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# Inflammatory response to parasitic helminths in the digestive tract of *Anguilla anguilla* (L.)

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ABSTRACT

The European eel, *Anguilla anguilla*, is a major warm water fish species cultured in North and South Europe. A total of 140 eels measuring 28–92 cm in total length (70.9 ± 14.7, mean ± SD cm), collected on 10 separate occasions during October 2005 to May 2006 from the Comacchio lagoons, were examined. Ninety-six (69%) harbored parasitic helminths. Of infected eels, 55% contained 3 digenean species, 2% a single cestode species, and 5% 2 nematode species. Intestinal pathology associated with digenean and cestode infection was minimal. The main damage caused by digeneans was destruction of the mucosal epithelium of the villi. Necrosis and degeneration of epithelial cells were also evident. At the site of digenean infection, a high number of rodlet cells (RCs) and mucous cells were observed in the epithelium, with both types of cells exhibiting discharge activity. The number of RCs per area (30,000 μm$^2$) in parasitized *A. anguilla* (10.83 ± 7.08, mean ± SD, $n = 40$) was significantly greater than in uninfected (2.18 ± 2.15, mean ± SD, $n = 40$, *t-test*, $P < 0.01$). The majority of RCs in both infected and uninfected intestine were mature cells and presented the typical cell cortex. The number of mucous cells per area (30,000 μm$^2$) was significantly higher in intestine of parasitized eels (70.58 ± 17.95, mean ± SD, $n = 40$) than in uninfected (27.18 ± 5.58, mean ± SD, $n = 40$, *t-test*, $P < 0.01$). Severe intestinal damage was caused by *Contracaecum rudolphii* A larvae encysted within the tunica propria and over the external surface of the stomach and intestine. At these sites, conspicuous granulomas showing chronic inflammatory responses characterized by infiltration of mast cells and fibroblasts were observed.



## 1. Introduction

Eels are important warm water fish species cultured in several European countries, among them Italy, Spain, Germany, Denmark, and the Netherlands, and in Asian countries including Japan, Taiwan, Malaysia, and China (Lee et al., 2003). In Italy, *Anguilla anguilla* (L.) is of high commercial value in the brackish Comacchio lagoons of the northern Adriatic Sea.

Avoiding disease is a major challenge in aquaculture, and there has been considerable interest in the overall health of the eel populations on farms. Knowledge of the eel immune system is crucial. Recent data on viral disease in eels has appeared in Chang et al. (2002) and Haenen et al. (2002); on bacterial disease in Austin and Austin (1999) and Haenen and Davidse (2001); on protozoan disease in Aguilar et al. (2005) and Kristmundsson and Helgason (2007); and on disease caused by helminths in Kirk (2003), Sures and Knopf (2004), and Kennedy (2007).

The gastrointestinal tract is a primary route of infection in fish as in other vertebrates (Ringo et al., 2007). This is likely due to ease of access for the pathogen, as well as the ready availability of attachment sites and nutrients and a relatively non-aggressive immune response (Secombes and Chappell, 1996). Infection of the alimentary canal by protozoa or helminths has detrimental effects on tissues and the digestive physiology of the host (Hoste, 2001), and intestinal helminths often induce inflammation in the area of infection.

In teleost fish, cellular involvement in the inflammatory response may be biphasic, beginning with an influx of neutrophils followed by the subsequent arrival of monocytes/macrophages (Reite and Evensen, 2006). Several investigations have attempted to elucidate the eel immune response to viruses, bacteria, and protozoan and metazoan parasites; nevertheless, observations of cellular and humoral immune responses of eels have been limited (Nielsen and Esteve-Gassent, 2006). In this study, at the site of cestode and digenean infection, a high number of mucous cells and rodlet cells (RCs) were observed in the intestinal epithelium. These cells showed discharge activity. The function of RCs has not been conclusively shown, but research has suggested that they contribute to host's

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defense against pathogenic organisms (Dezfuli et al., 2000, 2007; Leino, 1996).

