Protective responses of intestinal mucous cells in a range of fish-helminth systems

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Abstract

Histopathological, immunofluorescence and ultrastructural studies were conducted on the intestines of four fish species infected with different taxa of enteric helminths. Brown trout (*Salmo trutta trutta*), eel (*Anguilla anguilla*), and tench (*Tinca tinca*) obtained from Lake Piediluco (Central Italy) were examined. Brown trout and eel were infected with two species of acanthocephalans, and tench was parasitized with a tapeworm species. In addition to the above site, specimens of chub (*Squalius cephalus)* and brown trout infected with an acanthocephalan were examined from the River Brenta (North Italy). Moreover, eels were examined from a brackish water, Comacchio lagoons (North Italy), where one digenean species was the predominant enteric worm. All the helminths species induced a similar response, the hyperplasia of the intestinal mucous cells, particularly of those secreting acid mucins. Local endocrine signals seemed to affect the production and secretion of mucus in the parasitized fish, as worms often were surrounded by an adherent mucus layer or blanket. This is the first quantitative report of enteric worm effects on the density of various mucous cell types and on the mucus composition in intestine of infected/uninfected conspecifics. We provide a global comparison between the several fish-helminth systems examined.

Key words: mucus, infected intestine, enteric worms, fish innate immunity.

Introduction

Fish have direct interaction with the immediate environment thus the study of teleost mucosal immunity is of particular interest (Esteban 2012; Lazado & Caipang 2014; Salinas 2015). The mucosal immune system has a key role in the defense mechanism and it is considered as a very active immunological site (Gomez, Sunyer & Salinas 2013; Salinas 2015). The luminal side of the mucosal layer of the digestive tract is covered by a mucus layer/blanket which protects the mucosa from mechanical damage and dehydration, the mucus constitutes a physical barrier between the underlying epithelium and luminal contents (Neutra & Forstner 1987; Kim & Khan 2013; Bosi & Dezfuli 2015). The mucus layer functions as a dynamic protective barrier as evidenced by studies in mammals (reviewed in Kim & Khan 2013) and fish (Dezfuli et al. 2010; Bosi & Dezfuli 2015; Bosi et al. 2015). Elevated mucus secretion within the infected intestines of fish (Dezfuli et al. 2010; Bosi & Dezfuli 2015; Bosi et al. 2015), increased expression of selective mucins in the intestines of *Trichinella spiralis*-infected mice (Shekels et al. 2001; Kim & Khan 2013), and differences in lectin staining between the goblet cells of germ-free and germ-exposed mice (Kandori et al. 1996; Kim & Kan 2013) were documented. Clearly, the mucus layer is amongst the most important of innate defenses located at mucosal surfaces (Gomez *et al.* 2013; Castro & Tafalla 2015).

Fish mucus is enriched with a variety of immuno-related factors such as mucins, lectins, toxins, immunoglobulins and antimicrobial peptides (Hasnain *et al.* 2013; Lazado & Caipang 2014). Indeed, in some fish species the mucous cells themselves are able to produce and release defensive substances in response to foreign intrusion or mechanical injury (Hasnain *et al.* 2010, 2013). The digestive tract undergoes significant alteration as a result of infection and inflammation (Khan 2008). The attachment organ of the helminth, the proboscis or scolex, often produces inflammation of the host gastrointestinal tract (Dezfuli *et al.* 2009, 2012; Buchmann 2012). Inflammation is the host response to physical injury or invasion by

foreign organisms and serves to protect the host by evoking specific chemical and morphological alterations to the injured cells and tissues (Sears *et al.* 2011; Johansson & Hansson 2014; Birchenough *et al.* 2015). The long lifespan of helminth parasites tends to produce chronic infections and indeed, the initial immune response mounted by hosts often progresses into a chronic condition characterized by pathological changes to the gut tissue (Wanstall, Robotham & Thomas 1986; Dezfuli *et al.* 2009).

Recently fluorescent probe labelling and ultrastructural analysis of infected fish revealed the relationship between enteric epithelial mast cell (MC) degranulation and excessive mucus secretion by mucous cells (Dezfuli *et al.* 2015a). A similar approach showed also close contact between enteric endocrine cells and mucous cell discharge in parasitized fish (Bosi *et al.* 2015). The main aim of the current investigation was to compare the number of intestinal mucous cells and their chemical contents in four fish species infected with different taxa of endoparasitic helminths. This is the first quantitative and comparative study on enteric mucous cells in different fish-helminth systems.

