Anguilla anguilla intestinal immune response to natural infection with *Contracaecum rudolphii* A larvae

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Abstract

The European eel, *Anguilla anguilla*, is a major warm water fish species cultured in North and South Europe. Seventy-one *A. anguilla* collected between 2010 and 2015 from the Comacchio lagoons were examined. Fish were infected and damaged by larvae (L3) of the nematode *Contracaecum rudolphii* A, which were encapsulated within the thickness of the intestinal wall and within the external visceral peritoneum (serosa). Conspicuous granulomas, visible at sites of infection, were arranged in a tri-layer, formed by a series of concentric whorls. The cells involved in the immune response and their distribution in the granuloma layers were assessed by immunohistochemical, immunofluorescence, and ultrastructural techniques. The outer part of the granuloma contained macrophages, macrophage aggregates, and mast cells (MCs) scattered among fibroblasts. This layer was vascularised, with degranulation of MCs occurring in close proximity to the capillaries. The middle layer was rich in MCs and fibroblasts. The inner layer, closest to the parasite larva, consisted mainly of dark epithelioid cells, some of which were necrotic. Non-necrotic epithelioid cells formed desmosomes between themselves or with fibroblasts. Within the granulomas numerous cells of different types were positive to proliferative cell nuclear antigen antibody, indicating a high degree of cellular proliferation around the larvae.

Keywords: European eel, fish nematodes, granuloma, immune cells, PCNA

Introduction

Contracaecum spp. is one of the largest ascaridoid genera with approximately 50 species. There are two genetically distinct sibling species, *C. rudolphii* A and *C. rudolphii* B (D'Amelio *et al.* 2007). *Contracaecum rudolphii* uses several fish species as paratenic host and infect cormorants as definitive host (Moravec 2009). Some papers have reported the histopathology associated with nematode infection in European eel (Molnar *et al.* 1993; Molnar 1994; Dezfuli *et al.* 2007; Kennedy 2007; Abdelmonem *et al.* 2010; Santoro *et al.* 2013). Moreover, evaluation by ELISA and immunoblotting of the humoral immune response in nematode-infected eel was reported by Knopf *et al.* (2000). Indeed, in a recent review, Buchmann (2012) provided three experimental models for the study of fish immune response against endoparasitic nematodes.

Endoparasitic helminths generally induce inflammation and changes of the structure and function of tissues (Castro 1992). The essential role of enteric immune cells in inflammatory processes induced by parasitic helminths was previously described in detail (Faiweather 1997; Dezfuli *et al.* 2000, 2013a). Fish response to extra-intestinal parasites in the formation of granuloma on the intestine and within the viscera for several parasite genera is well described (Abdelmonem *et al.* 2010; Dezfuli *et al.* 2013a, 2015a). Granulomas are chronic inflammatory lesions that appear as nodules in one or more organs (Adams 1976). Granulomas resulting from helminths have been reported (Karanis & Taraschewski 1993; Molnar 1994; Dezfuli *et al.* 2013a; Dezfuli, Manera & Giari 2015b).

Proliferating Cell Nuclear Antigen (PCNA) is a nuclear non histone protein and is an accessory protein for DNA polymerase delta (Walsh & Eckert 2014). PCNA is necessary for DNA synthesis, both in replication and repair (de Oliveira *et al.* 2008; Mailand, Gibbs-Seymour & Bekker-Jensen 2013; Walsh & Eckert 2014). PCNA rises during the G1/S phase of the cell cycle, and accordingly in quiescent and senescent cells, there is very low PCNA expression (Kelman 1997). Because proliferating cells remain a longer time in the G1/S phase, PCNA expression may be used as a marker of cell proliferation. PCNA methodology was developed for and applied mostly to mammalian tissues (Foley *et al.* 1991) and it is widely used as a tumoural marker in human pathology (Bologna-Molina *et al.* 2013), but also in fish (Manera & Biavati 1994). Expression of PCNA can provide an early indication of changes in cell proliferation and can be observed via immunohistochemical staining (Mathews *et al.* 1984; Ortego *et al.* 1994). In the current study the expression of PCNA was investigated in an attempt to further clarify host cellular proliferation within the granuloma.

Innate immunity of vertebrates relies on various types of immune cells and one of the most common is the eosinophilic granule cell, the piscine equivalent of the mast cell (MC) (Reite &

Evensen 2006; Da'as S *et al.* 2011; Dezfuli *et al.* 2015c). It is generally accepted that MCs are involved in the induction of inflammatory responses by their effects on vasodilation, neutrophil attraction and macrophage activation (Vallejo & Ellis 1989; Dezfuli *et al.* 2012a, 2015a). The present study utilized immunohistochemistry and ultrastructural analysis to evaluate the occurrence and nature of the immune cells involved in defense of eel intestine against nematode larvae.

