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Cell types and structures involved in tench, *Tinca tinca* (L.), defence mechanisms against a systemic digenean infection

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Abstract

Histopathological and ultrastructural investigations were conducted on 36 tench, *Tinca tinca* (L.), from Lake Trasimeno (Italy). The gills, intestine, liver, spleen, kidney and heart of 21 individuals were found to harbour an extensive infection of larvae of an unidentified digenean trematode. The eyes, gonads, swim bladder and muscles were uninfected. The parasites in each tissue type were embedded in a granulomatous proliferation of tissue, forming a reactive fibroconnective capsule around each larva. Most of the encysted larvae were metacercariae, in a degenerative state, but on occasion some cercariae were found. Many of the granulomas were either necrotic or had a calcified core. Within the granuloma of each, the occurrence of granulocytes, macrophages, rodlet cells and pigment-bearing macrophage aggregates was observed. Hearts bore the highest parasitic infection. Whilst the presence of metacercariae within the intestine was found positioned between the submucosa and muscle layers, metacercariae in the liver were commonly found encysted on its surface where the hepatocytes in close contact with the granuloma were observed to have electron-lucent vesicles within their cytoplasm. Metacercariae encysting adjacent to the cartilaginous rods of gill filaments were seen to elicit a proliferation of the cartilage from the perichondrium. Rodlet cells, neutrophils and mast cells were frequently observed in close proximity to, and within, infected gill capillaries. Given the degenerated state of most granulomas, a morphology-based identification of the enclosed digeneans was not possible.

Keywords: fish infection, granuloma, helminth larvae, immune cells, inflammation.

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Introduction

Despite the popularity of tench, *Tinca tinca* (L.), as trash fish for angling and its' wide geographic range, the pathological effects their helminth parasites exert have received very little attention. The life cycles of several digeneans are complex, with those of most species requiring two intermediate hosts and a definitive host (Poulin & Cribb 2002). In fish, which act as the second intermediate host for several digenean families, the metacercariae are found to encyst in a range of different body organs (see Olson & Pierce 1997; Wang, Yao & Nie 2001; Ogawa, Nakatsugawa & Yasuzaki 2004).

Following infection, the extent of the subsequent host reaction can vary considerably. Typically, a fibrous capsule of collagen, associated with host fibroblasts, is produced surrounding the invading parasite with, according to some authors, little, if any, host immune response (Stein & Lumsden 1971; Galaktionov *et al.* 1997). There is a widely held belief that the main function of the host immune system is to protect the organism against infection in order to minimize the fitness costs of being infected (Rohlenová *et al.* 2011).

The current study sets out to detail the cellular inflammatory reaction launched by tench in

Journal of Fish Diseases

response to an infection of digenean larvae within the gills and several visceral organs. The intense inflammatory response documented involves mast cells (MCs), neutrophils, macrophage aggregates (MAs), macrophages, epithelioid cells, collagen fibres and rodlet cells (RCs).

Mast cells, also referred to as eosinophilic granular cells (Reite & Evensen 2006), in fish are reported to occur at the sites of inflammation caused by parasitic infection (Reite & Evensen 2006; Dezfuli *et al.* 2009). MCs degranulate when exposed to a variety of known degranulation agents (Manera *et al.* 2011) and pathogens (Dezfuli & Giari 2008).

Neutrophils are the first cell type recruited to the site of an acute inflammatory response (Mollinedo, Borregaard & Boxer 1999), and their chemotaxis, phagocytosis and destruction of intracellular and extracellular pathogens demonstrate their important role in innate immunity against pathogens and parasitic infection (Secombes 1996; Stakauskas *et al.* 2007).

MAs or melanomacrophage centres may also be found within tissue responses encapsulating many foreign bodies and parasites (Ferguson 2006). MAs form from aggregations of pigment-containing cells (for a review, see Agius & Roberts 2003), and their functions have been reported to include the focal destruction, detoxification and recycling of endogenous and exogenous materials (Ellis 1980; Vigliano *et al.* 2006).