Materials and methods

A total of 572 fish belonging to 4 species were examined (Table 1). Table 1 shows the host-parasite systems considered in this study regarding the morphometric data of the fish species and details on parasite infection. The fish were obtained from three separate localities in Italy (Table 1); two freshwater sites, the Lake Piediluco in the Province of Terni (42° 31´ N; 12° 45´ E) and the River Brenta (45° 32' N; 11° 47' E) in the Province of Padua and the Comacchio coastal lagoons (Northern Adriatic Sea, 44º 36´ N, 12º 10´ E). All fish species for the current study were sampled on several occasions during 2014 and 2015 by professional fishermen using gill net, fyke nets or electrofishing. Fish were euthanised with an overdose of 125 mg L^{-1} MS222 (tricaine methanesulfonate, Sandoz, Basel, Switzerland) and pithing.

Immediately after euthanasia, a complete necropsy was performed on each fish with particular interest paid to the gills, heart, gonads, liver, kidney, spleen and swimbladder for the presence of parasites. Fresh impression smears were prepared from each tissue and screened for protozoa. The digestive tract and associated organs were removed and the intestine cut open longitudinally and searched for helminths.

Pieces of intestine from infected and uninfected fish of all species were fixed in 10% neutral formaldehyde for 24 h and were processed as usual to obtain histological sections which were stained with alcian blue 8GX pH2.5 followed by periodic acid Schiff (see Bosi *et al.* 2015). For quantification of the different stained mucous cells, 3 areas from each fish specimen (20 infected and 20 uninfected) were examined with a Nikon Microscope ECLIPSE 80i (Nikon, Tokyo, Japan) at 40x magnification. For each fish-helminth system, the 20 fish selected for the cell counting had similar intensity of infection (see Table 1). In both uninfected and infected fish, the mucous cells counting was done in the anterior part of the intestine which in all four fish species appeared to be the most infected region of the digestive tract. The mean number of mucous cells per 100,000 μm^2 of epithelial area in sections taken from uninfected and infected fish were compared using the Mann-Whitney test. The level of significance was set at $p = 0.01$. The co-occurrence cases of parasites were excluded from the counting in each fish-helminth system.

Dual immunofluorescence staining was performed on sections of parasitized intestine for each fish, as indicated previously in Bosi *et al.* (2015), to visualize the relationship between endocrine and mucous cells. Endocrine cells were detected with rabbit polyclonal anti-leu-enkephalin (code CA-08-235, Genosys Biotechnologies Inc.; 1:200 in TBS for 24 h at room temperature) and intestinal mucous cells were marked with biotinylated *Dolichos biflorus* agglutinin (DBA, code, Vector Lab.; in 10 mM HEPES pH 7.5, 0.15 M NaCl, 0.08% NaN_3, 0.1 mM $CaCl_2$ for 3 h at RT).

For transmission electron microscopy (TEM), representative pieces of infected and uninfected intestines were processed as reported in Bosi *et al.* (2015).

Results

Table 1 shows the prevalence and intensity of infection of enteric helminths for each fish species. Data on a few parasite species (protistan and metazoan) in other organs are in preparation and will be published elsewhere.

In all fish species the total number of mucous cells was higher in infected specimens in comparison to uninfected ones, and the differences were highly significant (Table 2, Figs. 1, 2). For instance, in *S. trutta* infected with the tapeworm *Cyathocephalus truncatus* the total number of mucous cells was 2.22 times more than the number of the same type of cells in uninfected brown trout (Table 2, Figs. 1a, 2a,d). Similarly, in brown trout with the acanthocephalan *Echinorhynchus truttae* the total number of mucous cells was 2.55 times greater than the number of these cells in uninfected conspecifics. Also in brown trout harboring acanthocephalan *Pomphorhynchus laevis* the total number of mucous cells was 2.99 times more than the mucous cells counted in uninfected *S. trutta* (Table 2, Figs. 1a, 2a,b,c). In the other 4 fish-helminth systems considered, there was a consistent increase in the total number of mucous cells in infected specimens, where the value of this increase ranged from 1.5 to 1.7 (Table 2, Figs. 1 b-d, 2 e-h).