Materials & Methods

Specimen collection and preparation

A total of seventy-one eels (40 yellow and 31 silver) were obtained by the Po Delta Park Administration from the Comacchio Lagoons (Northern Adriatic Sea, Italy, 44º 36´ N, 12º 10´ E) on 11 occasions from March 2010 to March 2015 (see details in Tab. S1 of Supporting Information). Fish were brought live to the laboratory of the Department of Life Sciences and Biotechnology, University of Ferrara, euthanised using a lethal dose of 300 mg L^{-1} MS222 (tricaine methanesulfonate, Sandoz, Basel, Switzerland) and pithing. Thereafter the eels were weighed (186-1500 g, min-max; 590.9 ± 300.3 g, mean ± SD) and measured (50-88 cm in total length, min-max; 65.3 ± 10.0 cm, mean ± SD). 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.

Parasite larvae molecular identification

The identity of nematode species found in the intestinal wall could not be determined based solely on morphology and required some genomic characterization. Therefore larvae (L3) were isolated and fixed in 90% ethanol for DNA extraction, PCR and restriction fragment analysis.

DNA was isolated from 10 larvae of a nematode using the Wizard® Genomic DNA purification kit (Promega, USA), according to manufacturer's protocol. Each DNA pellet was air-dried for 20 min and dissolved in 100 μl of DNA rehydration solution.

The ITS (Internal Transcribed Spacers 1 and 2, plus intervening 5.8S rRNA gene) was amplified by PCR using 4.0 μl of template DNA. 4.0 ml of template DNA (20–40 ng), 10 mM Tris-HCl (pH 8.3), 50 mM KCl (Applied Biosystem, USA), 3 mM $MgCl_2$ (Applied Biosystem, USA), 1 mM of dNTPs (Promega, USA), 50 pM of each the forward primer NC5 (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and the reverse primer NC2 (5'-TTAGTTTCTTCCTCCGCT-3') (Zhu *et al.* 2000) and 0.5 U of AmpliTaq Gold™ (Promega, USA) in a final volume of 50 μl. The PCR was performed under the following conditions: 10 min at 95ºC

(initial denaturation), 30 cycles of 30 sec at 95ºC (denaturation), 40 sec at 52ºC (annealing) and 75 sec at 72ºC (extension), and a final elongation step of 7 min at 72ºC. The amplification of the *rrn*S (small subunit of the mitochondrial ribosomal DNA) was performed using 4.0 µl of template DNA, 5.0 µl of 10x PCR buffer II (Promega, USA), 3 mM $MgCl_2$ (Applied Biosystem, USA), 1 mM of dNTPs (Promega, USA), 50 pM of the forward primer MH3, 5'-TTGTTCCAGAATAATCGGCTAGACTT, 50 pM of the reverse primer MH4.5, 5'-TCTACTTTACTACAACTTACTCC) and 0.5 µl of AmpliTaq Gold™ (Promega, USA) in a 50 µl final volume of reaction. The conditions of PCR were as follows: 10 min at 95ºC (initial denaturation), 35 cycles of 30 sec at 95ºC (denaturation), 30 sec at 55ºC (annealing) and 30 sec at 72ºC (extension), and a final elongation step of 7 min at 72ºC. A negative control, was included in each amplification.

Aliquots (5 µl) of individual PCR products were detected on agarose gels (1%), stained with ethidium bromide (10 mg/ml) and detected upon ultraviolet transillumination. Gel images were captured electronically and analyzed using the program MULTI-ANALYST (v.1.1, Bio-Rad).

For each DNA locus, amplicons were digested with the restriction endonuclease *Tsp509*I (for the ITS, Promega, USA), or *Rsa*I or *Dde*I (for *rrn*S, Promega, USA). Digests were resolved by electrophoresis in 2% agarose gels, stained with ethidium bromide (10 mg/ml), detected upon transillumination and the sizes of fragments determined by comparison with 100 bp ladder (Promega, USA) as size marker.

Histology and immunohistochemistry

Pieces of infected-uninfected intestine (15 × 15 mm) and of all other organs (gills, heart, gonads, liver, kidney, spleen and swimbladder) were fixed in 10% neutral buffered formalin for 24 h and then were rinsed in several changes of 4ºC 70% ethanol before being stored in the same medium until processed for histology. The fixed tissues were dehydrated through an alcohol series and then paraffin wax embedded using a Shandon Citadel 2000 Tissue Processor (Shandon, UK). After blocking out, 5 µm thick sections were taken from each tissue block, stained with haematoxylin and eosin (H&E), alcian blue 8 GX pH 2.5 combined with periodic acid Schiff's reagent (AB/PAS), Giemsa and / or Masson's Trichrome. Multiple histological sections were taken from each tissue block, examined and photographed using a Nikon Microscope ECLIPSE 80i (Nikon, Tokyo, Japan).