Rodlet cells are characterized by a thick subplasmalemmar fibrillar capsule, by a basal nucleus and by the presence of conspicuous rodlets (for a review, see Manera & Dezfuli 2004). Evidence for the possible function of these cells, as immune cells, results from their reported increase in fish that are infected with parasites (Reite & Evensen 2006; Matisz, Goater & Bray 2010).

Certain cells, which are involved in the chronic inflammatory response of teleost fish, have many features that are typical of mammalian epithelioid cells (Noga, Dykstra & Wright 1989; Ferguson 2006). In the presence of a foreign antigen, this cell type becomes activated and avidly phagocytic (Johnson 1988).

Materials and methods

In September 2011, 36 specimens of tench from Lake Trasimeno (Province of Perugia, Umbria Region, Central Italy) were provided by the Trasimeno Fish Consortium. The fish were transferred alive to the Consortium's facility and killed using 125 mg L^{-1} MS222 (tricaine methanesulfonate, Sandoz), and their spinal cords severed before being lengthed, 41.85 ± 12.24 cm (mean total length ± SD) and weighed, 1463.6 ± 816.2 g (mean weight ± SD). The tench were dissected and sexed before the internal organs and gill filaments from each were removed and screened for parasitic infection. From each infected organ, a 15 × 15 mm piece of tissue was excised and then fixed in either chilled (4 °C) Bouin's or in 10% neutral buffered formalin for 24 h. The samples were then processed routinely for paraffin embedding, sectioned (5 μm thick) and then stained with haematoxylin and eosin and/or Alcian Blue 8 GX pH 2.5 and periodic acid Schiff's (AB/PAS) stains. Multiple histological sections were taken from each tissue block, examined and photographed using an optical microscope (Nikon Eclipse 80i).

For transmission electron microscopy (TEM), 7 × 7 mm pieces of tissue from infected organs were fixed in chilled 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 3 h. The fixed tissues were then 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). Semi-thin sections (1.5 μm) were cut on a Reichert Om U 2 ultra microtome (Reichert-Jung) and stained with toluidine blue. Ultrathin sections (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. For both light and transmission electron microscopy, corresponding pieces of organs from uninfected tench were also processed for comparison with the parasitized organ.

In addition, 5 × 5 mm pieces of tissue were excised from the infected organs and then fixed in 90% ethanol for DNA extraction, PCR and sequencing.

Results

Light microscopy

Of the 36 tench that were examined, the gills and viscera (*i.e.* intestine, liver, spleen, kidney, heart) of 21 fish (58.3%) were found to be infected with larvae of an unidentified digenean trematode. No digenean larvae were encountered in the eyes, gonads,

swim bladder and muscles of tench. In all the infected organs, a granulomatous inflammatory tissue surrounded the parasite larvae which varied markedly in size and structural organization (see below for the electron microscopy description). The vast majority of granulomas contained necrotic material or a calcified core; as such, the enclosed parasite stages were in a degenerated state with no discernible taxonomic features upon which to base a definitive identification. In a small proportion of granulomas, general metacercarian features were evident and in a few granulomas, cercarial-like stages were seen. Whilst suggestions as to the possible digenean species involved (e.g. *Asymphylodora tincae* (Modeer, 1790)) can be made by cross-referencing host and geographic location details contained in open-source, online parasite databases (i.e. http://www.nhm.ac.uk/research-curation/research/projects/host-parasites/database/index.jsp), a definitive identification, as determined by base sequencing of informative genomic regions, is preferred. Although a parallel molecular study is ongoing, no definitive identification has, as yet, been possible.

In the following sections, the host reaction within each tissue to the parasitic infection is described.

Gills. Figure 1a shows the gill filaments of an uninfected tench, whilst those presented in Fig. 1(b) shows that the presence of encysted metacercariae adjacent to the cartilaginous rods of the gill filaments elicits a proliferation of the cartilage from the perichondrium. In all the infected tench that were examined, the gill cartilage harboured the highest number of larvae that were found in the gills (Fig. 1c). In a few cases, hyperplasia of the gill epithelium and fusion of the lamellae were seen in filaments that were close to the sites of metacercarial infection. Frequently, the metacercariae induced detachment of the gill filaments from the cartilaginous rods (Fig. 1b). Numerous RCs were commonly observed inside the efferent blood vessels of infected gills (Fig. 1d).

