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Short Communication

Fine structure and cellular responses at the host–parasite interface in a range of fish–helminth systems

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ABSTRACT

A series of ultrastructural-based studies were conducted on the interface region in different fish–helminth systems: (a) an intestinal infection of the cestode *Monobothrium wageneri* in tench, *Tinca tinca*; (b) an extensive intestinal submucosa and mucosal infection in tench by metacercariae of an unidentified digenean trematode; (c) an intestinal infection in brown trout, *Salmo trutta*, by the acanthocephalan *Dentitruncus truttae*; (d) an extraintestinal infection by larvae of the acanthocephalan, *Pomphorhynchus laevis* in three-spined sticklebacks, *Gasterosteus aculeatus*; and (e) an infection in the livers of Eurasian minnow, *Phoxinus phoxinus*, by larvae of the nematode *Raphidascaris acus*. Endoparasitic helminths frequently cause inflammation of the digestive tract and associated organs, inducing the recruitment of various immune cells to the site of infection. In each of the fish–helminth systems that were studied, a massive hyperplastic granulocyte response involving mast cells (MCs) and neutrophils in close proximity to the helminths was documented. The current study presents data on the interface region in each fish–helminth system and documents the penetration of mast cells granules within the tegument of *P. laevis* larvae. No extracellular vesicles containing tegumental secretions from any of the four different taxa of endoparasitic helminths species at the host–parasite interface region were seen.

1. Introduction

Fish which include over 27,000 species are, phylogenetically, the oldest vertebrate group representing more than one-half of the vertebrates on the planet (Toledo-Ibarra et al., 2013). Understanding the immune systems of fish, therefore, is of great relevance as it provides information on the evolution of immunity in vertebrates (Rauta et al., 2012).

The innate immune system of fish comprises: (1) cytotoxic (i.e. natural killer) or phagocytic (i.e. macrophages, granulocytes) cells; (2) proteins that mediate the responses to helminth infection and (3) the use of physical (e.g. epithelial) and chemical (e.g. anti-microbial peptides) barriers to minimise the likelihood of parasitic infection (Dixon and Stet, 2001). In fish, neutrophils are the first cell type recruited to the site of an acute inflammatory response (Secombes, 1996; Katzenback and Belosevic, 2012) and their chemotaxis, phagocytosis and destruction of intracellular and extracellular pathogens demonstrate their important role in innate immunity (Secombes, 1996; Stakauskas et al., 2007; Katzenback and Belosevic, 2012).

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Mast cells (MCs), a type of granulocyte, are potent inflammatory cells that are present in most tissues and are commonly strategically positioned in close proximity to blood vessels (Reite and Evensen, 2006). In helminth-infected fish, MCs have been observed to migrate and accumulate in large numbers at the site of parasitic infection (Reite and Evensen, 2006; Dezfuli et al., 2008, 2011a, 2013a, 2014). In fish as in other vertebrates, MCs are very active and their role in the early orchestration of an immune response against a range of disease agents, including parasites, has been documented in several studies (Abraham and St John, 2010; Prykhozhi and Berman, 2014; Sfacteria et al., 2015). Mast cells in nonmammalian vertebrates contain a wide range of compounds (i.e. histamine, heparin, neuropeptides, proteases) and, in bony fishes, also antimicrobial peptides (AMPs) (Baccari et al., 2011; Masso-Silva and Diamond, 2014).

Recently the investigation of host–parasite interactions has increased considerably, numerous studies focusing on the identification of mammalian helminth excretory/secretory (ES) proteins (Marcilla et al., 2012; Smith and Maizels, 2014). Knowledge on the occurrence and effects of helminth ES proteins on the immune systems of fish, however, is still limited (Buchmann, 2012; Bahlool et al., 2013).

For the current study, transmission electron microscopy is used to study and comment on the interface region in four different taxa of endoparasitic helminths and their hosts.