Granulomas in fish may result from viruses, bacteria, protozoa, or foreign bodies (Anders and Möller, 1988; Gardiner and Bunte, 1984), and granulomas resulting from helminths have been reported (Karanis and Taraschewski, 1993; Taraschewski, 1988, 1989). Granulomatous tissue in infected eel has been found to be composed predominantly of fibroblasts with interspersed mast cells and a few scattered macrophages (Karanis and Taraschewski, 1993 and present survey).

The aim of this study was to describe and evaluate histopathology of the digestive tract of eel induced by parasitic helminths. The main goal of the investigation was to identify the cells involved in eel inflammatory response.

## 2. Materials and methods

Eels ($n = 140$) measuring 28–92 cm in total length ($70.9 \pm 14.7$, mean $\pm$ SD cm) were obtained by the Po Delta Park Administration from the Comacchio lagoons (Northern Adriatic Sea, Italy) on 10 occasions from October 2005 to May 2006. Fish were brought live to the laboratory, anesthetized with MS222 (Sandoz), and killed by severing the spinal cord. The digestive tract and associated organs were removed and the intestine cut open longitudinally and screened for helminths. Pieces of stomach and intestine measuring up to $15 \times 15$ mm with attached parasites were excised from sixty infected *A. anguilla* and fixed in chilled (4 °C) Bouin fluid for 8 h. The samples were processed routinely for paraffin embedding, cut in 5 µm thick sections, and stained either with Harris Haematoxylin–Eosin or Alcian Blue/PAS.

For light and electron microscopy, infected eel intestine and duodenum pieces measuring up to $7 \times 7$ mm were fixed 3 h in chilled (4 °C) 2% glutaraldehyde solution buffered to pH 7.2 with 0.1 M sodium cacodylate. The samples were rinsed for 12 h with 0.1 M sodium cacodylate buffer containing 6% sucrose, then post-fixed in 1% osmium tetroxide in the same buffer for 2 h, dehydrated through a graded ethanol series, transferred to propylene oxide, and embedded in an Epoxy-Araldite® mixture (Fluka, Switzerland). Semi-thin sections (1.5 µm) were cut on a Reichert Om U2 ultramicrotome and stained with toluidine blue. Ultra-thin sections (90 nm) were stained with a solution of 4% uranyl acetate in 50% ethanol and Reynold's lead citrate and examined using a Hitachi H-800 electron microscope. For comparison, pieces of intestine of 20 uninfected eels were also processed. Light photomicrographs of intestine sections were obtained, using a Nikon Eclipse 80i microscope, showing the overall distribution of rodlet cells, mucous cells, and mast cells, as well as cell images. The dimensions of 100 cells of each type were obtained using computerized image analysis software (Nis Elements AR 2.30). To quantify differences in the number of the three cell types in intestines, thick resin sections were analyzed at 400× magnification.