Heart. This was the most heavily parasitized organ. Of the 21 parasitized hearts, both the bulbus arteriosus (Fig. 1e) and the ventricle (Fig. 1f) harboured single or aggregations of larvae. RCs were the dominant cells found in the heart, and those encountered in the ventricle either partially or completely surround the larvae (Fig. 1g). Some of the granulomas seen within the heart contained necrotic tissue and/or a calcified core (Fig. 1g). RCs were also found in abundance in tissues located at a distance away from the sites of infection (Fig. 1h). RCs were found to co-occur with MCs and neutrophils, whilst the number of the granulocytes recorded was remarkably low. Macrophages were also commonly encountered within the vicinity of metacercariae; these cells, which possessed indefinable inclusions, appeared large in comparison with the other cell types that were observed (see the descriptions provided in the electron microscopy section).

Intestine. Larvae were observed in the submucosal and muscle layers (Fig. 2a) and within the thickness of the serosa where they induced a hyperplastic response (not shown). MAs, some RCs, MCs and neutrophils were seen in close proximity to the metacercariae; most notably, numerous MAs were seen in the muscle layer (Fig. 2a). Again, granulomas with necrotic and/or calcified centres were seen (Fig. 2a, right side of the photograph).

Liver. Metacercariae were observed throughout the liver (Fig. 2b), and not only on the surface (Fig. 2c). MAs were frequently observed in intimate contact with parasites (Fig. 2d). MCs and RCs were observed in infected tissues, and the hepatocytes close to the metacercariae had electronlucent vesicles within their cytoplasm (see below).

Spleen. The spleen had a lower number of larvae compared to those observed in the other visceral organs. The larvae were seen in both the deeper tissues (Fig. 2e) and on the surface of the organ (Fig. 2f). MAs were occasionally observed in contact with larvae (Fig. 2f), and RCs (Fig. 2f), neutrophils and MCs were all found to co-occur within the vessels of infected splenic tissue.

Kidney. The pathology and cellular responses displayed by infected kidneys were similar to those of the spleen. Parasite larvae were found throughout the kidney (Fig. 2g), as well as close to (Fig. 2h) or on the surface. MAs were commonly observed in contact with the metacercariae (Fig. 2h); MCs and neutrophils were notably abundant throughout infected kidneys (see below). Again, granulomas containing necrotic or calcified material were seen (Fig. 2g).