2. Materials and methods

In 2013, a total of 28 specimens of tench, *Tinca tinca* (L.) (47.36 ± 4.55 cm, mean total length TL ± standard deviation [SD]) and 40 specimens of brown trout, *Salmo trutta* (L.) (28.9 ± 7.48 cm, mean TL ± SD) were processed from Lake Piediluco situated in the Province of Terni, Central Italy (42°31′01″ N; 12°45′00″ E). The fish were caught by gill net that was deployed on three occasions by professional fishermen operating within the lake. Twenty-five specimens of Eurasian minnow, *Phoxinus phoxinus* (L.), (60.96 ± 3.73 mm, mean ± SD), and 39 three-spined sticklebacks, *Gasterosteus aculeatus* (L.) (47.80 ± 4.62 mm, mean ± SD), were sampled by electrofishing a tributary of the River Brenta, North Italy.

After capture, the fish were transported live to the laboratory, euthansed using an overdose of 125 mg L^{-1} MS222 (tricaine methanesulfonate, Sandoz, Basel, Switzerland) and thereafter, the spinal cord was severed. The fish were lengthed and weighed and a complete necropsy was performed, with particular interest to gills, gonads, liver, kidney, spleen and the alimentary canal which was completely dissected and opened.

For light and electron microscopy, small pieces (i.e. 7 mm × 7 mm) of the following tissues were excised and fixed in chilled (4 °C) 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer, pH 7.3 for 3 h: parasite-infected intestines from brown trout and tench, parasite-infected liver from minnows, encysted larval acanthocephalans on the outer surface of the intestine of three-spined sticklebacks. Thereafter the fixed tissues were post-fixed in 1% osmium tetroxide for 2 h and then rinsed and stored in 0.1 M sodium cacodylate buffer containing 6% sucrose for 12 h. Then, the samples were dehydrated through a graded acetone series and then embedded in epoxy resin (Durcupan ACM, Fluka, Buchs, Switzerland). Semi-thin sections (i.e. 1.5 μm) were cut on a Reichert Om U 2 ultra microtome (Reichert-Jung, Austria) and stained with toluidine blue. Ultra-thin sections (i.e. 90 nm) were stained with a 4% uranyl acetate solution in 50% ethanol and Reynold's lead citrate and observed using a Hitachi H-800 transmission electron microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany).

Corresponding pieces of intestine and liver were prepared from uninfected fish for comparison with parasite-infected tissues. The absence of parasites in uninfected fish was established by the necropsy and trough fresh microscopic smears which were performed on all the examined organs to rule out microparasites and related lesions. Histological sections confirmed that tissues of these control fish were parasites-free.

3. Results

Table 1 summarises the main information on host–parasite systems including fish and helminth species, prevalence and intensity of infection, parasite tissue location, host cell types and pathology.

3.1. *T. tinca* and the cestode *M. wageneri* (Table 1)

The attachment of the *M. wageneri*, typically in tight clusters of variable number, resulted in the formation of a raised, surrounding, inflammatory swelling. Cestode attachment to its host was effected by means of a simple, rounded scolex inserted deep into the intestinal wall, extending into the *mucosa* and *submucosa* as far as the *muscularis* layer. While these inflammatory swellings consist primarily of fibroblasts, there are also a large number of two different granulocytes, i.e. neutrophils and MCs. Interestingly, rodlet cells (RCs) were also found to co-occur with these granulocytes within the *submucosa* of the resultant nodule. Neutrophils and MCs were also recorded within the connective tissue surrounding capillaries and within the blood vessels within the *submucosa* and *muscularis* layer. MCs were observed to be irregular in shape with an eccentric, polar nucleus, and a cytoplasm characterised by numerous large, electron-dense, membrane-bounded granules (Fig. 1a). The cytoplasm typically contained two to three mitochondria and an inconspicuous Golgi apparatus. MCs were frequently surrounded by collagen fibres of the *submucosa* or by fibroblast-like unsheathing cells. Within the nodule, there were numerous neutrophils which appeared round to oval in shape though their outline was commonly irregular. These cells also contained a round nucleus and a cytoplasm with dark, elongated granules which were fibrous in appearance (Fig. 1b). Only a small number of mitochondria and some fragments of rough endoplasmic reticulum were seen within the cytoplasm.

