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Infectious disease

White spots amidst the gold: ultrastructural and histological aspects of the chronic inflammatory response of goldfish with ichthyophthiriasis

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ABSTRACT

Ichthyophthirius multifiliis, the causative agent of white spot disease, is a ciliated protozoan parasite that infects freshwater fish and induces high mortality. Outbreaks occur both in natural and production sites. The aim of the present study was to describe the lesions caused by chronic infection by *I. multifiliis* in goldfish (*Carassius auratus*) from an ornamental fish farm, highlighting important ultrastructural aspects of this protozoan. Damaged skin and gills, collected from fish with white or ulcerative skin lesions, were routinely processed for histological analysis and transmission electron microscopy. The parasitic forms present in the skin were associated with an inflammatory infiltrate consisting of macrophages, lymphocytes and other polymorphonuclear cells. The lesions associated with the presence of the parasite were organized in the form of granulomas, with macrophages in the layers closest to the parasites. A trophont-thickened membrane and induction of granulomatous inflammation were identified in this study as mechanisms for evasion of the immune response. We concluded that the presence of *I. multifiliis* trophonts resulted in the formation of granulomatous inflammation, whether associated or not with pathogen lysis, suggesting that the parasite can use an inflammatory response to evade the immune response.

Ichthyophthirius multifiliis is one of the most common protozoan pathogens of freshwater fish. It has a significant economic impact on aquaculture, due to its low host specificity and high morbidity and mortality [1], causing outbreaks both in natural habitats [2] and in fish farms and leading to millions of dollars in losses [3]. This protozoan has three life stages, all ciliated: theront, trophont and tomont [4]. The theront infectious form swims freely, reaching a host, where it penetrates through the skin or gills. On entry, it quickly develops into a feeding trophont. This parasitic form spends several days in the host, depending on environmental conditions, until it reaches maturity. Next, it leaves the host, now as a tomont, and swims for about 1 h before adhering to a substrate, where it forms a cyst. Within this cyst, tomites are formed resulting in the infecting theronts starting a new cycle [5].

The trophont is found within the epidermis and gills of fish, where it may reach up to 1 mm in diameter and is apparent as a characteristic white spot, which is why the condition is called 'white spot disease' [4]. The physical integrity of the epithelium is compromised by invasion of the trophonts. However, it is the inflammatory response subsequent to the invasion that characterizes the lesions caused by this parasite [6]. Our knowledge of immune responses against fish parasites has greatly increased in recent years, but many questions regarding parasite infections remain unanswered [7]. The formation of granulomas determines the encapsulation and isolation of parasitic stages by epithelioid cells and connective tissue, yet the pathophysiological response can be part of the sometimes fatal manifestation of the disease [8]. However, there is a lack of descriptions of the chronic inflammatory response against this parasite, which could help in better understanding the pathogenesis of this infection. In addition, comprehending the relationship between host response and parasite evasion mechanisms could encourage adoption of measures to prevent these pathogens.

The market for ornamental fish is steadily increasing worldwide, leading to increasing production each year [9], and is a significant source of income in some countries [10]. One of the most common

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genera of ornamental fish is *Carassius* spp, especially *Carassius auratus* (goldfish), with thousands of tons being produced annually through fish farming [11]. However, due to the conditions that the fish are subjected to, this form of production can lead to the emergence of various diseases, including parasitic diseases, which benefit from a variety of abiotic and biotic factors [12]. *Ichthyophthirius multifiliis* causes disease outbreaks in goldfish [13] and, therefore, understanding the pathogenesis of this disease could lead to improved management practices to reduce economic losses caused by this protozoan.

In this study, which aimed to better understand the pathogenesis of the chronic inflammatory response against *I. multifiliis* infection in goldfish, ultrastructural and histological aspects of *I. multifiliis* inflammatory host cells were investigated using light microscopy and transmission electron microscopy (TEM).