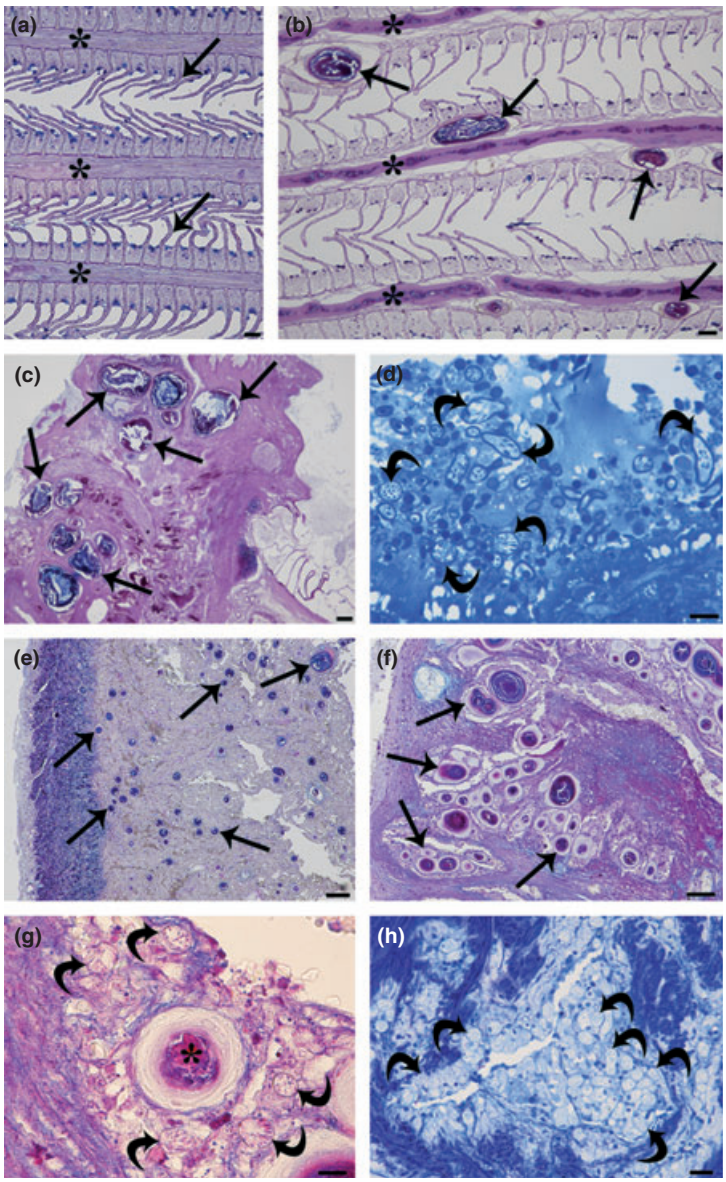

Figure 1 (a) Uninfected tench gills. The micrograph shows the typical appearance of normal lamellae (arrows) arising from a single filament with evident gill rods (asterisks), scale bar = 50 μm. (b) Metacercarial cysts (arrows) lie adjacent to the gill rods (asterisks) and to filaments detached from the gill rods, scale bar = 50 μm. (c) Several metacercariae (arrows) are visible inside the gill cartilage, scale bar = 50 μm. (d) Numerous RCs (curved arrows) inside the efferent blood vessel of an infected gill, scale bar = 10 μm. (e) A high number of metacercariae (arrows) can be seen inside the bulbus arteriosus of an infected heart, scale bar = 200 μm. (f) In the ventricle, aggregations of metacercariae (arrows) can be seen, scale bar = 100 μm. (g) Numerous RCs (curved arrows) encircling a metacercaria (asterisk) encysted within the ventricle, scale bar = 10 μm. (h) Part of the ventricle lumen in an infected tench is filled by a high number of RCs (curved arrows), scale bar = 20 μm.

Electron microscopy

Electron microscopy was used to detail the cellular responses elicited by the tench in response to the larval infection. 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 granulomas were composed of collagenous fibres with different types of immune cells scattered among them. Neutrophils appeared as large polymorphic cells with an irregular outline and spherical nuclei; the cytoplasm of each neutrophil was rich in elongated granules with lamellar, electron-dense cores (Fig. 4g). Within the cytoplasm, fragments of rough endoplasmic reticulum and a few mitochondria were evident. With regard to the MCs, they were voluminous and irregular in shape, the nucleus was eccentric, and the cytoplasm contained numerous, large, membrane-bound granules with a homogeneous, electron-dense content (Fig. 3a). The cytoplasm had an inconspicuous Golgi apparatus and very few, round mitochondria.