The most significant finding of this study is that in each fish-parasite system, the number of mucous cells containing acidic glycoconjugates (i.e. staining with AB) were significantly higher in comparison to the number of the same cells in uninfected conspecifics (Table 3, Figs. 1, 2). It is interesting that, with reference to the number of alcianophilic mucous cells (AB+), brown trout showed more sensitivity to the occurrence of the enteric helminth. Accordingly, in this species, the ratio between number of mucous cells staining

positively for acidic glycoconjugates in infected/uninfected intestine ranged from 3.86 to 4.80 (Table 3). In the other fish-helminth systems examined, the ratio between cells with acidic mucins in infected versus uninfected fish is at the minimum (1.55) in eels from Lake Piediluco infected with *A. rhinensis*, and is maximum (1.80) in chub from River Brenta parasitized with acanthocephalan *P. laevis* (Table 3, Fig. 2e,h).

A similar and highly significant increase was observed in the number of mucous cells staining positively for neutral secretions (i.e. staining with PAS) in infected fish as compared to uninfected fish (Table 4). An exception was found in chub harbouring the acanthocephalan *P. laevis* in which there was no difference between infected and uninfected fish (Table 4, Figs. 1b, 2e). In eel a consistent increase in number of PAS positive mucous cells was noticed, and accordingly the ratio of the number of PAS positive mucous cells between infected and uninfected intestine in eels from Lake Piediluco harboring *A. rhinensis* (Acanthocephala) was 2.52 and in eels from Comacchio lagoons with *Helicometra fasciata* (Platyhelminthes) was 3.16 (Table 4, Figs. 1c, 2g,h). However, our data also showed that in all fish-helminth systems examined, eel infected with acanthocephalan and/or with *H. fasciata* are the species with lowest number of mucous cells with neutral mucins (Table 4).

In 3 of the 4 fish species examined in this survey, generally, in infected intestines, a significantly higher number of mucous cells staining for mixed glycoconjugates (i.e. stain violet with AB/PAS) was observed as compared to the number found in uninfected fish (Table 5, Figs. 1, 2). Moreover, the highest ratio of mucous cells with mixed glycoconjugates in infected versus uninfected tissue was recorded in brown trout parasitized with *P. laevis* (2.59, Table 5, Figs. 1a, 2c) and followed by tench infected with *M. wageneri* (1.99 Table 5, Figs. 1d, 2f). In contrast in *S. trutta* from Lake Piediluco harboring *C. truncatus*, no significant difference was found in the number of mucous cells with mixed glycoconjugates (AB/PAS positive) in comparison to the same cells in fish with no tapeworm (Table 5).

It is interesting to note that in comparing the proportion of different sub-populations of mucous cells of uninfected brown trout and infected conspecifics the intestinal mucous cells with acidic glycoconjugates increase percentage-wise with respect to the total number of mucous cells, independently of parasite taxon (Table 6).

Furthermore, in *S. trutta* a slight decrease in percentage of PAS positive mucous cells and those with mixed glycoconjugates was found (AB/PAS positive, Table 6). In chub from River Brenta the above trend for percentage of alcianophilic mucous cells was observed but the proportion of PAS and AB/PAS positive mucous cells were quite similar in infected and uninfected fish (Table 6). Nonetheless, in infected eel, independently from taxon of helminth, there was an increase in percentage of neutral glycoconjugates (PAS positive) mucous cells, and the percentage of the other two sub-populations of mucous cells were almost the same in infected vs uninfected eels (Table 6). A similar situation was also observed in tench harbouring the cestode *M. wageneri* (Table 6).

The Confocal Laser Scanning Microscope observations of sections treated for immunofluorescence clearly showed a relationship between a sub-population of mucous cells and a sub-population of endocrine cells. In all fish examined, DBA-rhodamine stained the mucus granules of mucous cells lined at the epithelial surface (Fig. 3). The polyclonal anti-leu-enkephalin coupled to fluorescein detected several ECs with their enlarged base near or leaning on basal membrane (Fig. 3). The ECs were often tightly apposed to the mucous cells, likely near the basally-located nuclear regions that were not readily identified in the tissue specimens used for immunofluorescence.