Degranulation of the MCs, which was common in the *submucosa*, was characterised by the conspicuous swelling of granules, with free granules frequently seen in close proximity to the capilliform filitriches or adjacent to or between the coniform spinitriches of the

Table 1
Prevalence (% hosts infected), intensity of infection (range and mean ± standard deviation), parasite tissue location, fish immune cells and pathology in the different host–parasite systems examined.

Fish species	Parasite species	Prevalence (%)	Intensity of infection (mean ± SD)	Parasite tissue location	Host cell types	Pathology associated with infection
Tinca tinca	*Monobothrium wageneri*	46.3	3–75 (38.69 ± 23.51)	Intestine (anterior region)	Fibroblasts, neutrophils, MCs (in degranulation), RCs	Inflammatory nodules
Tinca tinca	*Digenean larvae*	39.3		Thickness of the intestine	Epithelioid cells, neutrophils, MCs	Hyperplastic response, granulomas
Salmo trutta	*Dentitruncus truttae*	77.5	2–77 (27.1 ± 24.25)	Intestine (anterior and regions)	MCs (in degranulation)	Intestinal layers disruption
Phoxinus phoxinus	*Raphidascaris acus*	44.0	1–8 (5.27 ± 2.28)	Liver	Epithelioid cells, macrophages, MCs, neutrophils	Nodules beneath the serosa
Gasterosteus aculeatus	*Pomphorhynchus laevis*	33.3	1–4 (2.38 ± 1.12)	Outer surface of the intestine	Epithelioid cells, MCs (significant degranulation)	Encapsulating reaction; epithelioid cells degeneration

MCs, mast cells; RCs, rodlet cells.

