UNIVERSITY OF FLORENCE

PhD IN EVOLUTIONARY BIOLOGY AND ECOLOGY

CURRICULUM: ETOLOGY AND GENETICS

XXXVIII CYCLE

COORDINATOR: Prof. Duccio Cavalieri

IMMUNE CELLS IN TELEOST AND ELASMOBRANCH FISH, INFECTED AND UNINFECTED

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Academic Year 2022-2025

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1) PREFACE

Since the emergence of humans on Earth, approximately 4.5 million years ago, the global population has steadily increased, reaching its current size of around 8 billion people. Along with the corresponding rise in food demand, food production has expanded, aquaculture has experienced substantial growth and global expansion, and compared to terrestrial organisms, fish offer several advantages, enabling even developing countries to become competitive with nations that have practiced aquaculture for centuries, such as Italy, which has been using it since Roman times.

Currently, most of the consumed fish comes from fish farms, which supply copious quantities of fish for sectors ranging from food production to recreational fishing. However, this high output often leads to overproduction and overcrowding, resulting in poor farm management and an increased risk of exposure to pathogens and parasitic diseases.

From an evolutionary perspective, parasitism is considered an effective survival strategy. However, parasitic infestations can cause severe damage, compromising the host's physiological integrity. Fortunately, organisms have evolved defense systems to counteract pathogenic threats. Gnathostomes, or jawed fishes, were the first vertebrates to develop both innate and adaptive immune systems, making them valuable models for understanding the evolution and origins of immunology in vertebrates. Most immunological studies have been conducted on higher vertebrates, while data on fish are limited. A more comprehensive understanding of fish defense mechanisms will serve as a foundation for the development of health management tools, thereby helping the growth of sustainable aquaculture and mariculture industries. This study will provide data from studies with the light microscope on the response of innate immune cells in a range of fish: *Anguilla anguilla* (European eel), *Dicentrarchus labrax* (seabass), *Salmo trutta* (trout), *Squalius tenellus* (chub), *Galeus melastomus* (black mouth shark), and the *Raja clavata* (ray), some infected with a range of different parasite genera.

The results of the present PhD thesis provided the data for publications in international journals and presentations at conferences:

- Franchella, E., Carosi, A., Lorenzoni, M., Bosi, G., Sayyaf Dezfuli, B. Histopathology and innate immune response of *Anguilla anguilla* infected with *Acanthocephalus rhinensis* (Acanthocephala). XXXIII Congress SOIPA, Padua (Italy), June 2024.

- Sayyaf Dezfuli, B., Franchella, E., Bernacchia, G., De Bastiani, M., Lorenzoni, F., Carosi, A., Lorenzoni, M., and Bosi, G. (2024a). Infection of endemic chub *Squalius tenellus* with the

intestinal tapeworm *Caryophyllaeus brachycollis* (Cestoda): histopathology and ultrastructural surveys. Parasitology, 151, 157-167.

-Sayyaf Dezfuli, B., Franchella, E., Depasquale, J. A., Lanzoni, M., and Giari, L. (2024b). Polyparasitism in sea bass: magnificence of parasites adaptation and host tolerance. Annals of Parasitology, 70, s38-s38.

-Sayyaf Dezfuli, B., Lorenzoni, M., Carosi, A., Bosi, G., Franchella, E., & Poddubnaya, L. G. (2024c). Glandular cell products in adult cestode: A new tale of tapeworm interaction with fish innate immune response. International Journal for Parasitology: Parasites and Wildlife, 25, 100991.

The following papers were produced during the PhD period and served as an inspiration for the thesis, although they were not directly applied within it:

- Bosi, G., Merella, P., Franchella, E., Sayyaf Dezfuli, B., & Giari, L. (2025). The Leydig organ of elasmobranchs: Shed light on an active and mysterious defense center with immunological characterization of its cells. Tissue and Cell, 93, 102755.

- Sayyaf Dezfuli, B., Franchella, E., Pironi, F., & Giannetto, D. (2025). A portrait of 2 nematodes in liver of their paratenic fish hosts, illustrating different immunological approaches. Parasitology, 1-9.

- Kumas, K., Sayyaf Dezfuli, B., Franchella, E., Duan, Y., Kania, P. W., & Buchmann, K. (2025). Cellular responses in rainbow trout *Oncorhynchus mykiss* to experimental *Anisakis simplex* infection. Parasitology Research, 124, 1-13.

2) INTRODUCTION

The global population continues to rise, along with the demand for aliments, leading to an increased need for food production. Over the past few decades, aquaculture has experienced significant growth, prompting a growing interest in understanding the fish immune system to enhance aquafarm management and improve fish health. To date, most studies on immunity have focused on mammals, neglecting other classes of vertebrates.