Some sections of intestine were processed for immunohistochemistry using a commercially available anti-Proliferating Cell Nuclear Antigen (PCNA) antibody (PC10 sc-56 mouse monoclonal antibody, Santa Cruz Biotechnology, Inc., Dallas, USA). After dewaxing in xylene and rehydrating through a graded alcohol series, the sections were treated for antigen retrieval in citrate buffer (pH

8.0) for 20 min in a steam bath at 95°C; the slides were then left for 10 min to cool to room temperature (RT). Endogenous peroxidase activity and non-specific staining were blocked, respectively, in 3% H_2O_2 for 10 min and then in horse normal serum (1:20, Elite Mouse IgG Vectastain ABC Kit, Vector, Burlingame, USA) for 30 min. Sections were then incubated with the primary antibody (anti-PCNA diluted 1:500) for 2 h at RT. After washing with PBS, the slides were incubated for 30 min with biotinylated horse anti-mouse serum (Mouse IgG Vectastain ABC Kit, Vector) followed by avidin-conjugated horseradish peroxidase (Mouse IgG Vectastain ABC Kit, Vector).

Additional sections of eel intestine were dewaxed and re-hydrated, then treated with 3% H_2O_2 in Tris-HCl buffer saline (TBS; 0.05 M, pH 7.4, 0.55 M NaCl) for 15 min at RT to block endogenous peroxidase. After rinsing in TBS, slides were incubated with 0.1% Trypsin in TBS for 15 min at RT for antigen retrieval. Sections were treated with a mouse monoclonal antibody specific to macrophage, diluted 1:50 (clone MAC387, code ab22506, Abcam, Cambridge, UK) in TBS for 3 days at 4 °C, washed in TBS, and treated with the Biotin-Avidin Blocking Kit (Vector Lab., code SP2001, USA). Slides were then incubated with 10 μg/ml biotinylated goat anti-mouse IgG (Vector Lab., code BA9200) in TBS for 45 min at RT. After rinsing in TBS, Streptavidin-Biotin/Horseradish Peroxidase Complex (Vectastain® ABC Kit, Vector Lab., code PK4000) was employed for detection.

For both (PCNA and MAC 387) immunohistochemical reactions, the immunoreactive sites were visualized using a freshly prepared solution of 4 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, code D5637, Italy) in 10 ml of TBS containing 0,1 ml of 3% H_2O_2 for 15 min. The sections were then dehydrated, counterstained with Alcian blue and Harris's or Mayers' haematoxylin. Sections were examined and photographed using a Nikon Microscope ECLIPSE 80i (Nikon, Tokyo, Japan).

Negative controls were obtained by the omission of the primary antibodies on sections from infected intestine. Sections of rat intestine and spleen, eel head kidney and spleen were used as positive controls. Both sets of controls gave the expected results. In spleen and head kidney of eels, in addition to macrophages and myeloid precursor cells, MCs were also stained with MAC 387 antibody (not shown). MCs positive to this antibody have previously been reported in the intestines of another fish species, chub *Squalius cephalus* L. (see Dezfuli *et al.* 2015c).

Immunofluorescence double staining

Sections (5-7 μm-thick) of parasitized intestines of the eel were dewaxed, re-hydrated, and then rinsed in Tris-buffered saline (TBS: 0.05 M Tris-HCl, 0.15 M NaCl) containing 0.1% Triton-X 100 (TBS-T). After each of the following steps, the slides were washed in TBS-T. Non-specific

antibody binding was inhibited by treatment with 1:20 goat normal serum (Vector) in TBS for 30 min in a humid chamber. For antigen unmasking sections were brought to a boil in 10 mM sodium citrate buffer pH 6.0, and then maintained at a sub-boiling temperature for 10 min. Slides were cooled on the bench top for 30 min before the treatment with the primary anti-body: 1:250 anti-PCNA (PC10 sc-56 mouse monoclonal antibody, Santa Cruz Biotechnology, Inc., Dallas, USA) in TBS for 3 days at 4°C. The sections were then treated with avidin-biotin blocking solutions (Vector), and incubated with 10 μg ml^{-1} biotinylated goat anti-mouse IgG (Vector) in TBS for 2 h at RT. Finally, immunological reactions were labeled with 10 μg ml^{-1} fluorescein avidin D (ex495 nm/em 515 nm ,Vector) in 0.1 M $NaHCO_3$ pH 8.5 with 0.15 M NaCl for 2 h at RT.