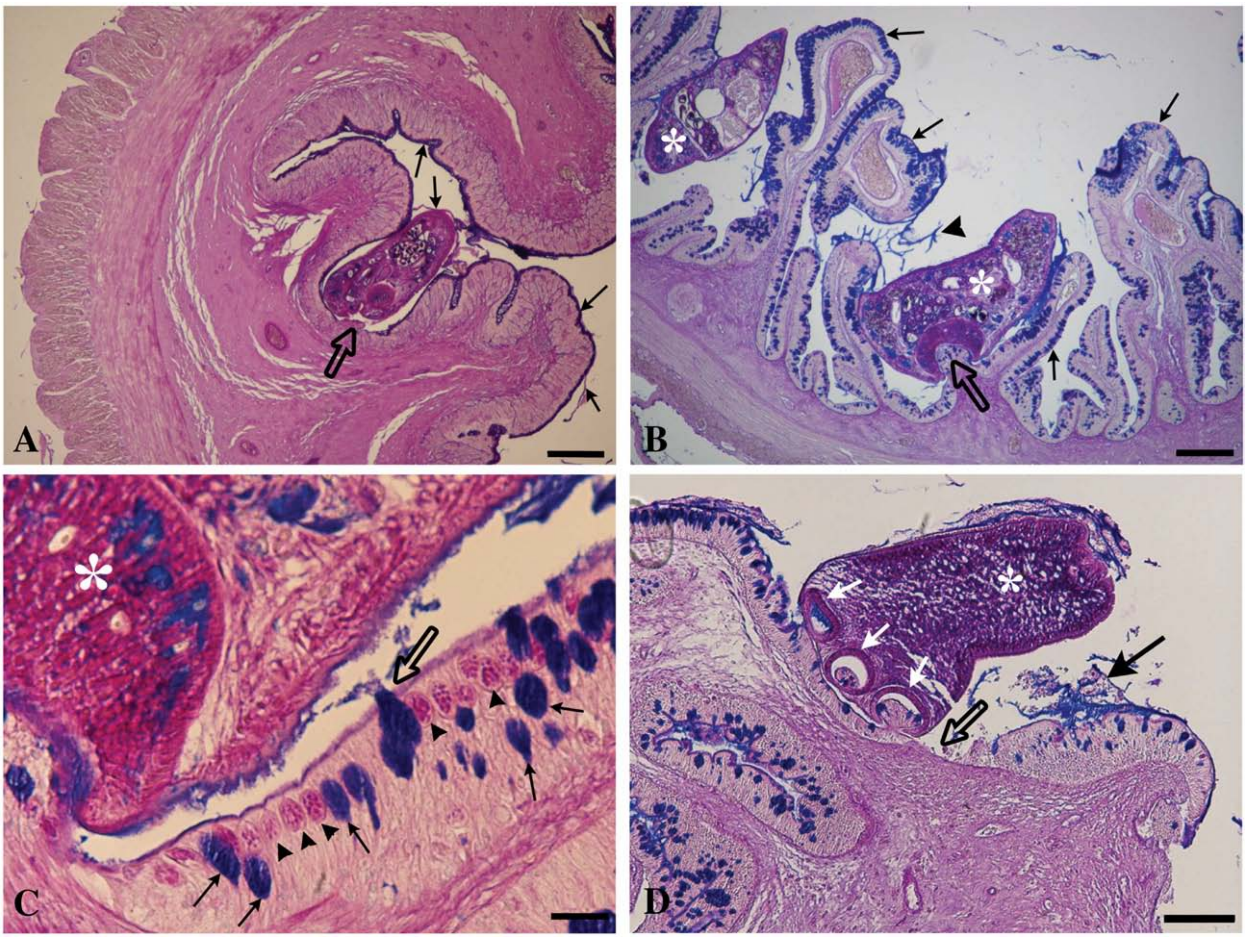


**Fig. 1.** Histological sections showing parasites and pathological effects in digestive tract of *Anguilla anguilla*. A) Micrograph shows *Helicometra fasciata* (empty arrow) within a stomach epithelium fold. A thick adherent blanket gel (arrows) envelopes parasite body and apex of villi (bar = 100 µm). B) Digeneans (asterisks) attached to the intestinal wall by single suckers; isolated fragment of epithelium (empty arrow); numerous mucous cells (thin arrows) within the epithelium. Arrowhead shows catarrh (bar = 100 µm). C) High magnification of *Deropristis inflata* sucker (white asterisk) within the depth of a fold; numerous strongly Alcian Blue positive mucous cells (thin arrows) scattered among the rodlet cells (arrowheads). Note mucous cell near the worm body (empty arrow) showing discharge activity (bar = 10 µm). D) Attachment of cestode larva Tetraphyllidea by three suckers (white arrows); erosion of the epithelium (empty arrow). Thick arrow shows catarrh near the parasite (bar = 50 µm).