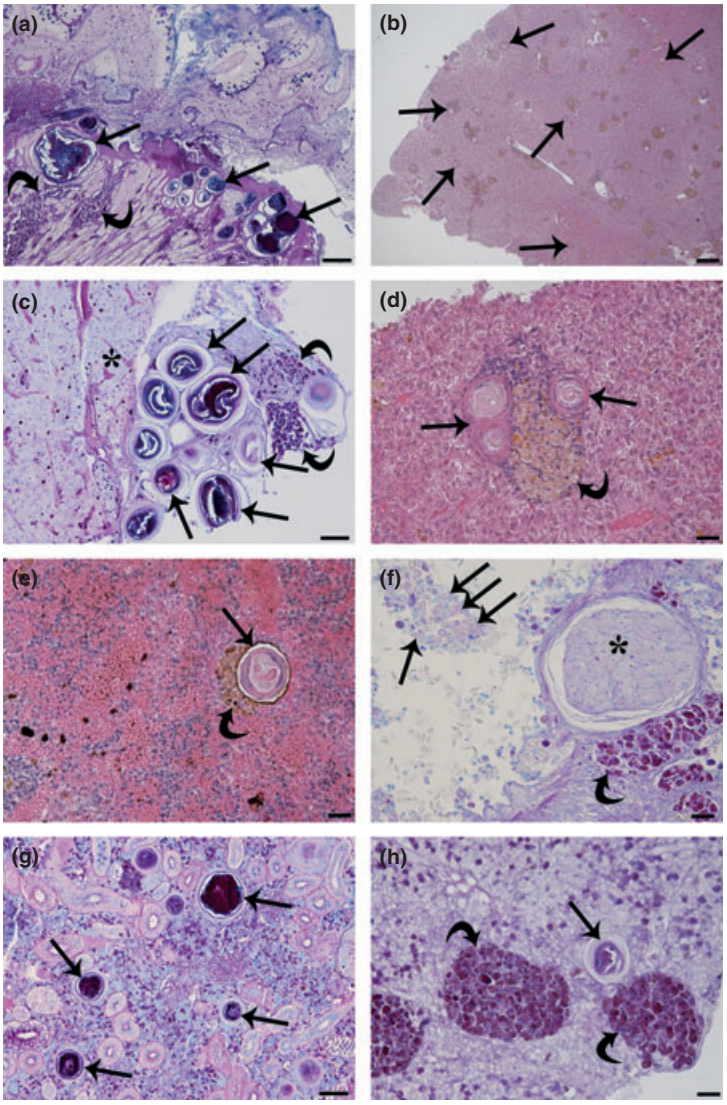

Figure 2 (a) Metacercariae (arrows) encysted in the muscle layer of the tench intestine; MAs (curved arrows) in close vicinity to the parasite can be seen, scale bar = 100 μm. (b) Histological section through an infected liver showing metacercariae (arrows) embedded within the tissue, scale bar = 200 μm. (c) Metacercariae (arrows) encysted on the surface of the liver (asterisk); MAs (curved arrows) in close proximity to the parasites are evident, scale bar = 50 μm. (d) Three metacercariae (arrows) in close contact with a hepatic MA (curved arrow), scale bar = 20 μm. (e) Histological section through an infected spleen showing the deep position of a metacercaria (arrow) encysted within. It can be seen that the parasite is in close contact with a MA (curved arrow), scale bar = 20 μm. (f) A parasite (arrow) embedded on the surface of the spleen with a MA (curved arrow) in contact with the external surface of the parasite. Several RCs (circle) inside the vessel can be seen, scale bar = 20 μm. (g) Histological section of an infected kidney with encysted metacercariae (arrows), scale bar = 50 μm. (h) A metacercaria (arrow) and two MAs (curved arrows) in close proximity to one another, scale bar = 20 μm.

The RCs that were seen were large in size, oval and contained several typical club-shaped formations ('rodlets'), part of which had a crystalline core. The cytoplasm of the RCs was vesicular and foamy (Fig. 3b,c), with no recognizable organelles within. The macrophages, by comparison, appeared large (Fig. 4d) and contained vesicular structures with electron-opaque contents (dense bodies) and electron-lucent vesicles.

Gills. Digenean larvae within the gills were surrounded by host neutrophils, MCs (Fig. 3a) and RCs (Fig. 3b). Numerous RCs, some of which were discharging their contents, were seen within the lumen of the efferent vessels of infected fish (Fig. 3b).

Heart. RCs were the dominant cells seen in association with larvae in the ventricle (Figure 3c), although RCs were also seen further away within the lumen of the ventricle where they were found to co-occur with MCs (Fig. 3d). Several RCs were observed in the process of discharging their contents. Macrophages were also present, frequently in close proximity to the metacercariae or, as seen in several instances, in contact with the external surface of a granuloma. These cells appeared large in size with indefinable electron-opaque inclusions (not shown).