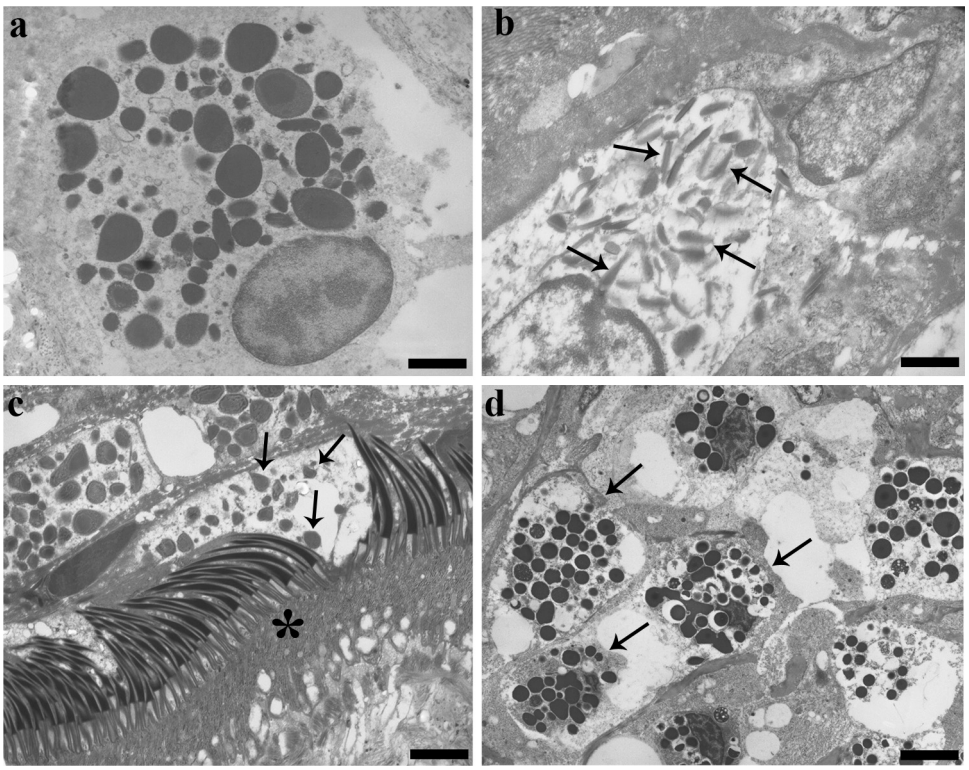

Fig. 1. (a) Transmission electron micrograph of a mast cell (MC) within the intestine of a tench, *Tinca tinca* (L.), infected with the cestode *Monobothrium wageneri* Nybelin, 1922, showing an eccentric nucleus and numerous electron-dense, membrane-bounded granules within the cytoplasm; scale bar = 1.0 μm. (b) Neutrophils are evident within the connective tissue of the submucosa of the tench's intestine. Note the aspect of the dark, elongated granules (arrowed) inside the cytoplasm; scale bar = 0.7 μm. (c) Interfacing region between host, i.e. tench tissue, and *M. wageneri*, where degranulation of the MCs is visible and where free granules (arrowed) were frequently seen in close proximity to the capilliform filitriches or adjacent to/between the coniform spinitriches of the scolex (asterisk); scale bar = 1.4 μm. (d) Intestine of a brown trout, *Salmo trutta* L., infected with the acanthocephalan *Dentitruncus truttae* Sinzar, 1955, showing MCs (arrows) in close proximity to the proboscis; scale bar = 4.0 μm.

scolex (Fig. 1c). Neutrophils were seen in close contact with the microtriches of the scolex. The MCs and neutrophils adjacent to the tegument of the parasite contained very few organelles and had a cytoplasm that appeared vacuolised, which were quite unlike the same cell types observed in zones approximately 1 cm away from the point of attachment of the cestode. In some tissue sections taken from *M. wageneri*-infected tench, focal loss of the apical plasmalemma of the cestode's microtiches were seen.

3.2. *T. tinca and digenean metacercariae (Table 1)*

Interestingly, within the thickness of the intestine of a small number of tench processed for this study ($n = 11$), a number of digenean larvae were found. The larvae, of the unidentified digenean, were encysted in the *submucosal* and muscle layers and within the thickness of the serosa where they induced a hyperplastic response. Each encysted digenean was surrounded by granulomatous tissue composed, mainly, of concentric layers of epithelioid cells forming a discrete spherical lesion. Epithelioid cells formed the inner layers of granulomas with cytoplasmic interdigitations and numerous desmosomes between adjacent epithelioid cells. The outer layers of the granulomas were composed of collagenous fibres with a variety of different immune cell types scattered among them. Some neutrophils and MCs were seen in close proximity to the metacercariae and, notably, several MCs were seen within the muscle layer.

3.3. *S. trutta and the acanthocephalan Dentitruncus truttae (Table 1)*

Although most *D. truttae* specimens did not cross the *stratum granulosum*, in several instances their proboscises were observed to have penetrated the *muscularis* layer. The *mucosa*, *lamina propria*, *stratum granulosum* and *muscularis* layer were disrupted at the point of proboscis insertion. Numerous MCs were seen in the host tissues in close proximity to the trunk/body of the acanthocephalan and around the proboscis (Fig. 1d). In both infected and uninfected brown trout, the *stratum granulosum* was rich in MCs. In both the *stratum granulosum* and in the *muscularis* layer, numerous MCs were in close contact with the capillaries; MCs were also seen in the outer layer of the endothelia as well as inside the blood vessels (Fig. 2a). Degranulation of the MCs within the *lamina propria* and the *stratum granulosum* was common (Fig. 2b); higher rates of degranulation were seen in the tissue in close proximity to the body of each acanthocephalan.