Specimens of goldfish were obtained from an ornamental fish farm in Guararema, São Paulo State, Brazil, where several fish species were growing together, separated only according to age. Fish of all ages (n = 30) with white or ulcerative skin lesions were collected and transported following standard procedures to the Molecular and Cellular Biology Laboratory of Paulista University. The fish were euthanized by immersion in a solution of 150 mg/L tricaine methanesulfonate (MS-222; Sigma, www.sigmaaldridge.com) until paralysis of the operculum [14]. The fish in this aquatic facility were maintained under the guidelines of the Brazilian National Council for Control of Animal Experimentation and all

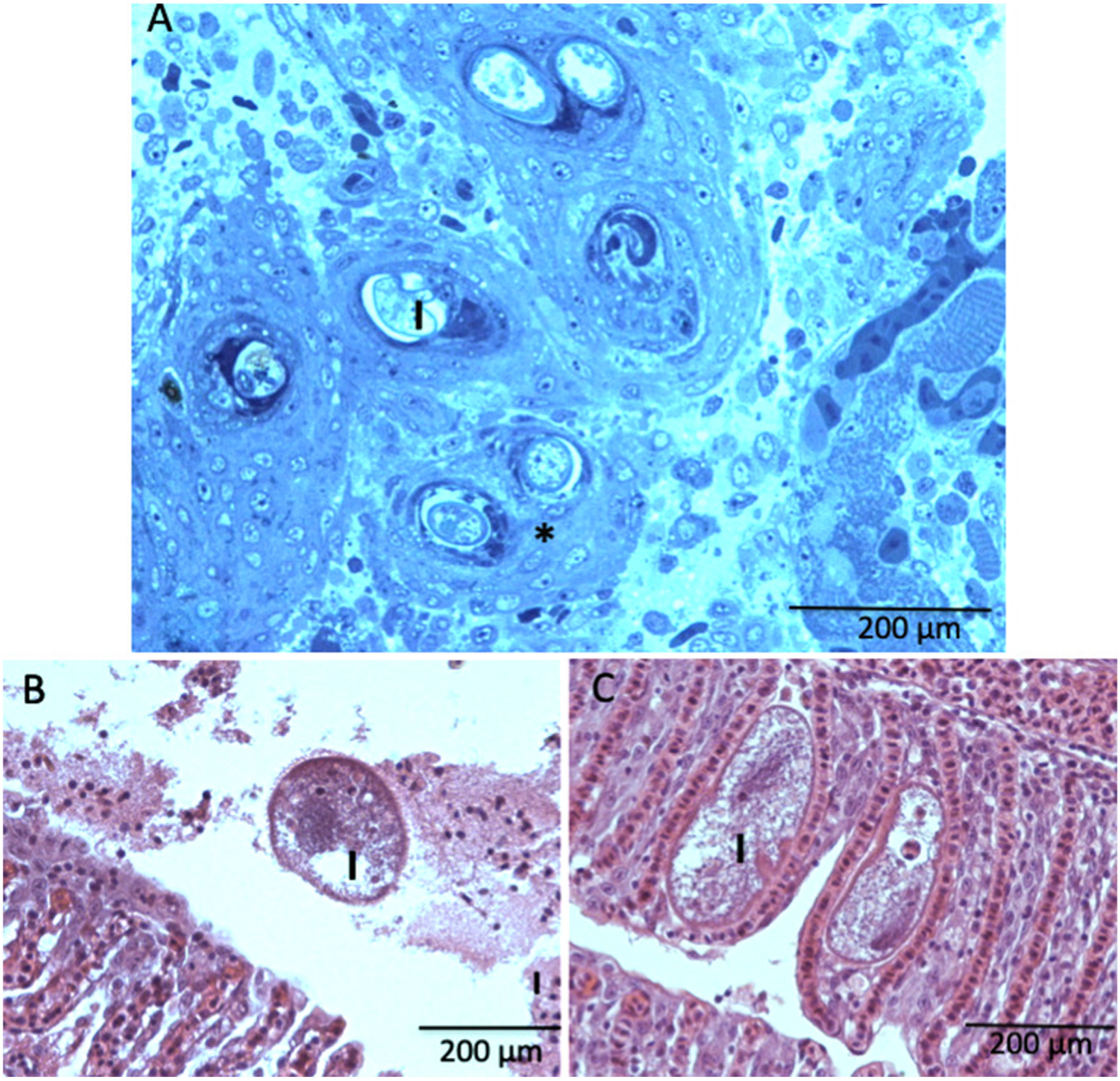

Fig. 1. *Ichthyophthirius multifiliis* infection, skin and gills, goldfish. (**A**) Granulomatous inflammation (*) around trophonts of *I. multifiliis* (I). Toluidine blue. (**B**) Free trophonts of *I. multifiliis* (I) in gill. HE. (**C**) Extensive proliferation of gill epithelium around invading parasites (I) and total lamellar fusion of secondary lamellae and blood vessels. HE.