After a brief washing in TBS-T, the same slides were treated with 1:50 mouse monoclonal antibody specific to macrophage, diluted 1:50 (clone MAC387, code ab22506, Abcam, Cambridge, UK) in TBS for 3 days at 4 °C. A further treatment with the avidin-biotin blocking solutions was performed at this time (Vector) then sections were incubated with 10 μg ml^{-1} biotinylated goat anti-mouse IgG (Vector) in TBS for 2 h at RT. After this treatment, the reactions were labeled with 10 μg ml^{-1} rhodamine avidin D (ex550 nm/em 575 nm, Vector) in 0.1 M $NaHCO_3$ pH 8.5 with 0.15 M NaCl for 2 h at RT. The stained tissue sections were mounted with Vectashield® mounting medium (Vector Laboratories, Burlingame, Ca) and examined on a Zeiss 510 confocal laser scanning microscope (CLSM) (Zeiss, Oberkochen, Germany). The CLSM was equipped with multi-argon and helio-neon-green lasers with excitation and barrier filters set for fluorescein and rhodamine. To avoid cross-contamination of the two signals in the displayed images, green and red fluorescent signals were obtained concurrently through alternate excitation (0.2 s^{-1}) at 488 nm and 540 nm, respectively.

The specificity of reactions was tested by incubating in parallel other sections of the same pieces with normal mouse serum, instead of the primary antibodies. Sections of rat (intestine and spleen) and eel (head kidney and spleen) organs processed as above, served as positive controls. Both positive and negative controls gave the expected results.

Transmission electron microscopy

For electron microscopy, representative pieces (7 × 7 mm) of nematode-infected and uninfected intestines were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 3 h at 4°C before being post-fixed in 1% osmium tetroxide in the same buffer for 3 h. The samples were then dehydrated through a graded acetone series before being embedded in epoxy resin (Durcupan™ ACM, Fluka, Sigma-Aldrich, Saint Louis, Mo). Semi-thin sections (*i.e.* 1.5 μm) were cut on a Reichert Om U 2 ultramicrotome (Reichert, Vienna, Austria) using glass knives and then were 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 then were examined using a Hitachi H-800 electron microscope (Hitachi Ltd, Tokyo, Japan).

Results

Histological examination showed the absence of parasites in gills, heart, gonads, spleen, kidney, and liver of *A. anguilla*. Six eels harboured 1 or 2 adult *Anguillicoloides crassus* specimens in the lumen of their swimbladder. Three Digenean species, namely *Helicometra fasciata*, *Deropristis inflata* and *Bucephalus anguillae* were encountered in the intestine of 39 eels (54%). Analysis of restriction fragment profiles, applied on nematode larvae isolated from intestinal granulomas, revealed that parasites belong to the species *Contracaecum rudolphii* A (Fig. S1 in Supporting Information). In particular, RFLP analysis of representative rrnS amplicons using *Rsa*I gave three fragments of 330, 110 and 49 bp, while digestion of the ITS amplicons with Tsp509I yielded fragments of 330, 220, 170 and 80 bp as described for *C. rudolphii* A (D'Amelio *et al.* 2007). *C. rudolphii* A larvae (L3) were found in 18 eels (25%) (see Tab. S1 in Supporting Information). The intensity of infection ranged from 3 to 39 larvae per host (16.8 ± 11.2, mean ± SD). Co-occurrence of both taxa (Nematoda and Platyhelminthes) in the intestine was observed in 10% of hosts.

Histology

Contracaecum rudolphii A L3 larvae were observed especially in the rectum region, encapsulated in the visceral peritoneal serosa on the outer surface (Fig. 1a,b) and within the thickness of the intestinal wall (Fig. 1a,c). The larvae appear to penetrate the rectum wall and migrate to the serosa. During migration and growth the larvae elicit a chronic inflammatory response which results in fibrosis and larval encapsulation (Fig. 1b,c). *In situ*, extra-intestinal larvae appear as loosely attached nodules (Fig. 1a,b). Histological sections of infected pieces of rectum revealed the presence of conspicuous granulomas (major axis 655.44 ± 127.65 μm, mean ± SD, n=42) (Fig. 1b,c) and within were often found fully developed, tightly packaged *C. rudolphii* A larvae (Figs. 1b, 2b).

The granuloma consists of three concentric layers: an outer layer of fibrous vascular granulation tissue containing macrophages and macrophage aggregates (MAs), which appeared as group of large oval-to-round shaped cells, and fibroblasts and collagen bundles (Fig. 1d,e). The outer layer had capillaries and around and/or inside them were MCs, some of which were undergoing degranulation (Fig. 1e). The middle layer of the granuloma was characterized by numerous MCs (Fig. 1f) scattered among the fibroblasts and epithelioid cells. The inner layer was formed mainly by fibroblasts and epithelioid cells.

