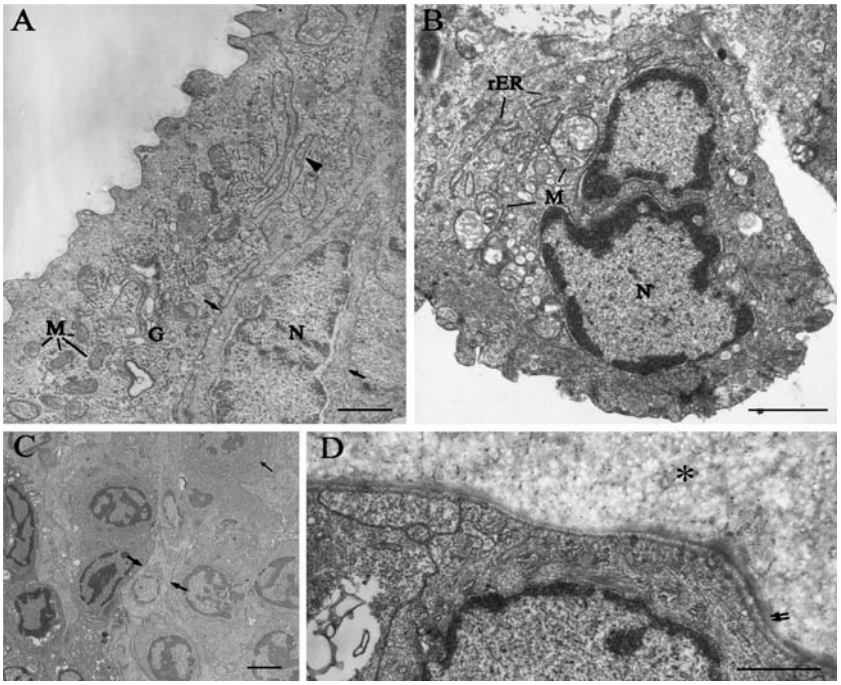

Fig. 1 Ultrastructure of the host outer tissue layer. **a** Within the outer host layer, several cellular organelles are visible together with a structural cell (between *arrows*): mitochondria (*M*) and convoluted membranes (*arrowheads*) are well visible as well as the Golgi complex (*G*), rough ER, and many vesicles of different shapes. **b** An excretory cell showing several organelles sparsely distributed within the cytoplasm. **c** A more internal view of the outer tissue layer showing the alternation of structural cells (between *arrows*) and excretory cells where large rows of rER are well visible (*arrow*). **d** At the end of the epithelial layer, a basal lamina (*double arrows*) divides the latter from an internal large area of collagen fibers (*asterisk*). (*M*, mitochondria; *rER*, rough endoplasmic reticulum; *N*, nucleus, *G*, Golgi complex). *Bars*: 1 mm in **a**, **b**, and **d**; 2 mm in **c**

rich part of the layer where collagen bundles are present, the tissue is interlarded with crescent-shaped elongated cells abundant with vesicles and small numerous cisterns (Fig. 2b, c).

Parasite body

The parasite body is in intimate contact with the inner parasite capsule layer. The thin (~4mm) tegument wall of the parasite body (Fig. 3a) is made of an external basal lamina in contact with a noncellular layer (cyst wall) composed of dense matrix of numerous secretory granules alternated to small vesicles and presumably thin microtubule filaments. Beneath this frame, another thicker basal lamina separates the external wall from a multicellular layer. The immediate deposit above the basal lamina seems to be composed by thin bundles of muscle or microfibrillar components disposed mostly parallel to the plasmalemma (Fig. 3a). The stratification of organelles extends with a wide multicellular layer containing a large number of rounded mitochondria together with lipid vesicles and, more deeply, large oval nuclei (Fig. 3b). This is the uterine wall that is composed of large and densely packed spherical parenchymal cells, centrally nucleated and abundant in cytoplasmic organelles. Cells are divided partially by membranous trabeculae perpendicular to the lumen where the number of vesicles increases (Fig. 3c) presumably ready to be secreted in the lumen. The last layer of uterine wall is made up of thin and long fibrous material where glycan granules of different sizes are accumulated.