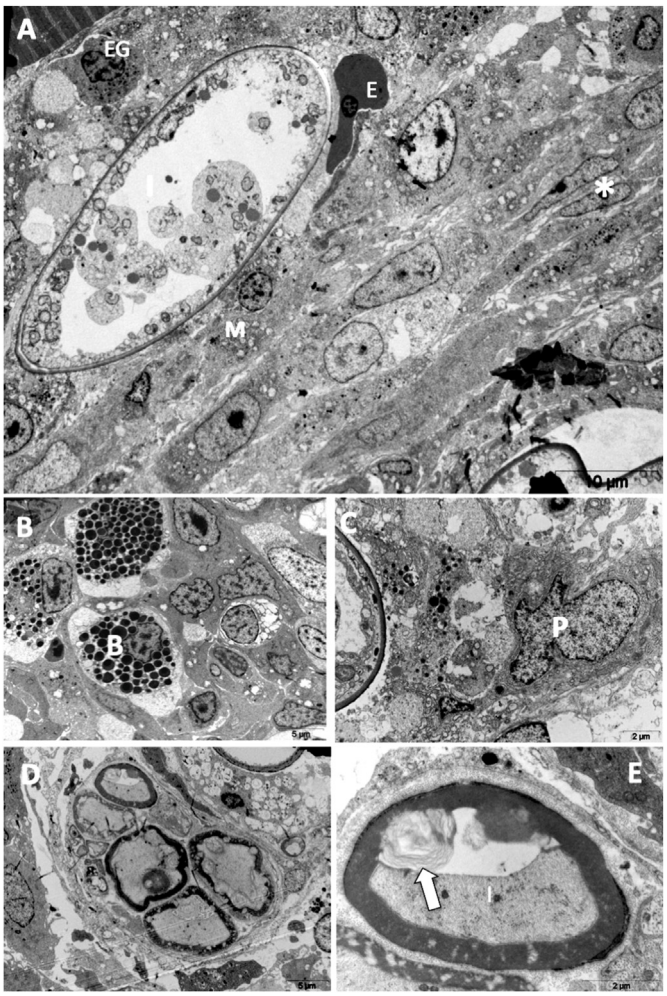

Fig. 2. *Ichthyophthirius multifiliis* infection, skin, goldfish. (**A**) Macrophages (M), eosinophilic granular cells (EG), erythrocytes (E) and reactive stromal cells (*) surrounding parasites (I). Bar, 10 µm. (**B**) Many infiltrated basophils (B). Bar, 5 µm. (**C**) Plasmocytes (P). Bar, 2 µm. (**D**) Parasites with evidence of lysis. Bar, 5 µm. (**E**) Membrane lysis with myelin figures (arrow) and absence of identifiable internal structures in parasite (I). Bar, 2 µm. TEM.