Intestine. Metacercariae within the submucosal layer were seen surrounded by MCs, neutrophils and RCs (Fig. 3e), where interestingly in some

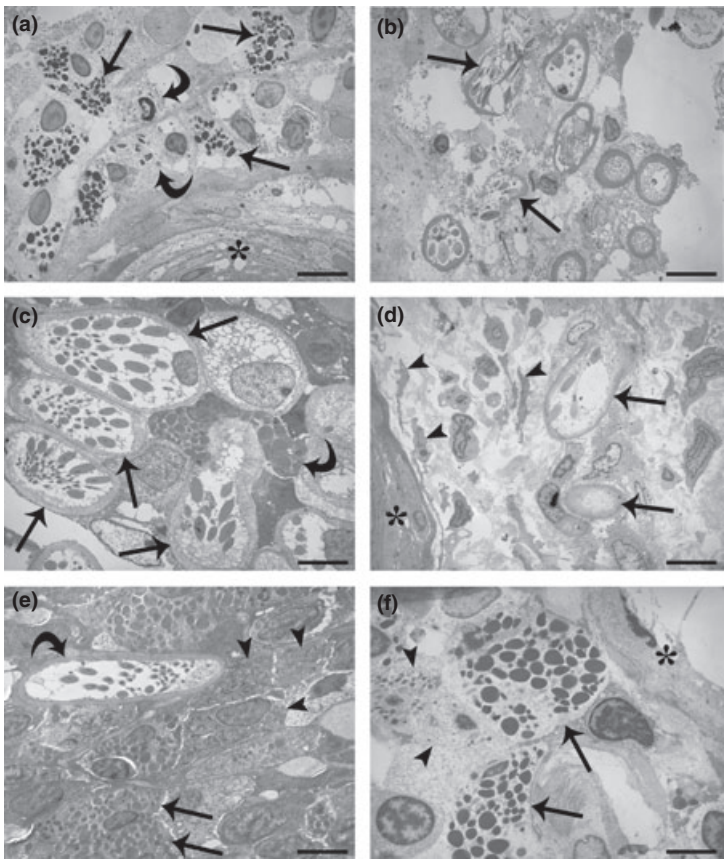

Figure 3 (a) Transmission electron micrograph of an infected tench gill showing a metacercaria (asterisk) surrounded by host MCs (arrows) and neutrophils (curved arrows), scale bar = 5.4 μm. (b) RCs within the lumen of an efferent vessel of an infected gill, where two RCs (arrows) are discharging their contents, scale bar = 4.8 μm. (c) RCs (arrows) and a MC (curved arrow) within an infected heart; a thick capsule, basal nucleus and several rodlets in the cytoplasm of the RCs are evident, scale bar = 3.9 μm. (d) A metacercaria (asterisk) in the heart of a tench surrounded by fibres (arrow heads) and RCs (arrows), scale bar = 4.5 μm. (e) Within the submucosal layer of an infected intestine, the co-occurrence of MCs (arrows), neutrophils (arrow heads) and RC (curved arrow) was frequently observed, scale bar = 2.8 μm. (f) An infected intestine where two MCs (arrows), one of which has adhered to the external surface of the parasite (asterisk), and neutrophils (arrow heads), are evident. Note the cytoplasm of the MCs is filled with numerous large, electron-dense, membrane-bound granules, scale bar = 2.9 μm.

TEM grids, the MCs were observed in contact with surface of the granuloma (Fig. 3f). MCs were also found within the submucosa, in close proximity to blood vessels and/or within the capillaries. Intense degranulation of MCs was encountered mainly within the submucosa; in some instances, electron-dense granules were swollen and were scattered among the fibroblast-like unsheathing cells and collagen fibres.

Liver. Numerous MCs and some neutrophils were observed in close proximity to granulomas (Fig. 4a). The hepatocytes close to the parasites frequently had electron-lucent vesicles (Fig. 4b).