Uterine lumen is filled with bean-shaped eggs at several developmental stages. Some eggs show a dense shell enclosing undifferentiated cells embedded in the amorphous mass (Fig. 4a) while others, in a more advanced stage, display few more cytoplasmic organelles (Fig. 4b). No operculum was noticed at this stage of development.

Discussion

In aquaculture, digenean impact on fish is usually limited to negligible or mild effect on the host. However, exceptions like sanguilicolid infections are able to deteriorate whole fish stocks and cause serious economic losses (Crespo et al. 1990; Ogawa and Fukudome 1994). The most common tissue reaction to trematodes is formation of a fibroconnective

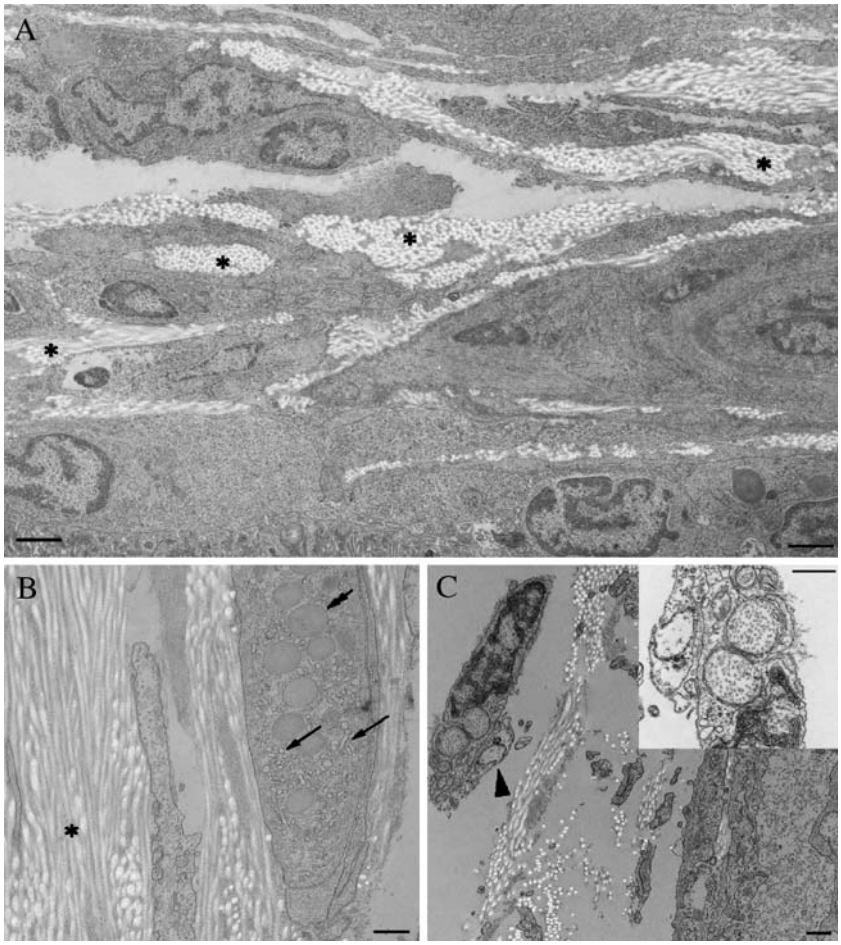

Fig. 2 Inner host tissue layer. **a** The outer layer of the host tissue and the beginning of the parasite body (*double arrow*) are separated by a large layer of a mixed collagen tissue (*asterisks*) and fibroblast. **b** Within the layer, collagen fibers are arranged in different orientation sometimes closely apposed to cells, abundant with lipid vesicles (*double arrow*) and small cisternae (*arrows*). **c** Together with collagen and fibroblasts, phagocytosis processes (*arrowhead* and *inset*) are frequently seen. *Bars*: 1 mm in **a**; 0.5 mm in **b** and **c**