3.4. *P. phoxinus and the nematode Raphidascaris acus (Table 1)*

Macroscopically, the encysted nematodes appeared as yellowish-white nodules beneath the serosa of the liver. There were no signs of acute reaction to the presence of the parasites, suggesting a well-established infection. The nodules contained one or more larvae which were surrounded by a concentric corona of epithelioid cells, 2–5 cells thick with typical epithelial features including tonofilaments and desmosomes. The cells surrounding the nematode larva appeared much darker than those in the outer part of the nodule. Ultrastructure examinations revealed that the innermost layer of cells of the epithelioid corona surrounding the nematode larvae were composed of elongated macrophages (e.g. epithelioid cells). The innermost epithelioid cell layer was electron dense with finger-like projections (i.e. filopodia), increasing the interface between host cells and the nematode cuticle. MCs were the most dominant cell type encountered around the larva, which were observed to encircle the epithelioid corona. These MCs had an eccentric nucleus and contained numerous polymorphic dense granules (Fig. 2c). The cytoplasm typically contained two to three mitochondria and several electron-lucent vesicles. Degranulation of these MCs was seen in nematode infected livers which were more frequent close to the nematode larva.

Neutrophils, also seen scattered among the MCs, had rod-shaped granules with an elongated, electron dense, lamellar core. Within the livers of infected minnows, in sinusoid lumen and within the parenchyma, frequent direct contact between MCs and neutrophils was observed (Fig. 2d).

3.5. *G. aculeatus and the acanthocephalan P. laevis (Table 1)*

The degree of acanthocephalan attachment varied although most parasites were embedded within the connective tissue with a loose connection to the intestines. In other fish, however, some larvae were firmly attached to the outermost part of the intestine. The host reaction encapsulating the parasite appeared to be a series of concentric whorls of fibroconnective elements. Among the fibres, there were partially degenerated or vacuolated epithelioid cells and numerous MCs in close proximity to the tegument of the larvae (Fig. 3a). There was significant degranulation of the MCs, notably among those adjacent to the acanthocephalan's tegument where the granules were commonly seen on the surface of the larvae (Fig. 3b and c). No acanthocephalan produced tegumental secretions were seen in the TEM sections taken through the interface region, however and interestingly, numerous MC granules appear to have moved towards the worm and penetrated into its tegumental pores (Fig. 3c and d). Electron-dense granules, beneath the striped layer, were very evident (Fig. 3d).

In each of the helminth–fish systems studied here, the damage to the host tissue was limited to the site of parasite attachment of parasite. The host's immune cells at these sites appeared to be normal/intact. Although numerous semi-thin and ultrathin sections from multiple hosts were used to study each host–parasite system, no calcified helminths were encountered.

4. Discussion

In fish, the innate defences responding to helminth infection are associated with inflammatory reactions (Secombes and Chappell, 1996; Bahlool et al., 2013) that are most frequently elicited by migrating parasite stages (Paperna and Dzikowski, 2006). Granulomas enclosing