Spleen. MCs, neutrophils and RCs (Fig. 4c) were observed in infected specimens; RCs were abundant within the lumens of vessels and frequently in the process of discharging their contents. Macrophages were found in close proximity to encysted larvae (Fig. 4d) and, in some instances, seen adhering to the surface of the granuloma.

Kidney. In the kidney, metacercariae were commonly found encircled by a range of host immune cells (Fig. 4e), with MCs and neutrophils being the most frequently encountered (Fig. 4f). The core of some granulomas contained necrotic or calcified tissue (Fig. 4e). Occasionally, flattened neutrophils adhering to the external surface of granuloma (Fig. 4g) were observed. Numerous desmosomes were seen between adjacent epithelioid cells (Fig. 4g).

Discussion

In the current study, a large number of unidentified digenean larvae were found encapsulated within the gills and viscera of an Italian population of tench. The tench cellular inflammatory reactions reported within each infected organ were found to be varied and to include the notable presence of either epithelioid cells, macrophages, MAs, MCs, neutrophils, RCs or fibres surrounding the parasitic larvae.

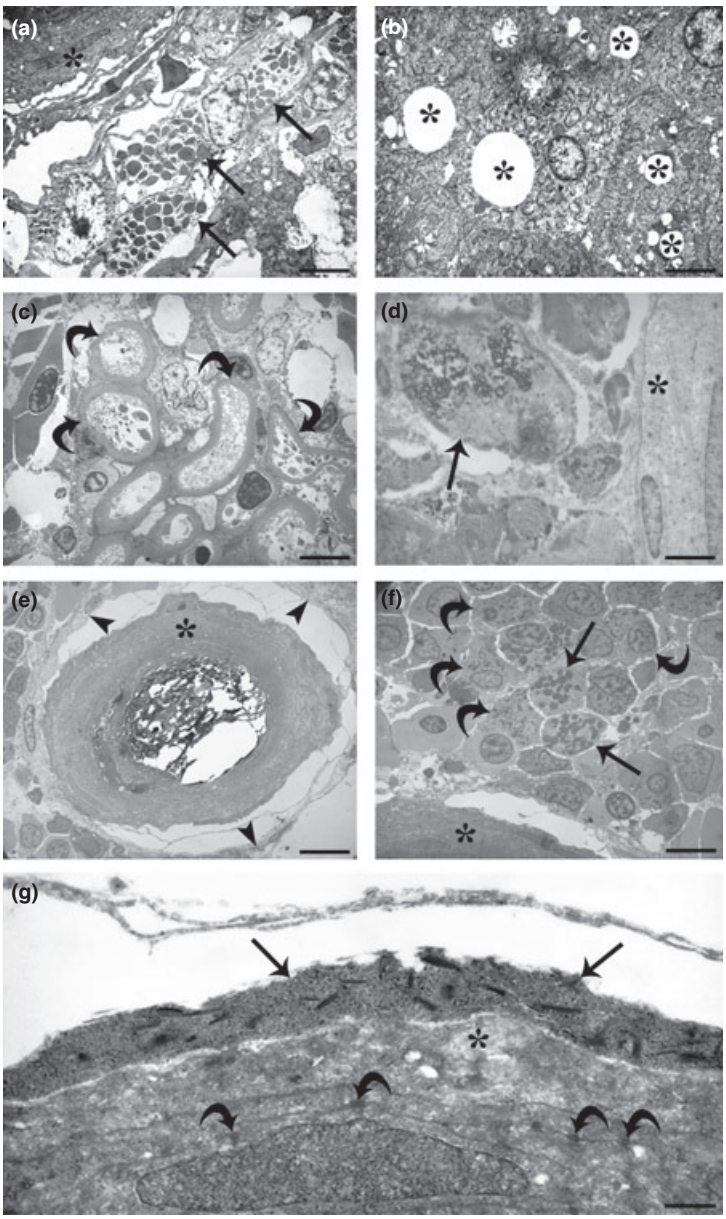

Figure 4 (a) Metacercaria (asterisk) encircled by MCs (arrows) in the infected liver of a tench, scale bar = 3.4 μm. (b) Electron-lucent vesicles (arrows) within the cytoplasm of hepatocytes of an infected liver were commonly seen, scale bar = 2.7 μm. (c) Micrograph shows the lumen of a vessel within a parasitized spleen, in which RCs (curved arrows) were abundant, scale bar = 4.4 μm. (d) Within an infected spleen, a macrophage (arrow) can be seen in close vicinity to the tegument of the parasite (asterisk), scale bar = 1.4 μm. (e) Transverse section through a metacercaria (asterisk) within the kidney; host cells (arrow heads) can be seen encircling the larva, scale bar = 5.6 μm. (f) Numerous MCs (arrows) and neutrophils (curved arrows) encircling a metacercaria (asterisk), scale bar = 5.1 μm. (g) High magnification of a section taken through an infected kidney where a flattened neutrophil (arrows) attached to the external surface of a granuloma (asterisk) can be seen, note numerous desmosomes (curved arrows) between adjacent epithelioid cells, scale bar = 0.6 μm.

Larval migration of digeneans and encapsulation within the viscera and body tissues of fish (Olson & Pierce 1997; Sitjà-Bobadilla 2008; Matisz *et al.* 2010) generally induces the development of fibrogranulomatous lesions (Balouet & Baudin-Laurencin 1986). Histologically, fish granulomas are inflammatory foci composed of concentric layers of epithelioid cells, and are very similar to mammalian granulomas (Noga *et al.