TEM provided details on intestinal mucosa of the four fish species examined. Accordingly, the intestinal mucosa is lined by a simple columnar epithelium with a sparse intermingling of mucous cells amongst the epithelial cells (Fig. 4a). Considerable heterogeneity exists between adjacent mucous cells of the intestine, and even between adjacent mucus

granules in the same cell (Fig. 4a,b). The nucleus of the mucous cell was observed to be elongated and basally placed (not shown). Well developed rough endoplasmic reticulum, Golgi apparatus and a few round-elongated mitochondria can be seen in the basal portion of the cell (not shown). Mucus granules were seen to occupy the entire supranuclear cytoplasm (Fig. 4a), appearing as spherules or polyhedrons surrounded by a single granule membrane. Within TEM sections, the mucus granules appeared electron-opaque and, in some instances, as electron-lucent granules (Fig. 4a,b).

Discussion

In recent decades, the immune response in fish against microparasites has received great attention (Piazzón, Leiro & Lamas 2014; Sitjà-Bobadilla, Estensoro & Pérez-Sánchez 2016). However major gaps still remain in our knowledge of how fish mount an immune response against endoparasitic helminths despite the widespread importance of these worms in fish health (Buchmann 2012). In fish, there are several accounts suggesting that the presence of a helminth parasite within the gut can induce the formation and/or recruitment of various inflammatory cells at the site of infection (Reite 2005; Reite & Evensen 2006; Dezfuli *et al.* 2015a,b, 2016a,b).

The first line of defense in the alimentary canal is the secretion of mucus and mucus-associated anti-microbial substances into the lumen, indicating an intimate coupling of the immune system with mucus production (Gomez *et al.* 2013; Kim & Khan 2013; Pelaseyed *et al.* 2014; Peterson & Artis 2014). The mucus is secreted continuously by specialized intestinal mucous cells, resulting in constant renewal of a gel coating that completely covers the mucosa (Lamont 1992; Hansson 2012; Dupont *et al.* 2014). Physiological stimulation or pathological challenge to the tissue triggers a rapid and abundant discharge of mucous cell contents into the lumen (Plaisancié *et al.* 1998; Bergstrom *et al.* 2010; Grootjans *et al.* 2016). A heavy

mucus production has also been described in several other fish-helminth systems (Chambers *et al*. 2001; Bosi *et al*. 2005a, 2015; Bosi & Dezfuli 2015; Dezfuli *et al*. 2010, 2016a). Neural, hormonal and paracrine signals are known to influence mucus secretion (Plaisancié *et al.* 1998; Artis & Grencis 2008; Bosi *et al.* 2015). A range of peptides regulate mucus release during inflammation (Antoni *et al.* 2013; Dupont *et al.* 2014; Cullen *et al.* 2015).

Vertebrate enteroendocrine cells (ECs) are distinguished by the expression of an assortment of regulatory molecules that modulate the mucosal immune response (Verburg-van Kemenade *et al.* 2009; Hernandez *et al.* 2012; Gomez *et al.* 2013; Nardocci *et al.* 2014). The role of mammalian ECs in regulating intestinal mucus secretion and discharge has been extensively characterized (see Lelievre *et al.* 2007; Lee May & Kaestner 2010; Saffrey 2014), while corresponding studies in fish are just beginning to emerge (Bosi *et al.* 2005b, 2015; Hur *et al.* 2013; Nardocci *et al.* 2014; Dezfuli *et al.* 2016a). Our present study represents only the second to document the potential effects of enteric neuromodulators on gut mucus secretion and discharge.

Opioid peptides have an integral role in regulating the mechanism of mucus discharge **by** goblet cells due to luminal stimuli (Zoghbi *et al.* 2006). Indeed leu-enkephalin is one such opioid that is frequently encountered within the gut-associated neuroendocrine system of teleosts (Domeneghini *et al.* 2000). In the current study infected intestines of different fish species displayed a significant increase in the number of anti-leu-enkephalin immunoreactive endocrine cells. Based on the evidence presented here and in earlier studies (Fairweather 1997; Palmer & Greenwood-VanMeerveld 2001; Bosi *et al.* 2005b, 2015; Barber & Wright 2006), it could be postulated that hyperplasia of mucous cells and intensified mucus discharge may be modulated by leu-enkephalin.