capsule surrounding juvenile (Mitchel and Crang 1976; Walker and Wittrock 1992; Wittrock et al. 1991) or adult stages (Taylor and Hall 1993; Taylor et al. 1994; Dezfuli et al. 1997), with additional aggregation of macrophages. Didymozoids, or so-called tissue flukes, are frequently isolated from pelagic and shelf fishes, particularly in scombroid species (Munday et al. 2003). Throughout diverse genera, there is often an enormous difference in body size and tissue localization. Histopathology can range from negligible host reaction to necrosis and sloughing of gill epithelium with consecutive secondary bacterial infections. *Didymocystis* spp. infestations rarely lead to gross pathologies (Mladineo 2007). In case of *D. semiglobularis* cysts, even though the size and shape of the parasite would imply host tissue activation at the site of infection, no changes in architecture of the superficial layers of tuna were observed. Other peculiarities in its cyst ultrastructure make this species stand out from common patterns of tissue reaction to trematodes.

The parasite is demarcated from the rest of host tissue in a sac of host origin, composed of active fibroblast and scattered bundles of collagen tissue, but no macrophages accumulations were noticed. Similar formation in terms of demarcation of the parasite with the tissue of host origin was described in the myxozoan *Myxobolus pendula* infection of cyprinid *Semotilus atromaculatus* gills. The striking difference however was that the parasite induced formation of a nonnecrotic epithelioid granuloma, where the capsule surrounding the parasite derived from macrophages is not detrimental to the parasite itself (Koehler et al. 2004). Authors have argued that the parasite expressed highly potent surface antigen that elicited a powerful immune response that failed to destroy the parasite, protecting both the tissue and the parasite by enclosing the cyst in a collagen-rich envelope. The lack of immune response to the *D. semiglobularis* could be related to the poor vascularization of the tissue at the infection site, consisting mainly of layers of connective fibers. Perera (1994) described cyst ultrastructure of a gill arch didymozoid in slimy mackerel (*Scomber australasicus*) showing a structure very coherent with the dermal and epidermal tissue where the parasite was embedded. The capsule was