Immunohistochemistry and immunofluorescence

Immunohistochemical staining with PCNA antibody showed numerous PCNA-positive cells inside the granuloma located within the outer visceral peritoneum (serosa) of the rectum (Fig. 2a) or within the thickness of the rectum wall (Fig. 2b). PCNA-positive cells were scattered among collagen fibres (Fig. 2c,d). In the middle layers PCNA-positive oval cells, most likely MCs, were observed (Fig. 2c). PCNA-positive, predominantly flat fibroblasts were found in the granuloma inner layer as were epithelioid cells situated in close proximity to the *C. rudolphii* A larva (Fig. 2d).
Immunohistochemical staining with antimacrophage antibody MAC 387 marked numerous macrophages scattered in the granuloma as singular elements or as aggregates as well as MCs (Fig. 2e,f), while the flat epithelioid cells close to the nematode larva were unstained by this antibody (Fig. 2e).
Figure 3a and 3b show the presence of granulomas in sub-serosa layer and in a sub-epithelial position, respectively. Immunofluorescent staining identified MAC 387-positive oval-shaped cells with or without cytoplasmic granules (Figs. 3, 4 column at left side), and numerous PCNA-positive round-oval or flat-elongate cells (Figs. 3, 4 central column). It is interesting that double immunofluorescence staining using the two antibodies demonstrates some oval cells are positive to both MAC 387 and PCNA, which are likely MCs and macrophages in proliferation (Figs. 3, 4 column at right side).
TEM observations confirmed the identification of the cells in the three regions of the granuloma (see below).

Transmission electron microscopy

Transmission electron microscopy showed details of the cells within the thickness of the granuloma. In the outer layer macrophages, MAs, fibroblasts, and collagen bundles were prevalent with some MCs scattered among them (Fig. 5a). The outer layer also showed vascularisation and frequent MCs in the vessel lumen or adjacent to it (Fig. 5b). MCs were numerous in the middle region of the granuloma (Fig. 5c). MCs were typically large and oval in shape, with an eccentric nucleus, and cytoplasm containing several large, membrane-bounded electron-dense granules (Fig. 5c). In the inner layer flattened cells with electron-lucent cytoplasm (Fig. 5d), few mitochondria and swollen rough endoplasmic reticulum (RER) were putatively identified as fibroblasts based on their consistent association with extracellular collagen. In close proximity to the parasite larva, a dark epithelioid cell layer was observed and it was formed mainly by necrotic cells (Fig. 5d). The epithelioid cells were flattened (Fig. 5d) with voluminous pale euchromatic nucleus, edematous and vacuolated cytoplasm. Ultrastructurally, epithelioid cells were similar to macrophages but with increased cytoplasmic and nuclear volume, few mitochondria and RER. These cells appeared highly compressed and cytoplasmic boundaries were indistinct. Non-degenerated epithelioid cells

frequently formed desmosomes between themselves (Fig. 5e) or with nearest fibroblasts. Interdigitation was also frequently observed around and between epithelioid cells. Neutrophils were not detected in any of the granuloma layers.

Discussion

Comacchio lagoons has a long history of eel culture and is one of the most important eel fisheries on the Mediterranean coast (Dezfuli *et al.* 2014a). Representatives of all taxa of helminths can occur in eels (Kennedy 2007). Nonetheless many parasitological investigations have centered on a pathogenic nematode species, *Anguillicoloides crassus,* which infects eel swimbladder (Taraschewski *et al.* 1987; Molnar 1994; Kennedy 2007; Abdelmonem *et al.* 2010). Eel and eight other fish species were experimentally infected with *Contracaecum rudolphii* A larvae and their role as paratenic hosts of this nematode was established (Moravec 2009). *C. rudolphii* A larvae also use *A. anguilla* as a paratenic host in the Comacchio lagoons. Occurrence of the same parasite in visceral cavity and gut wall in eels and in four other fish species was also reported in a lagoon in Sardinia (South Italy) by Culurgioni *et al.* (2014) but no histopathological data were provided in that study. Information on the status of the eel immune system is of importance to maintain good health throughout the grow out period in eel fisheries. Nevertheless there is limited knowledge in terms of both cellular and humoral immune responses of *A. anguilla* to heminths (Knopf *et al.* 2000; Nielsen & Esteve-Gassent 2006). The immunological implications (immunity versus immune evasion mechanisms) in relation to the life cycle strategy of the nematode were illustrated very well in review by Buchmann (2012). The author emphasised on tissue dwelling and luminal stages of nematodes, and only the third stage larvae of genus *Contracaecum, Anisakis and Pesudoterrnova* can be found in fish. This would suggest that the cellular reactions to the larva are likely one of the stimuli inducing coiling (reducing the exposed surface area of the parasite) and production of a protective wall formed of an inner layer of dead host cells and outer layers of leucocytes and fibroblasts (Larsen *et al.* 2002).