The role of excessive mucus secretion in helminth-infected vertebrate intestines is not well understood. In mammals it is believed that increased mucus secretion aids in the

expulsion of intestinal nematodes (Artis & Grencis 2008; Zaph, Cooper & Harris 2014; Peterson & Artis 2014; Grencis 2015). However this may not be sufficient to dislodge helminths as the proboscis of many acanthocephalan species, and the scolex of many cestodes, guarantee a strong and secure anchoring to the host intestinal wall. Indeed, dislodged helminths have not been detected in post-mortem examinations. The current study, combined with our earlier works, compels us to agree with the general consensus that the main role of mucus is to protect the intestinal mucosa from mechanical and biochemical damage due to parasite invasion (Guzman-Murillo, Merino-Contreras & Ascencio 2000; Schroers *et al.* 2009; Dezfuli *et al.* 2010; Bosi & Dezfuli 2015; Castro & Tafalla 2015).

A main finding of this study is mucous cells hyperplasia, or cellular proliferation, in infected fish intestine which has been extensively documented here through histomorphometric and immunohistochemical assays and as well as previously reported in some of our studies (see references cited above). Cell counting revealed a direct consequence of cellular proliferation was the significantly higher number of mucous cells in the intestinal folds close to the sites of attachment of the worms when compared to the numbers found in uninfected intestines. Consistent with this finding, the hyperplasia of mucous cells within the intestine of *Salmo trutta* infected with *Pomphorhyncus laevis* appeared in Bosi *et al.* (2005a) and with *Echinorhynchus truttae* in Dezfuli *et al.* (2010), respectively. The results of the current investigation using different host-helminth systems, combined with our previous studies, indicate that the intestinal reaction is independent of the fish species. Quantitative changes of the intestinal mucous cells are the result of an increased secretion due to the non-specific innate immune response to the parasite or its products (Theodoropoulos *et al.* 2001). Instead, the qualitative modifications in mucus secretion concern changes in the way of mucins glycosylation, which should provide an adaptive barrier to colonization of the mucosa by pathogens (Theodoropoulos *et al.* 2001; Gomez *et al.* 2013). Mucins represent a dynamic

component of the mucosa that interact with and are regulated by both innate and adaptive immunity during infections (McGuckin *et al.* 2011). Changes in mucin production and glycosylation are reported in many mammal-helminth systems: mice intestines infected with *Trichuris muris* (Hasnain Thornton & Grencis 2011) and *Gymnophalloides seoi* (Guk *et al.* 2009), mice and rat intestine with *Echinostoma caproni* (Cortes *et al.* 2015), rat airway and small intestine with *Nippostrongylus brasiliensis* (Tsubokawa *et al.* 2012), lamb gut with *Haemonchus contortus* or *Teladorsagia circumcincta* (Hoang, Williams & Simpson 2010; Simpson *et al.* 2016), cattle abomasum with *Ostertagia ostertagi* (Rinaldi *et al.* 2011) and cattle intestine with *Cooperia oncophora* (Li *et al.* 2009). The modifications of mucus composition may alter its permeability and viscosity, facilitating the worm trapping, limiting parasite motility and feeding capacity and finally leading to successful expulsion (Carlisle, McGregor & Appleton 1991; Webb, Hoque & Dimas 2007; Thornton, Rousseau & McGuckin 2008). Qualitative changes of mucus in mammals might play a role in the establishment and/or rejection of helminths, but the effect of these changes is still not completely clear and could vary based on the host/parasite species, tissue, host age and susceptibility, and stage of infection/reinfection (Linden *et al.* 2008; McGuckin *et al.* 2011; Rinaldi *et al.* 2011; Simpson *et al.* 2016).

The present study demonstrates that the intestinal mucous cells of 4 fish species secrete acidic and neutral glycoconjugates. Our findings agree closely with those on the same fish species previously published (Fiertak & Kilarski 2002; Domeneghini *et al.* 2005; Marchetti *et al.* 2006). Qualitative changes in mucus have been shown to occur in parasitized fish intestines (Bosi *et al.* 2005a; Díaz, García & Goldemberg 2008; Schroers *et al.* 2009; Dezfuli *et al.* 2010). Several studies on fish have observed an increase in acidic mucins which is associated with an increased viscosity of secreted mucus, providing enhanced protection against pathogens (Abaurrea-Equisoaín & Ostos-Garrido 1996; Tibbets 1997; Díaz *et al.* 2008). The

altered intestinal mucus composition in infected fish reported here are suggestive of a defensive role, possibly as an integral part of the host protective response. However, further research using physiological experimental systems is needed to clarify the functional significance of the altered mucus composition in helminth-infected fish intestines.