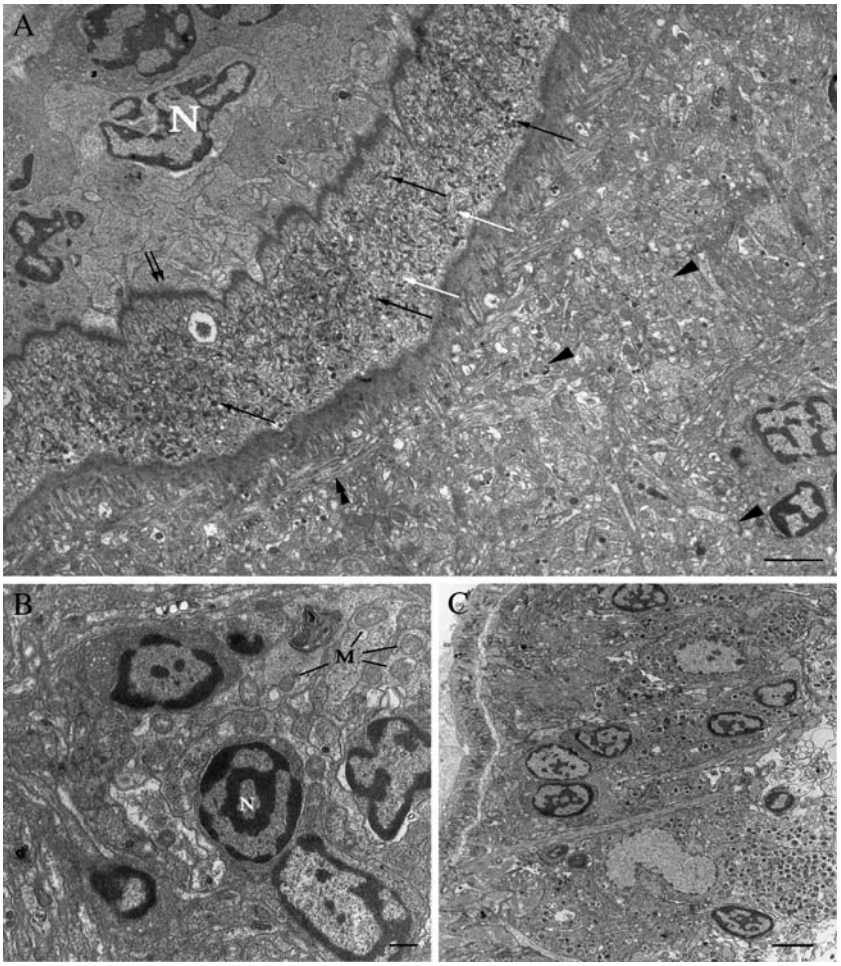

Fig. 3 Ultrastructure of the parasite body. **a** At the cross-section of the parasite cysts, several tissue layers are clearly distinguishable at electron microscope level. In the upper left corner, the collagen layer (*asterisk*) is still visible followed by the multicellular organization of excretory cells with large nuclei (*N*) and dense matrix. Right above that layer, an opaque basal lamina (*double arrow*) separates the parasite cyst wall from the external tissue. Inside, a dense tegumental wall contains numerous excretory granules (*black arrows*) as well as possible microtubule filaments (*white arrows*). The following layer consists of another dense multicellular compartment where several cellular elements are shown. Particularly, microfibrillar components (*double arrowheads*) are disposed right above the inner membrane and mitochondria (*arrowheads*) are sparsely disposed within the cytoplasm. **b** Further deep in the parasite body, large cells with big mitochondria (*M*) and large nuclei (*N*) form the most interior part of the outer parasite body wall. **c** The uterine wall is composed of large and densely packed cells, containing a large central nuclei (*N*) and a large variety of cytoplasmic organelles and electron-dense vesicles of glycan origin. *Bars*: 2 mm in **a** and **c**; 0.5 mm in **b**

surrounded with dermal tissue abundant with collagen fibers that did not show any particular pathway. In contrast with our findings, there was a noticeable space between the parasite body and host tissue and an elevated number of cellular elements, particularly lymphocytes. In comparison with other didymozoids encysted in vascularized tissues, like *Didymocystis wedli* in gill filaments and *Koellikerioides intestinalis* in intestinal submucosa, lymphocytic infiltration was described as well (Mladineo 2007). Moreover, observation of dehydrated black cyst isolated in subserosa of pyloric ceca that harbor leftovers of the didymozoid state an evidence of a very powerful and efficient destruction of *Coeliodidymocystis abdominalis* cysts in tissue rich with blood vessels (Mladineo, personal observations). Within poorly vascularized tissue like tuna gill rakers, the capsule has been formed by layers of fibroblasts, reinforced by bundles of collagen without the involvement of macrophages or other immunity mediators. Even though venula was observed on one occasion (figure not shown) in the deep inner host tissue layer in the intimate contact with the parasite capsule, no anastomosis or diapedesis from the vessels into the parasite capsule were observed, addressing the incapacity of development of inflammatory process. The complexity of the reaction to parasites in highly vascularized tissue was described by Dezfuli et al. (2005). The authors noticed that the heart-infecting metacercariae