Two intestine sections were observed from each of 20 uninfected and 20 infected eels. Cell counts were based on the assessment of similar sized areas of tissue (30,000 μm$^2$) from the same region of the intestine excised from infected and uninfected eels. Data were analyzed using Student's two tailed *t*-test, where significance was set at $P<0.01$.

## 3. Results

Three Digenea, *Helicometra fasciata*, *Deropristis inflata* and *Bucephalus anguillae*, a tetraphyllidean cestode larva, 2 nematodes *Contracaecum rudolphii* A, and *Anguillicoloides crassus* were found in the samples. Co-occurrence was observed in 38% of hosts.

The most heavily parasitized segments of eel digestive tract were the intestine and duodenum. The digeneans were the predominant species and occurred throughout the length of the alimentary canal. They and the cestode larvae were often immersed in copious yellowish mucus. Histological sections showed the anterior portion of the digeneans to penetrate deeply into folds in the stomach epithelium (Fig. 1A). They were also often attached to the intestinal wall by the oral sucker which detached particles of epithelium (Fig. 1B). Epithelial cells in various stages of degeneration were observed inside the sucker. Detached and damaged epithelial fragments were also seen in proximity to the parasite's body and within the gut lumen. Intestinal sections of infected *A. anguilla* showed a mild catarrhal enteritis formed by mucus, leucocytes, and epithelial cell debris (Figs. 1D, 2A). In sections, almost all digeneans (Fig. 1A, B, C) and cestodes (Fig. 1D) attached to the stomach and intestinal linings were associated with a thick adherent mucus blanket that was strongly Alcian Blue positive. Numbers of mucous cells counted in 30,000 μm$^2$ areas were significantly higher in intestine of infected eels (Figs. 1B, C, D, 3A) ($70.58 \pm 17.95$, mean ± SD, $n=40$) than in uninfected specimens ($27.18 \pm 5.58$, mean ± SD, $n=40$) (*t-test*, $P<0.01$). Alcian Blue–PAS staining revealed the vast majority of mucous cells were Alcian Blue positive.

Within the intestinal epithelia of infected eels, high number of RCs were scattered among the mucous cells (Fig. 1C). The number of RCs per 30,000 μm$^2$ area in parasitized *A. anguilla* ($10.83 \pm 7.08$, mean ± SD, $n=40$) was significantly greater than in uninfected ($2.18 \pm 2.15$, mean ± SD, $n=40$) (*t-test*, $P<0.01$). The majority of RCs in infected (Figs. 1C, 3A) and uninfected intestinal epithelia were mature cells and presented the typical cell cortex (Fig. 3A, B). In some instances, discharge activity of RCs located near the digenean and cestode bodies was documented (Fig. 3B).

At the site of attachment of the tetraphyllidean larvae, the intestinal fold lost its structural integrity (Fig. 1D). Damage to the epithelium increased from the apex of folds toward the base of the villi and was more pronounced at the site of attachment (Fig. 1D). Erosion of the epithelium was frequently observed near the attachment site (Fig. 1D).

Thirty-three eels contained encysted larvae of *C. rudolphii* A within the tunica propria of the intestinal wall (Fig. 2A) and/or on the outer