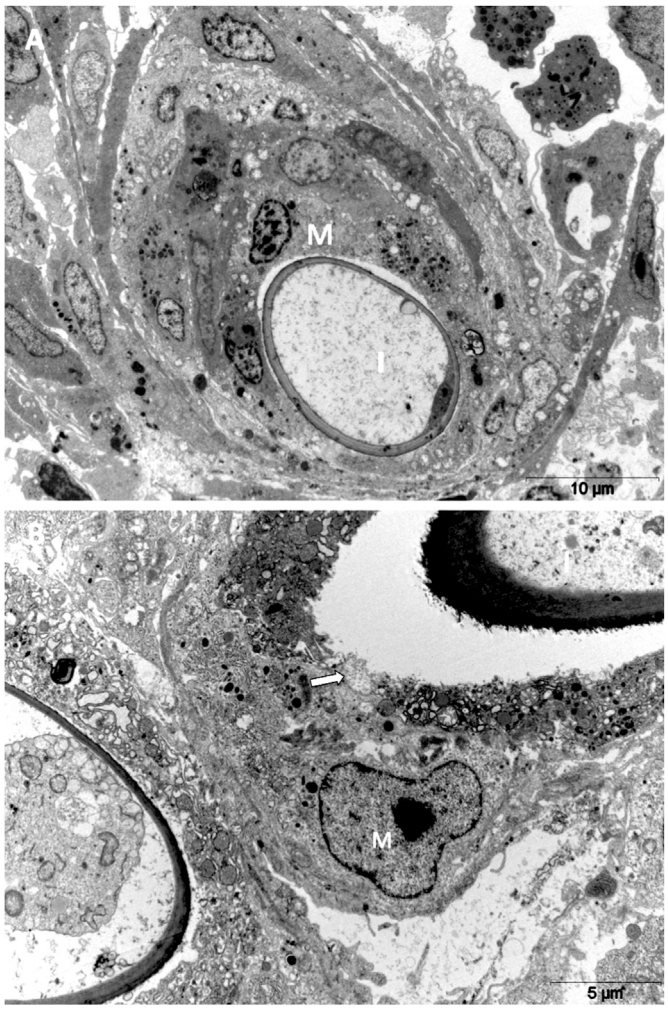

Fig. 3. *Ichthyophthirius multifiliis* infection, skin, goldfish. (**A**) Granulomas contain macrophages (M) in inner layers around parasites (I). (**B**) Released lysosomal content (arrow) in parasitic vacuole. TEM.

procedures were approved by the ethics committee of Paulista University (No. 1632015). Damaged skin and gills were fixed in Bouin's solution for 8 h and kept in 70% alcohol until embedding in paraffin wax for histological analysis. Sections were cut and stained routinely with haematoxylin and eosin (HE) and Giemsa. For TEM, small samples of skin and gill lesions from 10 fish were fixed in 2% glutaraldehyde in 0.2M cacodylate buffer (pH 7.2) at 4°C for 10 h, post-fixed in 1% OsO_4, buffered for 2 h and routinely processed. The samples were dehydrated in a graded ethanol series and embedded in Epon 812 resin. Semithin sections were stained with toluidine blue and photographed under an optical microscope. Ultrathin sections were double stained with aqueous uranyl acetate and lead citrate and observed under a Zeiss EM 109 TEM (Zeiss Group, www.zeiss.com) operated at 80 kV.

The skin lesions were extensive and affected the musculature with the presence of *I. multifiliis* trophonts (Fig. 1A). The number of parasites was high and damaging to the gills, as indicated by the fusion of lamellae (Fig. 1B and C). In the gills, there were some areas in which presence of the parasite was associated with lesions and others in which the parasites were not associated with lesions, thus indicating recent infection (Fig. 1B and C). The tissue damage is caused by the perforatorium, an apical membrane that the parasite uses for penetration into epithelia [4]. The protozoan parasitizes skin and gills, causing systemic inflammatory responses [15,16]. In the present study the lesions produced by parasitic infection were associated with a chronic inflammatory process, with extensive tissue damage.

Various types of cells were identified in inflamed areas of skin, such as macrophages (Fig. 2A), eosinophilic granular cells (Fig. 2A), basophils (Fig. 2B), lymphocytes, plasmocytes (Fig. 2C) and neutrophils. Lesions associated with the presence of the parasite were organized in the form of granulomas, with macrophages in the inner layers around the parasites (Figs. 2A and 3A). Other leucocytes, such as basophils and eosinophilic granular cells, were located in the outermost layers of the granulomas (Fig. 2A). The inflammatory response frequently led to formation of granulomas, in which the parasite and its products are encapsulated, and this is one of the recognized mechanisms for immune evasion [17,18]. This mechanism was recognized in this study as many parasites involved in the granulomatous response had external and internal structural integrity, indicating that *I. multifiliis* uses this immune evasion mechanism to resist the host's defenses. The presence of granulomatous lesions indicates a more chronic pattern of infection, in contrast to acute inflammation in which neutrophils and eosinophilic granular cells predominate [19].