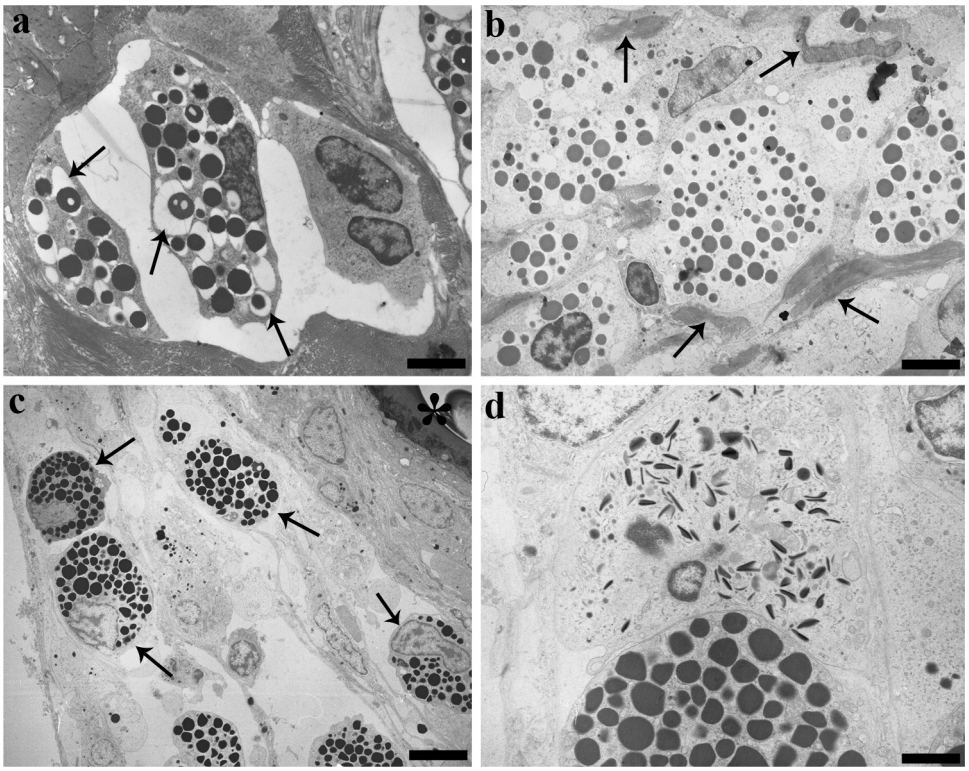

Fig. 2. (a) MCs inside a blood vessel within the intestinal submucosa of a brown trout, *Salmo trutta* (L.), infected with *Dentitruncus truttae*, where there is a reticulated appearance to the granules and electron-lucent halos (arrows) around the granules can be seen; scale bar = 2.5 μm. (b) Degranulation of *S. trutta* MCs in close proximity to the proboscis is evident; note the collagen fibres (arrows); scale bar = 3.3 μm. (c) The liver of a minnow, *Phoxinus phoxinus* L., with an encysted larval nematode of *Raphidascaris acus* (Bloch, 1779) (asterisk), where several MCs (arrows) close to the parasite can be seen; scale bar = 5.0 μm. (d) An infected liver of *P. phoxinus* where a MC and a neutrophil are in contact with one another; note the aspect of the granules in the two cell types; scale bar = 1.4 μm.

parasites, preventing their migration and development within the host's tissues can form within a number of sites including the visceral organs, on the outer intestinal surface or within the muscles of vertebrates (Moreau and Chauvin, 2010). From the current studies, granulomas were found encapsulating the unidentified digenean metacercariae in *T. tinca*, the extraintestinal larvae of *P. laevis* in *G. aculeatus*, and, the larval nematodes of *R. acus* in the livers of *P. phoxinus.* Within the granulomas in each host, numerous MCs, neutrophils, some macrophages and a small number of RCs were seen.

Evidence for the involvement of granulocytes i.e. MCs (Silphaduang and Noga, 2001; Prykhozhi and Berman, 2014; Sfacteria et al., 2015) and neutrophils (Katzenback and Belosevic, 2012; Toledo-Ibarra et al., 2013) in the immune system of fish is growing where they have been reported to play a critical role in the defence against pathogenic agents (Jones, 2001; Katzenback and Belosevic, 2012) including parasites (Reite and Evensen, 2006; Alvarez-Pellitero, 2008; Dezfuli et al., 2013a,b, 2014). Mast cells or eosinophilic granule cells (Reite and Evensen, 2006), serve a critical role as sentinels of the immune system. At the site of parasitic infection, these cells release their contents which, in fish, are various tryptases, lysosyme and antimicrobial peptides including piscidins (Silphaduang and Noga, 2001; Campagna et al., 2007; Dezfuli et al., 2010; Fernandes et al., 2010; Baccari et al., 2011; Masso-Silva and Diamond, 2014).