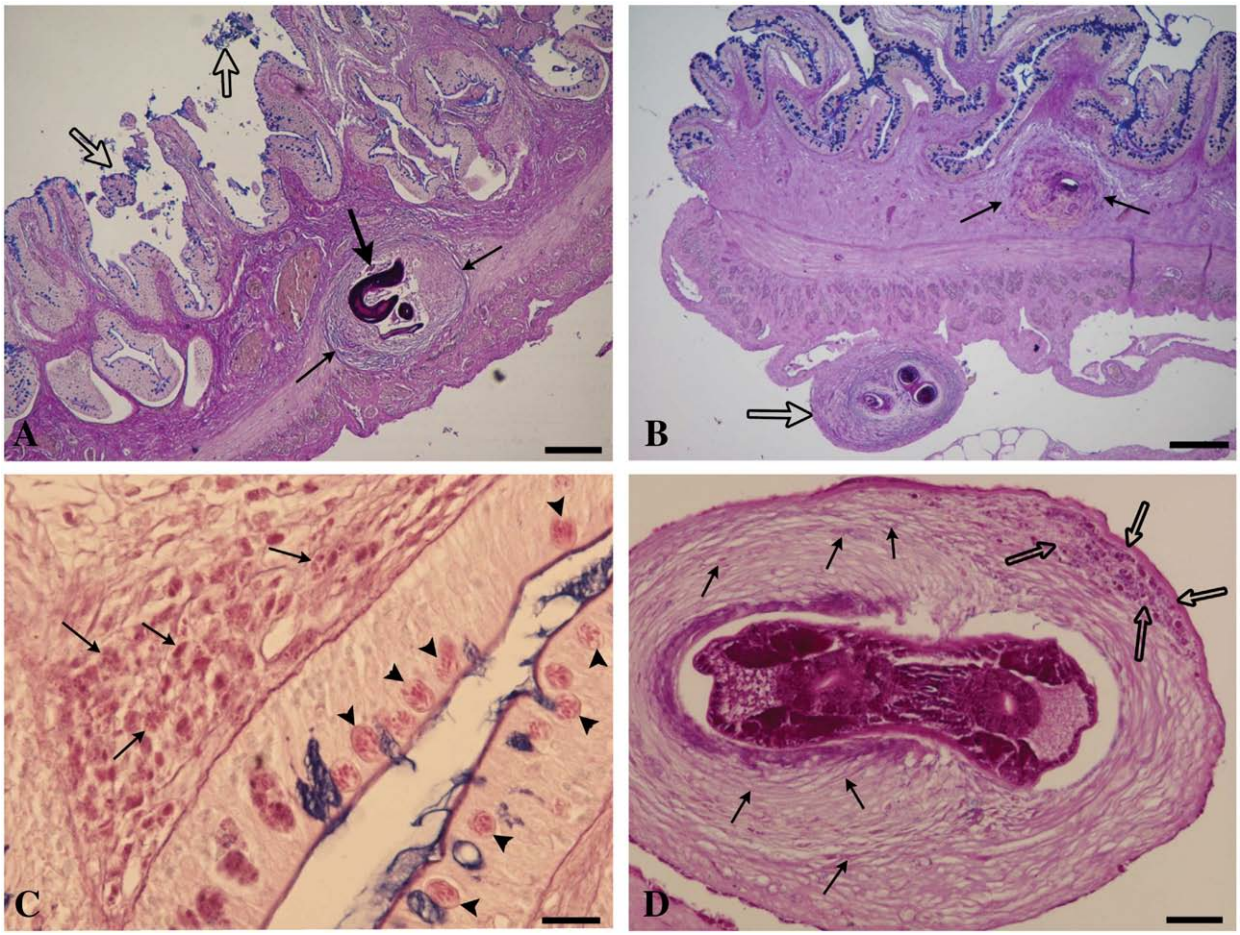


**Fig. 2.** Histological sections showing enteric helminths in intestine of *Anguilla anguilla*. A) *Contracaecum rudolphii* A larva (arrow) within the intestinal wall; granulomas (thin arrows), and catarrh (empty arrows) within the lumen (bar = 100 μm). B) Conspicuous granulomas on external surface of the intestine (empty arrow) surrounding *C. rudolphii* A larva; granulomas within the wall of the intestine (arrows) with central highly condensed multilaminated substance (bar = 100 μm). C) Mast cells (arrows) within the outer area of granulomas; rodlet cells (arrow heads) in epithelium (bar = 10 μm). D) Micrograph showing granuloma surrounding *C. rudolphii* A larva on external surface of eel intestine. Mast cells (empty arrows) in periphery and numerous fibroblasts (thin arrows) occupying the inner area of the granulomas (bar = 20 μm).