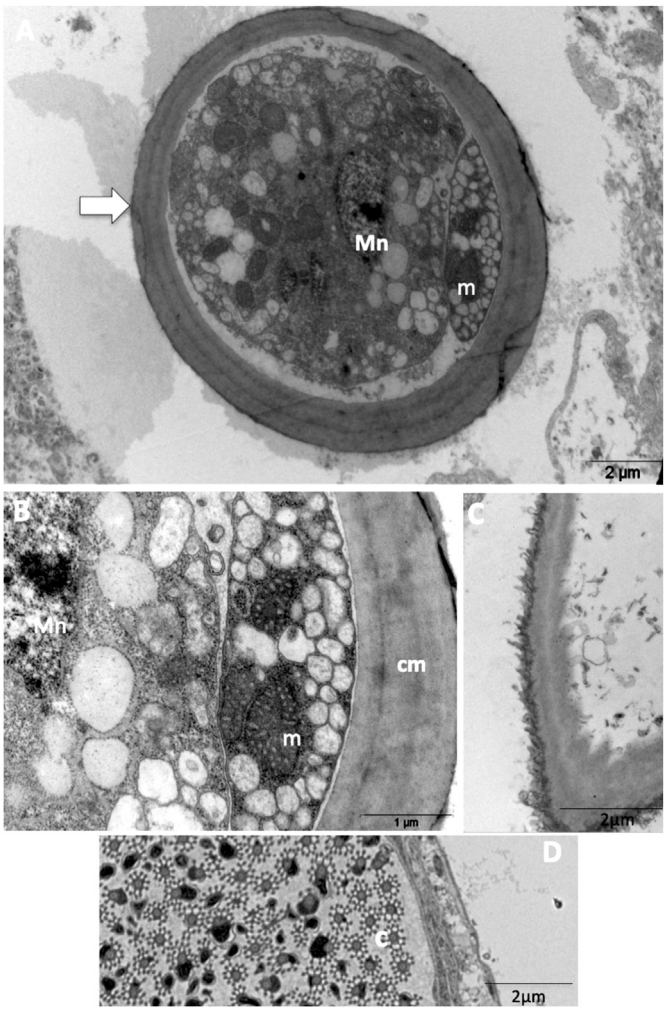

Fig. 4. *Ichthyophthirius multifiliis* infection, skin, goldfish. (**A**) Trophont in transverse section has thick cell membrane (arrow), macronuclei (Mn) and mitochondria (m). (**B**) Detail of thick cell membrane (cm), macronuclei (Mn) and mitochondria (m). Bar, 1 µm. (**C**) Numerous cilia (c) in membrane. Bar, 2 µm. (**D**) Section slightly oblique to surface of parasite. Basal bodies have ninefold symmetry of cilial microtubules (c). TEM.

Fins from fish have been used in studies to demonstrate involvement of the various macrophage activation pathways that are known in mammals [20]. M1 macrophages with a proinflammatory profile and high microbicidal capacity are activated by Th1 cytokines, whereas a more anti-inflammatory profile induced by Th2 cytokines is associated with M2 macrophages with repair activity and these cells are essential for host survival in situations of infections such as schistosomiasis [21]. In the present study, parasitic structures in some areas had clear evidence of hydrolysis in response to phagocytosis and lysosomal enzyme action (Fig. 2D and E) associated with vacuolation and formation of myelin figures in the parasite membrane. At this site, a lytic response compatible with and suggestive of proinflammatory activity was evident. However, in other areas, parasites with morphological integrity and without signs of lysis were present (Fig. 3B) indicating asynchronous parasitic developments as infections could occur at different times.

Ichthyophthirius multifiliis trophonts had various morphologies. They were mostly slightly flattened in the middle region and tapered towards the apical end, thus resulting in a spheroidal to pyriform shape with a somewhat pointed anterior tip (Fig. 3C), as described in previous studies on both *I. multifiliis* and *Cryptocaryon irritans*, a ciliate of marine fish [4]. The cell membrane of the trophonts was thick and highly electrondense (Figs. 3C and 4A).

The surface of the parasite has an undulating appearance associated with the presence of numerous cilia (Fig. 4B and C). The surface membrane system is composed of three membrane units [22]. In this study, two membrane structures were identified: one layer that was more continuously external to the cilia and another internal (alveolar) layer that was very thick and electrondense. These features indicate the nature of the parasite's physical protection mechanisms. As in other ciliates such as *Paramecium tetraurelia*, basal bodies had ninefold symmetric microtubule blades adjacent to the cell membrane [23]. Food vacuoles containing host cells or cell debris were present more frequently in mature trophonts. Mitochondria were uniformly spheroid and predominantly underlaid the plasmalemma (Figs. 3 and 4).

In this study the presence of *I. multifiliis* mobilized an inflammatory response that involved macrophages, lymphocytes and polymorphonuclear cells in the infected goldfish. The presence of a thickened membrane in trophonts and induction of granulomatous inflammation are characteristic features associated with evasion of the immune response. Further investigations to better understand the pathogenesis of ichthyophthiriasis are required to ensure improved control of the disease and, therefore, the consequent economic loss.

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Statement of author contributions

C.R.S. Moyses: Conceptualization; Methodology; Investigation; Data curation: Writing – original draft. **M.A. Lallo:** Conceptualization; Project administration; Funding acquisition; Supervision; Methodology; Investigation; Data curation; Writing – review & editing. **B.de L. Araújo, D.D. Spadacci-Morena, J.G. Xavier:** Investigation; Data curation; Review and editing. All the authors discussed the results and contributed to the final manuscript.

Declaration of competing interests

The authors declared no conflicts of interest relating to the research, authorship or publication of this article.

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