* 1989; Roberts 2012). Epithelioid cells are so-called because of their morphological similarity to epithelial cells (Cotran, Kumar & Collins 1999). It is assumed that these epithelioid cells are typically transformed macrophages, which have the principal role of engulfing foreign agents (Ferguson 2006). In each metacercariae-infected organ examined in the current study, the parasites were found encircled by epithelioid cells. Numerous macrophages were found within the hearts of infected tench and, in some instances, were seen adhering to the external surface of the larvae. Almost all the macrophages that were encountered possessed vesicular structures with electron-opaque contents (dense

bodies) and electron-lucent vesicles, suggesting that they were actively engulfing particles.

A prominent feature of chronic inflammation is the presence of melanin and/or other pigment-containing macrophages, also known as MAs (Agius & Roberts 2003). In the present study, MAs were predominantly seen in close proximity to metacercariae in the liver, spleen and kidney; these results are in agreement with the active role of these centres in retaining resistant pathogens such as parasitic spores or bacteria, and in antigen processing during the immune response (Agius & Roberts 2003).

Mast cells in fish are reported to occur at sites of inflammation that are induced by parasitic infection (Reite & Evensen 2006; Dezfuli *et al.* 2009). The presence of MCs is closely linked to the ability to rapidly recruit neutrophils and macrophages to the site of infection (Lamas *et al.* 1991; Secombes 1996). Several records document the degranulation of MCs following their exposure to a variety of known degranulation agents (Manera *et al.* 2011) and pathogens (Dezfuli & Giari 2008). In infected tench, the degranulation of MCs was frequently observed within the connective tissue surrounding capillaries and within the blood vessels of the spleen, intestine and gills.

According to Roberts (2012), when a common acute inflammatory response is elicited, it is characterized by the presence of neutrophils and monocytes in the blood and by an accumulation of neutrophils and macrophages at the site of infection. Several researchers have reported that instead of degranulating, neutrophils have been seen tightly bound to the surface of the parasite (Butterworth 1984). The latter was seen in the current study, with the neutrophils present in certain infected organs binding tightly to the surface of the granulomas with no evident degranulation.

Numerous RCs were observed within the gills, spleens and hearts of infected tench, where interestingly, an intense migration of RCs was seen within the circulatory system of each organ. The finding of a high number and recruitment of RCs provides further evidence regarding their potential function and lends additional support to the findings from the observational studies on helminth–fish interactions (Reite 2005; Matisz *et al.* 2010).

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