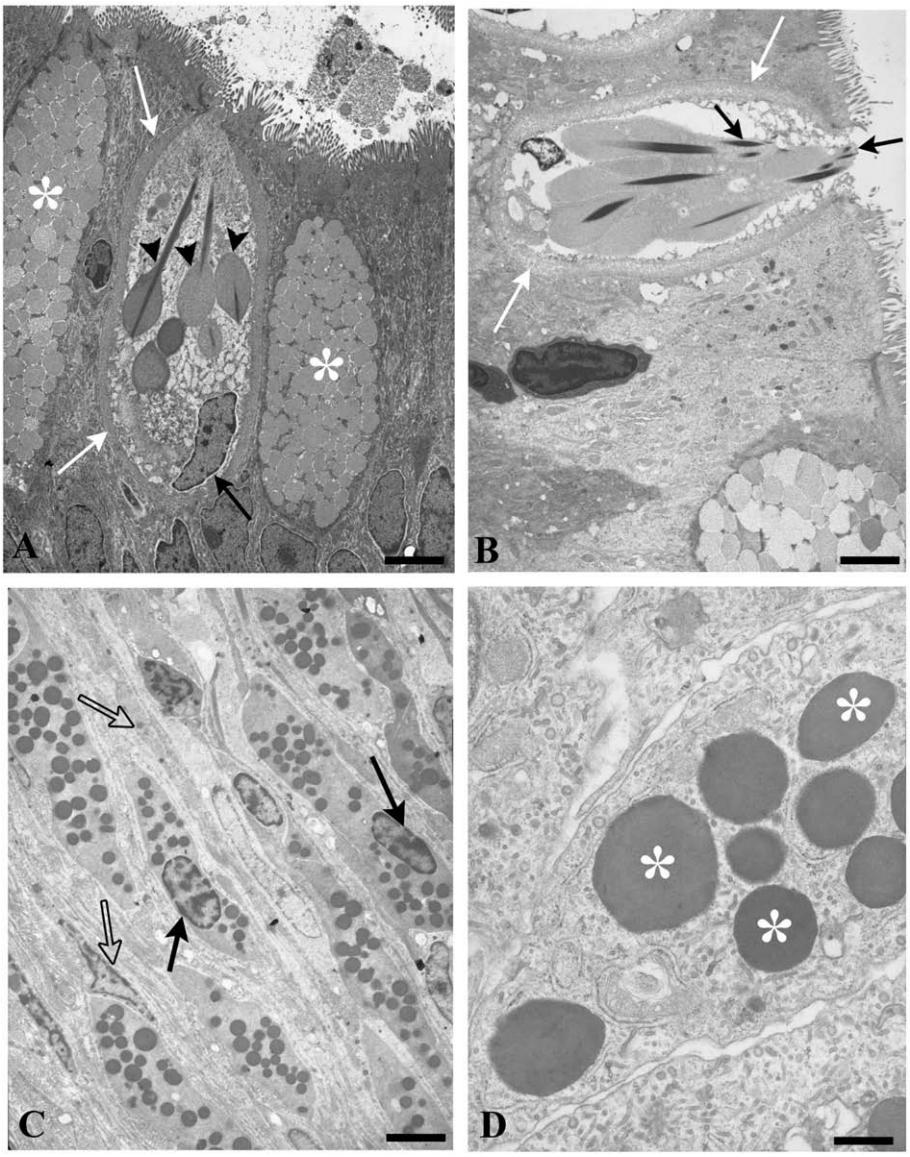


**Fig. 3.** Transmission electron microscopy showing cell types in *Anguilla anguilla* intestine. A) Mature elongated rodlet cell flanked by mucous cells (white asterisks). Note basal nucleus (thin arrow), cortex (white arrows) and club-shaped rodlets (arrowheads) inside the rodlet cell (bar = 3.3 μm). B) A rodlet cell discharging contents (arrows) into interface region between a trematode and epithelium; note the thickness of the cell cortex (white arrows) (bar = 2.5 μm). C) Several elongated mast cells in periphery of a granuloma on external surface of the host intestine; eccentric polar nuclei (arrows); electron-dense granules and fibroblasts (empty arrows) (bar = 3.3 μm). D) Micrograph showing several electron-dense granules (white asterisks) of a mast cell in a granuloma within the intestinal wall (bar = 0.7 μm).