In the current study, the larvae of *C. rudolphii* A were surrounded by granulomas. Granulomas are chronic inflammatory lesions that appear as nodules in one or more organs (Adams 1976) and the extent of the subsequent host reaction can vary considerably. Granulomas in fish may result from viruses, bacteria, protozoa, foreign bodies and helminths (Gardiner & Bunte 1984; Anders & Möller 1988; Molnar 1994; Gauthier, Vogelbein & Ottinger 2004; Dezfuli *et al.* 2013a, 2015b). The granulomas associated with *C. rudolphii* A larvae infection have similar ultrastructural features described for *Mycobacterium marinum* granuloma by Noga, Dykstra & Wright (1989). Fish innate immune defenses responding to parasite or pathogen infection include MCs (Reite &

Evensen 2006; Buchmann, 2014; Dezfuli *et al.* 2012a, 2013b; Sfacteria, Brines & Blank 2015), neutrophils and fibroblasts (Secombes & Chappell 1996; Stakauskas *et al.* 2007; Dezfuli *et al.* 2012a; Roberts 2012), macrophage aggregates (Wolke 1992; Passantino *et al.* 2014; Dezfuli *et al.* 2015a) and epithelioid cells (Noga *et al.* 1989; Gauthier *et al.* 2004).

The occurrence of MAs in the outer layer of granulomas within parasitized eel intestine was evaluated in the current study. The presence of MAs in association with helminth larvae have been reported previously (Ferguson 2006; Dezfuli *et al.* 2013a, 2014b, 2015a). Several roles for MAs in fish have been described. For example MA proliferation has been associated with both physiological and pathological factors such as aging, starvation, and infectious diseases **(**Wolke 1992; Ferguson 2006; Kharraz *et al.* 2013; Passantino *et al.* 2014; Dezfuli *et al.* 2015a). The nature of MAs and their role in fish pathology was reviewed by Agius & Roberts (2003). Our observations lend support to the view that MAs may be linked to parasite infections and, in all likelihood, represent an inflammatory response different from the typical granulomatous reaction (Vogelbein, Fournie & Overstreet 1987).

MCs are found in all vertebrates and several studies have highlighted critical roles for these cells in the early orchestration of immune responses (Abraham & St. John 2010; Da'as *et al.* 2011; Dezfuli *et al.* 2014b, 2015a; Prykhozhi & Berman 2014; Sfacteria *et al.* 2015). MCs are suggested to have the potential to directly influence fibroblasts or indirectly influence other cells, leading to a profibrotic response (Puxeddu *et al.* 2003; Dezfuli *et al.* 2013a, 2015a). MCs frequently are strategically positioned at perivascular sites to regulate inflammatory responses (Mekori 2004; Dezfuli *et al.* 2013b; as well as the current study). We observed MCs within or in close proximity to capillaries inside the granuloma (Vallejo & Ellis 1989; Reite & Evensen 2006; Dezfuli *et al.* 2008). Based on numerous available studies it is likely that fish may have two populations of MCs, resident and circulating (Powell, Wright & Burka 1990; Murray, Leggiadro & Douglas 2007; Dezfuli *et al.* 2013a) and the development of parasite infections likely induces MC recruitment and proliferation in infected tissue (Reite & Evensen 2006; Dezfuli *et al.* 2013a,b).

One of the most interesting findings of the current study is the occurrence of PCNA-positive MCs within the granulomas. The expression of PCNA, which is a protein directly involved in DNA synthesis, has been shown to be directly related to proliferative rate (Linden *et al.* 1992). The gene sequence and functions of PCNA are remarkably conserved among eukaryotes (Leung *et al.* 2005). PCNA-positivity has been reported in several cell types in mammalian tissues and from a number of different organs in fish (Ortego *et al.* 1994; Wiesniewska *et al.* 2013). Changes in the expression of PCNA has recently been used to study fish health (Blas-Machado, Taylor & Means 2000; Chikwati *et al.* 2013) as well as parasite-infected fish, where immunohistochemical staining of sections of

infected tissue reveals the distribution and abundance of different types of host cells which are in a state of proliferation and regeneration (Dezfuli *et al.* 2012b, 2014b, 2015a). The presence of MCs positive for PCNA was previously documented in intestine of *Salmo trutta* at the site of acanthocephalan infection (Dezfuli *et al.* 2012b) and in liver of *Perca fluviatilis* parasitised with a tapeworm (Dezfuli *et al.* 2014b).