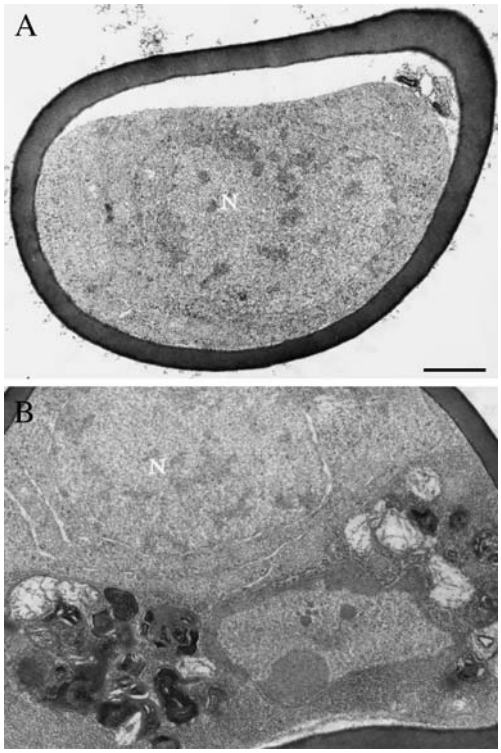

Fig. 4 Successive stages of embryonated eggs inside the parasite body. **a** Early developmental stage of the eggs where only the nuclei (*N*) is distinguishable. **b** More advanced embryonic stage where cytoplasmic organelles are embedded in to an amorphous mass. *Bar*: 1 mm

of the trematode *Ichthyocotylurus erraticus* are able to induce the formation of a new network of nervous fibers at the inflammatory site, which serves to stimulate blood flow through vascularization of the developing cysts. A similar finding was associated with the acanthocephalan *Pomphorhynchus laevis* infection in *Salmo trutta* where neuropeptides were assumed as putative neurotransmitters in the neo-formed network of nervous fibers at the site of tissue inflammation (Dezfuli et al. 2002).

There is striking variety of adaptations among didymozoids as well, aimed to the colonization of the host tissues. *Unitubulotestis sardae* (Trematoda, Didymozoidae) is found parasitizing the gill of the Atlantic bonito *Sarda sarda*, in a formation know as *aneurisma verminosa saccata* (Marino et al. 2003). The parasites elicit neovascularization and hyperplasia of the gill epithelium, leading to the formation of a double stratified cystic wall that surprisingly lacks connective capsule. This finding reinforced the hypothesis that some didymozoids reach the predilection tissue via blood vessels, penetrating their walls and starting to grow close to the blood flow, eventually inducing neoangiogenesis at the site (Grabda 1991). However, this process seems to be unique only for some didymozoids, whereas species isolated from tuna unvascularized tissue do not show intimate engagement of the blood vessels around their capsule and lack local inflammatory reaction. Little has been documented so far regarding other possible strategies of didymozoid colonialization or the fate of metacercariae if they do not reach appropriate infection site. First account of metacercarial colonialization was given by Ishii (1935), who experimentally observed that larvae of *Didymocystis katsuwonicola* adhere to appropriate place on the gill epithelium, entering tissue head first. After arriving at their destination, larvae band round up and produce a membrane afterwards.

In *D. semiglobularis*, a multilayered membrane with strong pinocytosis activity was formed above the fibroblast layer of parasite capsule, channeling the exchange of nutrients and metabolites between the parasite and host tissue. No degeneration or necrosis was observed in the following upper layer of the host tissue, implying that no detrimental metabolites were excreted from the cyst. The lack of the noncellular layer of parasite origin, which is a result of secretion from the parasite body and is common in many digenean metacercariae (Paperna 1995), is another feature characteristic to *D. semiglobularis*.

The fact that some didymozoids evoke inflammatory reactions and others do not, as in the case of *D. semiglobularis*, might be partially related to the level of vascularization of the infected tissue. Elevated levels of immunoglobulin E and eosinophile granulocytes as well as mast cell hyperplasia and secretion of inflammatory cytokines are the hallmarks of helminthic infection in mammals and lead to the development of more or less pronounced inflammatory reaction. In highly vascularized tissues, like gut or gill epithelium, parasites are more quickly detected by those immunity components circulating in the blood than species secluded in poorly vascularized tissues. Some parasites also evolved a variety of sophisticated mechanisms to evade effective host immune responses, like seclusion within a host cell, production of molecules that inhibit nonspecific host defenses in the case of protozoans, shielding in a layer of host-derived molecules, or displaying an ever-changing repertoire of surface antigens to elude antibody-mediated destruction (Allen and Liu 2004). Which strategy was develop by *D. semiglobularis* still remains an open question, since it is controversial that such a large species, belonging to moderately pathogenic genus, inflicts no ultrastructural changes at its parasitization site.

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