surface of the intestine (Fig. 2B). Several macroscopic externally encysted nematode larvae were visible. Intensity of infection ranged from 1 to 45 (12.5 ± 10.3, mean ± SD). In this group of eels, conspicuous granulomas were observed within the host intestinal wall (major axis 563.26 ± 32.95 μm, mean ± SD, $n = 10$) or on the outer surface of the intestine (Fig. 2A, B). Intestinal granulomas were formed by host cells and, in some instances, contained a central, highly condensed, multilaminated substance (Fig. 2B). At the outer region of the granulomas a large number of mast cells, with large nuclei and several electron-dense granules/inclusions, were observed (Figs. 2C, 3C, D). Fibroblasts filled most of the inner region of the granulomas (Fig. 2D).

Three eels each harbored a single adult *A. crassus* specimen in the lumen of their swimbladder.

With transmission electron microscopy, RCs appeared elongated (major axis 15.44 ± 2.17 μm, mean ± SD, $n = 100$) (Fig. 3A). The thickness of the cell cortex (Fig. 3B) ranged from 0.6 to 0.9 μm, the nucleus was in the basal position, spherical–oval with an irregular border. The nucleus was characterized by randomly distributed zones of heterochromatin (Fig. 3A). In each RC, several club-shaped rodlets were present, appearing as electron-dense inclusions, with the head oriented toward the basal nucleus and the narrower "tail" toward an opening in the cytoplasmic border at the apex of the cell (Fig. 3A). In parasitized eels, RCs were frequently seen at the apex of intestinal folds near the digenean/cestode body (Fig. 3A).

Mature mucous cells were common at the epithelial apex, next to the RCs (Fig. 3A). Mucous cells (major axis 16.16 ± 3.17 μm, mean ± SD, $n = 30$) had a basal nucleus, and the cytoplasm was filled with numerous electron-lucent vesicles (Fig. 3A). Discharge activity of mucous cells was common at the site of infection, especially near the digenean/cestode body (Fig. 1C).

Mast cells were seen mainly in the intestine harboring *C. rudolphii* A larvae. They were found in the outer part of the granulomas within the intestinal wall (Figs. 2C, 3D) and/or in the periphery of the granulomas on the outer surface of the intestine (Figs. 2D, 3C). Mast cells were elongated (major axis 9.12 ± 1.30 μm, mean ± SD, $n = 100$),

with an eccentric polar nuclei having an irregular border (Fig. 3C). Mast cell cytoplasm was filled with numerous electron-dense, membrane bound, granules (Fig. 3C, D). A degranulation phase was not common.

## 4. Discussion

The major damage induced by digeneans and cestodes consisted of necrosis and sloughing of stomach and intestine epithelium. At the site of infection, numerous RCs and mucous cells were seen in the epithelium. Rodlet cells are exclusive to fish, and their ultrastructure is well known. Data from several recent surveys of wild and farmed fish support the suggestion that RCs are an immune cell type closely related to other piscine inflammatory cells such as mast cells (Dezfuli et al., 2008; Jordanova et al., 2007; Reite, 2005; Reite and Evensen, 2006; Vigliano et al., 2009).