The main results of this study can be summarized as follow: (a) in fish intestinal parasites induce an increase in the number of mucous cells, particularly of those secreting acid mucins; (b) concerning this increase, some fish are more responsive than others to the presence of parasite, i.e. the brown trout regardless of the type of parasite that harboured; (c) each host species seems to display a further species-specific action against parasites, i.e. the eel and the tench in which there was a marked increases of PAS positive mucous cells; (d) local endocrine signals could affect the production and secretion of mucus in the parasitized fish.

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Figure captions

Fig. 1. Uninfected intestinal sections of four fish species stained with Alcian blue and periodic acid-Schiff's (AB/PAS). In all sections, mucous cells (arrowed) can be seen distributed among the epithelial cells. (a) Sagittal section through a *Salmo trutta* intestine; scale bar = 100 µm. (b) Section of the intestinal folds in sampled from *Squalius cephalus*; scale bar = 100 µm. (c) Micrograph shows intestinal folds of *Anguilla anguilla*; scale bar =50 µm. (d) Some intestinal folds of *Tinca tinca*; scale bar = 50 µm.

Fig. 2. Intestinal sections of four fish species infected with different taxa of helminths and stained with Alcian blue and periodic acid-Schiff's (AB/PAS). (a) Villi of *Salmo trutta* close to the site of acanthocephalan attachment. The thin arrows highlight the majority of mucous cells which stain positively for acid glycoconjugates while the curved arrows indicate mucous cells containing neutral glycoconjugates, which are less numerous; scale bar = 3 µm. (b) *Echinorhynchus truttae* (asterisk) *in situ* within the intestinal folds of *S. trutta*, mucous cells (thin arrows) are numerous in the epithelium in close proximity to the site of parasite attachment; scale bar = 200 µm. (c) Intestine of *S. trutta* infected with *Pomphorhynchus laevis* (asterisk), note the mucus layer/blanket (thick arrows) interposed between parasite (asterisk) and host intestinal folds; scale bar = 50 µm. (d) *Cyathocephalus truncatus* (asterisk) attached to the brown trout intestine: epithelial destruction is evident below the scolex (thick arrow). The thin arrows highlight the abundance of alcianophilic mucous cells at the site of infection; scale bar = 50 µm. (e) Micrograph shows *Squalius cephalus* intestine infected with *P. laevis* (asterisk): numerous mucous cells (arrows) among the epithelial cells are visible; scale bar = 100 µm. (f) Intestine of *Tinca tinca* with attached *Monobothrium wageneri* (asterisk): the parasite scolex which destroyed the epithelia and a high number of mucous cells (arrows) can be seen; scale bar = 100 µm. (g) Intestine of *Anguilla anguilla* infected with

the digenean *Helicometra fasciata*, note the mode of attachment of the sucker (thick arrow) to the apex of the fold. Numerous mucous cells (arrows) scattered among epithelial cells; scale bar = 25 µm. (h) *A. anguilla* infected with *Acanthocephalus rhinensis* (asterisk), mucus layer/blanket encircled the parasite body, in some parts (curved arrows) the mucus layer is very thick, arrows show mucous cells; scale bar = 100 µm.

Fig. 3. (a) Mucous cells within intestinal epithelium of acanthocephalan infected *Anguilla anguilla* are positively stained with DBA (arrows), while the endocrine cells are immmunoreactive with the Leu-enkephalin antiserum (arrow heads); scale bar = 100 µm. (b) Some endocrine cells within the gut epithelium of an infected *A. anguilla* that are immunofluoropositive to the Leu-enkephalin. Arrows show DBA-reactive mucous cells; scale bars = 50 µm. (c) Mucous cells marked with DBA (arrows) in intestine of *Tinca tinca* infected with *Monobothrium wageneri*, some endocrine cells (arrow heads) are positive to the Leu-enkephalin antiserum; scale bar = 50 µm.

Fig. 4. (a) A TEM micrograph of the intestine of *Anguilla anguilla* infected with digeneans showing some mucous cells (arrows) in the apex of the epithelium. The arrow head points to the basal portion of a mucous cell, mucus granules occupied supernuclear part of the mucous cell; scale bar = 4.1 µm. (b) Two mucous cells containing numerous mucus granules of various size and with different electron densities that are positioned close to the surface of the epithelium; scale bar = 2.5 µm.