The degranulation of MCs in response to parasite presence has been reported in several recent studies, notably Dezfuli et al. (2011b), Rieger and Barreda (2011), and Prykhozhi and Berman (2014). The secretions that MCs in teleosts produce may have a role in attracting other types of granulocytes such as neutrophils, a key component of the inflammatory immune response, to the site of parasitic infection (see further). Neutrophils are involved in the inflammatory process, especially during the period of initial pathogen challenge, migrating to and accumulating at the site of parasitic infection or injury (Sharp et al., 1991; Secombes and Chappell, 1996; Matsuyama and Iida, 1999; Katzenback and Belosevic, 2012; Dezfuli et al., 2013b). Fish neutrophils have also been shown to phagocytise small foreign particles (Alvarez-Pellitero, 2008; Katzenback and Belosevic, 2012) and to degranulate, releasing the contents, in close proximity to parasites (Sears et al., 2011). The involvement of neutrophils and macrophages in fish in response to helminth infections is well documented, however, what is less clear is whether these phagocytes have the ability to directly kill helminths. *In vitro* adherence assays with immune serum has shown that the tegument of cestodes can be damaged (Hoole and Arme, 1986; Sharp et al., 1991). Tegumental damage was

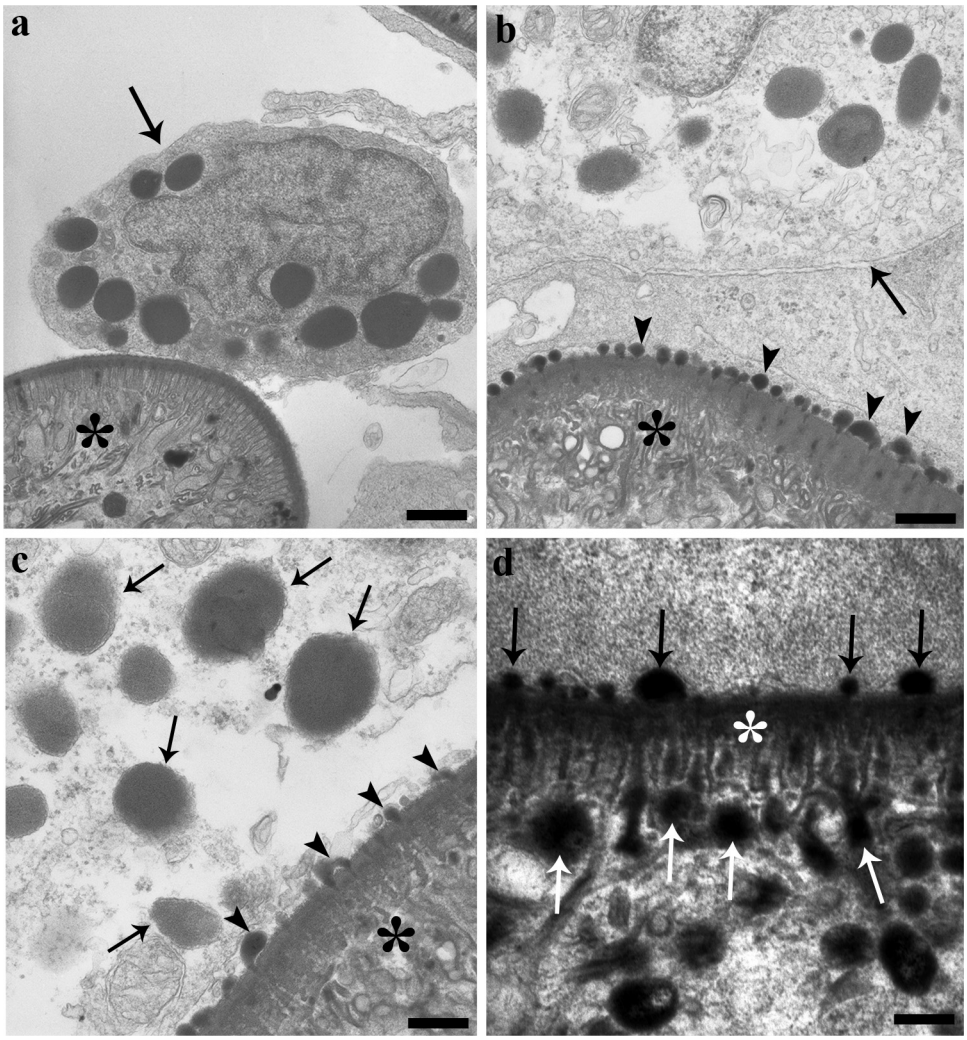

Fig. 3. The interface between the intestine of a three-spine stickleback, *Gasterosteus aculeatus* (L.), and an encysted larvae of the acanthocephalan *Pomphorhynchus laevis* (Zoega in Müller, 1776). (a) A MC (arrow) in contact with the parasite's tegument (asterisk); scale bar = 0.8 μm. (b) A transmission electron micrograph of a MC (arrow) in close proximity to a specimen of *P. laevis*, where numerous free granules (arrow heads) adhering to the tegument (asterisk) can be seen; scale bar = 0.5 μm. (c) MCs granules (arrows) close to the tegument of *P. laevis* (asterisk), where a number of granules (arrow heads) appear to have penetrated the tegumental pores; scale bar = 0.3 μm. (d) Higher magnification of the granules (arrows) adhering to the tegument of *P. laevis*. Beneath the striped layer (white asterisk), electron-dense granules (white arrows) are visible; scale bar = 0.2 μm.

in the form of microtrich shedding, focal loss of the apical plasmalemma and release of labelled ^{14}C-cycloleucine from larvae (Hoole and Arme, 1986). From the current study of tench-*M. wageneri* material, MCs and neutrophils were frequently observed adjacent to the tegument of the cestode's scolex in the process of degranulating in close proximity to the capilliform filitriches or adjacent to/between the coniform spinitriches of the scolex. These particular findings concur with the damage described in other cestode–fish systems (see for example Hoole and Arme, 1986; Sharp et al., 1991). By comparison, in brown trout infected with the acanthocephalan *D. truttae*, massive hyperplasia of MCs in the submucosal layer was seen at the site of proboscis insertion, where the MCs in the tissues immediately surrounding the proboscis were in a state of degranulation.