Several investigations have demonstrated how MCs degranulate in response to exposure to a variety of known degranulating agents (Powell, Wright & Burka 1991; Manera *et al.* 2011) and pathogens (Vallejo & Ellis 1989; Dezfuli *et al.* 2008, 2013b; present study) with release of their contents. Mast cell products are pivotal in mediating leucocyte recruitment into inflammatory sites (Prykhozhi & Berman 2014). In the paper we report immuno-labeling of eel macrophages and MCs with MAC 387 antibody at sites of nematode infection. Chub MCs have been previously shown to cross react with the same antibody (Dezfuli *et al.* 2015c) and with another anti-macrophage antibody (clone no. LN-5, code M-1919; Sigma, St Louis, MO, USA) (Dezfuli *et al.* 2002). MAC 387 antibody was properly clustered as anti-myeloid at the Third International Workshop and Conference on Human Leucocyte Differentiation Antigens (Jones, Flavell & Wright 1984). Recently, a cell population termed basophil/MC common progenitor, has been described in the mouse spleen and is derived from granulocyte/macrophage progenitors in the bone marrow (Arinobu *et al.* 2005), supporting the hypothesis of a joint granulocyte/macrophage progenitor. The findings of our study of *A. anguilla*, combined with the observation of Dobson *et al.* (2008) on zebrafish MCs, leads us to suggest a bone marrow-like origin also exists for fish MCs.

Numerous fibroblasts were noticed within the granuloma which surrounds *C. rudolphii* A larva. Fibroblasts are intimately involved in the initiation of wound repair and regeneration processes (Castillo-Briceno *et al.* 2011). Fibroblasts modify the quality, quantity and duration of the inflammatory infiltrate and play a critical role in the transition from acute to chronic persistent inflammation (Parsonage *et al.* 2005); indeed, fish inflammation caused by helminths is of the chronic type (Noga 2010; Dezfuli *et al.* 2013a). Fibroblasts and MCs cooperate together in tissue repair in mammals (Kharraz *et al.* 2013; Van Linthout, Miteva & Tschöpe 2014) and in fish (Rocha & Chiarini-Garcia 2007; Dezfuli *et al.* 2008, 2013a). The presence of numerous MCs and fibroblasts in granulomas in infected eel intestines leads us to suggest that this association is probably necessary to mediate intestinal remodelling after tissue injury induced by *C. rudolphii* A larvae.

Granulomas are characterized by concentric cellular layers (Molnar 1994; Gauthier *et al.* 2004) that form due to the sequential development of epithelioid cells from free macrophages (Papadimitriou & Spector 1971; Noga *et al.* 1989; Gauthier *et al.* 2004; Ferguson 2006). The

epithelioid cells in the inner layer of *C. rudolphii* A larva (L3) induced granuloma observed here were sometimes necrotic, consistent with findings reported by Noga *et al.* (1989) and also by Gauthier *et al.* (2004) in Mozambique tilapia *Oreochromis mossambicus* and in striped bass (*Morone saxatilis*), both infected with *Mycobacterium marinum*. The occurrence of normal and/or necrotic epithelioid cells were found in the deep layer of nodules around *Anguillicoloides crassus* larvae in the intestine of eels (Molnar 1994). It seems that there is a transition from metabolically active cells such as macrophages and, to a lesser extent, epithelioid cells to structural sequestering elements (Noga *et al.* 1989; Gauthier *et al.* 2004; Ferguson 2006).

Neutrophils were not observed in granuloma encircling *C. rudolphii A* larvae. Neutrophils commonly appear concurrently with macrophages that readily engulf small extracellular pathogens, such as viruses and bacteria (Ellis 2001; Katzenback & Belosevic 2012; Roberts 2012), or parasites of small size, such as the migrating diplostomules of *Diplostomum spathaceum*, which infects the eyes of fish (Whyte, Chappell & Secombes 1989). Neutrophils were reported to be abundant in the intestine of tench infected with digenean larvae (Dezfuli *et al.* 2013a). The reasons for the absence of neutrophils in the granuloma of infected eel intestines are unknown, but two possible explanations are: a) neutrophils are associated with acute inflammation, particularly where there is tissue destruction (Ferguson 2006). *C. rudolphii*, like most parasitic helminths, instead induces a chronic inflammation; b) *C. rudolphii* A larvae are too large to be engulfed by host neutrophils.

Our study demonstrates the presence of various types of innate immune cells in the granulomas encasing the nematode larvae, and examines the relationship between innate immune cells within granuloma situated around nematode larvae which use eel as paratenic host. This paper provides further evidence supporting the proliferation of MCs and macrophages at sites of helminth infection in a fish system.