The attachment organ of endoparasitic helminths often provokes inflammation of the host gastrointestinal tract. Inflammation is a protective reaction of the host in response to injury, resulting in specific chemical and morphological alterations in cells and tissues (Suzuki and Iida, 1992). The first level of defense consists of the substances secreted into the lumen, including mucus, bicarbonate, acid, immunoglobulins, and other antibacterial and surface-active phospholipid materials (Martin and Wallace, 2006; Wallace and Ma, 2001).

It appears that several peptides involved in the regulation of intestinal mucus secretion are released during inflammation (Fairweather, 1997; Lamont, 1992; Plaisancié et al., 1998). Our previous data on other fish parasite response systems (Dezfuli et al., 2002, 2003) are in accordance with this. Fish mucus is involved in a wide range of functions, including respiration, reproduction, excretion, feeding, ionic and osmotic regulation, and protection against, and resistance to, disease (Schroers et al., 2009; Shephard, 1994; Smirnova et al., 2003; Yan et al., 2007). It has been reported that, in some fish species, mucous cells produce and release defensive materials (Cho et al., 2002; Nakamura et al., 2001). In brown trout naturally infected with an acanthocephalan, the number of mucous cells increased significantly, and copious mucus secretion appeared as an adherent blanket around the worm body, at the site of infection (Bosi et al., 2005). In a comparison of uninfected to infected brown trout intestines, helminths were associated with increased thickness of the mucus layer (Bosi et al., 2005). In the present study, in parasitized eels, hyperplasia of intestinal mucous cells and enhanced mucus secretion were documented. Digenean and cestode bodies were often covered with an adherent mucus blanket. Our data are in agreement with the suggestion that the mucus gel layer protects the underlying epithelium as a physical barrier against pathogens and their toxins (Lamont, 1992; Schroers et al., 2009).

*C. rudolphii* A larvae induced severe damage within the tunica propria and on the external surface of the stomach and intestine, with conspicuous granulomas. Data on fish granulomas provoked by helminths have been reported by Taraschewski (1988, 1989) and Karanis and Taraschewski (1993). Cellular composition and zonation of fish granulomas appear to be similar to that of granulomas observed in other vertebrates (Boros, 1978). In *A. anguilla* granulomatous tissue was formed mainly by fibroblasts which were interspersed with mast cells and a small number of scattered macrophages. It is widely accepted that mast cells in fish, in view of their homology with mammalian mast cells and granular leukocytes, are inflammatory cells (Reite, 2005; Reite and Evensen, 2006; Silphaduang and Noga, 2001). It has been reported that mast cells are major effector cells in the immune response to helminth infection (Dezfuli et al., 2008; Sharp et al., 1989) and suggested that mast cells or their products are pivotal in mediating leukocyte recruitment to inflammatory sites (Mekori, 2004). Mast cells have been associated with defense against bacteria (Wedemeyer et al., 2000) and metazoan parasites (Dezfuli et al., 2000, 2008; Dezfuli and Giari, 2008; Reite, 2005). Their primary function is considered to be stimulating the activation of cells such as neutrophils to kill pathogens (Reite and Evensen, 2006), but some evidence suggests that they may also participate directly in killing microbes (Murray et al., 2007; Silphaduang et al., 2006; Silphaduang and Noga, 2001). Recently it has been reported that the mast cells of Perciformes, the largest and most evolutionarily advanced order of teleosts, contain histamine, which regulates the fish inflammatory response (Mulero et al., 2007, 2008).

A close relationship between mast cells and fibroblasts in various fish species has been reported (Flaño et al., 1996; Kent et al., 1993). In mammals and fish, several lines of evidence indicate that mast cells are involved in the fibrotic process and tissue remodeling (Dezfuli et al., 2008; Hrckova et al. 2006; Metcalfe et al., 1997; Rocha and Chiarini-Garcia, 2007). Data presented in this paper contribute to the understanding of *A. anguilla* immune defenses against endoparasitic helminths. Due to their high commercial value, knowledge of the efficiency of the eel immune system is crucial to maintaining the health of eel populations on farms.

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