In this investigation, the degranulation of MCs close to the tegument of the acanthocephalan *D. truttae*, the cestode *M. wageneri*, the nematode *R. acus*, and encysted digenean metacercaria was documented. Only the MCs in association with the extraintestinal infections of the acanthocephalan *P. laevis* in *G. aculeatus*, were observed lying on the surface of the parasite or their granules had penetrated the tegument (Fig. 3c and d). Acanthocephalans lack tegumental glands and so the electron-dense granules seen beneath the striped layer in *P. laevis* are those released by the MCs in close association. These results are among the first to document the penetration of MC granules into the tegument of a helminth. The MC granules, which contain piscidins have been shown to be involved in the permeabilization of bacterial membranes by toroidal pore formation (Campagna et al., 2007). It is reasonable, therefore, to presume that the

products released from the MCs observed here, may have the same pore forming mechanism against *P. laevis*.

Parasitic helminths excrete or secrete (ES) a variety of molecules into their hosts. The ES products of trematodes, cestodes and nematodes contribute to immune evasion strategies of the parasites trough different mechanisms (Lightowlers and Rickard, 1988). There is an extensive body of work on the excretory/secretory proteins produced by helminths infecting mammals including a helminth secretome database which provides information on ES products from at least 78 helminth species (Garg and Ranganathan, 2012). ES products can be passively released from the parasite soma, or actively excreted/secreted from the worm tegument, either within vesicles or not (Marcilla et al., 2012). Research into the ES substances produced by helminths infecting fish is still very much in its infancy with only a few scattered observations on nematode-fish models (see Buchmann, 2012; Bahlool et al., 2013). From the fish–helminth studies conducted here, or from earlier studies conducted by the authors, no tegumental secretions packaged into extracellular vesicles were observed, however it does not exclude the possibility that a fraction of ES proteins, not packaged in vesicles, may be produced by parasite. Sadly our current knowledge on the ES substances produced by fish helminths and their effects on their host's immune systems are too limited for definitive statements and conclusions to be made at this time (Bahlool et al., 2013). We concur, therefore, with the statement made by Buchmann (2012) that the challenges in fish immunology lies in the creation of different types of host–parasite model that are able to address the range of responses that are seen.

Conflict of interest statement

All authors disclose any financial and personal relationships with other people or organisations that could inappropriately influence (bias) their work.

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