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Figure captions

Fig. 1. Histological sections showing intestine of *Anguilla anguilla* infected with *Contracaecum rudolphii* A larvae. (a) Section of *A. anguilla* rectum, three larvae (black arrows) are encysted within the visceral peritoneum (serosa) and one larva (white arrow) within the thickness of the rectum, Masson's trichrome stain, bar = 200 μm. (b) Conspicuous granuloma surrounding a larva (asterisk) on external visceral peritoneal surface of the rectum appears as loosely attached, peduncolated nodule (arrow), Masson's trichrome, bar = 50 μm. (c) Granuloma (arrow) within the wall of the intestine in sub-epithelial position, Alcian Blue/PAS, bar = 100 μm. (d) Outer layer of the granuloma around *C. rudolphii* larva (asterisk): one large macrophage aggregate is visible (arrows), Giemsa, bar = 20 μm. (e) A capillary (curved arrow) in outer layer of granuloma, mast cells (arrows) near it, three mast cells (arrow heads) in degranulation are evident, Giemsa, bar = 10 μm. (f) Numerous mast cells (arrows) and fibroblasts (curved arrows) can be seen in the middle layer of a granuloma, Giemsa, bar = 10 μm.

Fig. 2. Sections of *Anguilla anguilla* intestine infected with *Contracaecum rudolphii* A larvae stained with proliferative cell nuclear antigen (PCNA) antibody (a-d) or antimacrophage antibody MAC 387 (e-f). (a) Granuloma loosely attached to the peduncolated outer surface of intestinal peritoneal serosa (arrows): occurrence of immunoreactive cells around the larva (asterisk) is evident, bar = 100 μm. (b) Granuloma in sub-epithelial position: *C. rudolphii* A larva (asterisk) is encircled by numerous PCNA-positive cells, arrow shows intestinal fold, bar = 50 μm. (c) High magnification of middle layer of granuloma, numerous mast cells (arrows) are positive to PCNA antiserum, bar = 10 μm. (d) Middle and inner layers of granuloma around the larva (asterisk): PCNA-positive mast cells (arrows), fibroblast (curved arrows) and, near to the larva, flattened epithelioid cells (arrow heads) are evident, bar = 10 μm. (e) Near the parasite larva (asterisk), mast cells (arrows) and one macrophage (arrow head) positive to MAC 387 antibody are visible; epithelioid cells (curved arrows) were not positive to the same antibody, bar = 10 μm. (f) Within the granuloma, mast cells (arrows) inside a vessel lumen (asterisk) and a few macrophage aggregates (arrow heads) display a positive reaction to MAC 387 antibody, bar = 10 μm.

Fig. 3. Confocal Laser Scanning Microscope images of the intestine of *Anguilla anguilla* infected with *Contracaecum rudolphii* A larvae. Each rows represent the same microscopic field excitated at 488 nm (first column), at 540 nm (second column), and with the two superimposed images (third column). (a) Granuloma in sub-serosa position with numerous macrophages and mast cells immunoreactive to the MAC 387 monoclonal antibody (green fluorescence), and some of them

positive to PCNA antibody (red fluorescence), bar = 100 μm (b) *C. rudolphii* A larva in sub-epithelial position with macrophages and mast cells positive to the MAC 387 antibody (green fluorescence), and several cells immunofluororeactive to PCNA antibody (red fluorescence), bar = 200 μm.

Fig. 4. Confocal Laser Scanning Microscope images of the intestine of *Anguilla anguilla* infected with *Contracaecum rudolphii* A larvae. Cells immunofluororeactive to the MAC 387 antibody (green fluorescence) and cells positive to PCNA antibody (red fluorescence). The bottom image shows the co-localisation of the two antibodies within the same mast cells (arrows) and presumably one macrophage (arrow head), bar = 50 μm.

Fig. 5. Transmission electron microscopy of granulomas around parasite larvae. (a) Outer layer of granuloma, two macrophages (thick arrows), collagen bundles (curved arrows) and mast cells (arrows) are visible, bar = 3.2 μm. (b) Inside the outer layer a big capillary with a mast cell (thick arrow) inside the lumen and mast cells (arrows) around the vessel. Curved arrow shows collagen bundles, bar = 3.2 μm. (c) Middle layer contains several mast cells (arrows), note their eccentric nuclei and numerous electron-dense granules in cytoplasm; fibroblasts (curved arrows) are scattered among the mast cells, bar = 3.2 μm. (d) Inner layer of granuloma, two dark flattened epithelioid cells (arrows) near a fibroblast (curved arrow) can be seen, bar = 1.1 μm. (e) Micrograph shows the occurrence of desmosomes (arrows) and interdigitation between non degenerated epithelioid cells, bar = 0.2 μm.

Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1. Numbers of yellow and silver eels examined and infected in each of the eleven sampling occasions.

Figure S1. A representative gel displaying the RFLP profiles following the digestion of the ITS amplicons with restriction endonuclease Tsp509I and of the rrnS amplicons with restriction endonuclease RsaI compared with a 100bp DNA ladder.