Fish represent the earliest group of vertebrates, and within them, Gnathostomata, including over 28,000 species such as Chondrichthyes (cartilaginous fish like sharks and rays) and Teleost (bony fish), were the first to exhibit both innate and adaptive immune systems (Magnadottir, 2010; Poynton & Nowak, 2020). Interestingly, elasmobranchs show a low incidence of disease and even possess the ability to inhibit tumour growth, making them particularly relevant for immunological research (Walsh et al., 2006).

One of the primary challenges in successful aquaculture is the presence of aquatic parasites, which can lead to high mortality rates, decrease product quality, and result in significant economic losses, affecting both wild and farmed fish populations (Poynton & Nowak, 2020). Usually in fish farming, chemical treatments and pharmaceuticals are commonly used to combat parasites or other pathogens and correlated diseases, but these methods present several drawbacks:

- High treatment costs
- Environmental contamination due to residual chemicals
- Variable drug efficacy depending on environmental conditions
- Emergence of drug-resistant pathogens, which may pose a risk to human health

(Toranzo et al., 2005; Samuelsen et al., 2006; Assefa & Abunna, 2018).

These challenges have led to the search for new strategies to control parasites and pathogens, driving growing interest in understanding the fish immune system. Among the most influential groups are metazoan parasites, including helminths —a broad term that encompasses worms from the phyla Platyhelminthes, Acanthocephala, and Nematoda —capable of infecting various fish organs. Most helminths are often found in the digestive tract, which acts as the primary entry point for pathogens, offering a nutrient-rich and favourable environment (Secombes & Chappell, 1996; Grondin et al., 2024).

Enteric helminths include species of digeneans, cestodes, acanthocephalans and nematodes, which cause inflammation in the digestive tract accompanied by leukocyte migration to the site of injury and, in some cases, they can induce structural alterations in host tissues and disrupt intestinal

physiology (Sayyaf Dezfuli et al., 2016). Beyond triggering local inflammatory responses, these parasites employ strategies to ensure their survival within the host. Their ability to establish successful infections relies on their capacity to manipulate or evade the host's immune system (Moreau & Chauvin, 2010; Franke et al., 2014).

Maintaining fish health is essential not only for fisheries and aquaculture, but also for the stability of aquatic ecosystems, where parasitic infections are highly prevalent and can influence population dynamics, trophic interactions, and biodiversity. In recent years, the global population has increased its consumption of raw or lightly processed fish, such as sushi, sashimi, ceviche, carpaccio, and smoked products. This trend has heightened the risk of exposure to zoonotic parasites (Bao et al., 2018; Golden et al., 2022), with humans acting as accidental hosts. A well-known example is the nematode *Anisakis simplex*, whose infective third-stage larvae (L3) can be present not only in the visceral cavity but also in the fish flesh intended for human consumption. Ingestion of raw or undercooked fish harbouring these larvae can lead to anisakidosis, a gastrointestinal and sometimes allergic condition of increasing relevance in public health (Mattiucci et al., 2018; Cipriani et al., 2024a). Anisakid larvae exhibit a remarkable ability to migrate through host tissues: they can penetrate the intestinal wall and access organs such as the gonads, liver, and musculature, eventually reaching the edible portions of the fish (Cipriani et al., 2024a; 2024b; Sayyaf Dezfuli et al., 2021a, 2025). Such migration not only reduces the commercial value of fish products but also poses a risk to consumers if proper processing measures (freezing, cooking) are not applied.

Fish occupy an important position in the study of the vertebrate immune system, as several key immune elements found in mammals such as macrophages, B and T lymphocytes, major histocompatibility complex (MHC), CD molecules, and cytokines, have also been identified in fish, suggesting a deep phylogenetic conservation (Rauta et al., 2012). The study of fish immunology represents a fundamental component of comparative immunology, offering new opportunities to understand the origin and function of immune molecules in higher vertebrates.

This PhD research project aims to conduct a comprehensive investigation of several immune cell types in both cartilaginous and bony fish, examining their morphology, distribution, and functional roles in healthy individuals as well as in those infected by metazoan parasites. The project seeks to unravel the cellular mechanisms underlying host defence in two evolutionarily distinct groups of vertebrates. Given that the immune system of cartilaginous fish remains comparatively underexplored and far less understood than that of teleosts, focusing on these species offers a particularly novel and scientifically relevant research avenue. Moreover, comparing immune responses between healthy and parasitized individuals will contribute to a deeper understanding of

how helminths and other metazoan parasites modulate host immunity, alter tissue homeostasis, and influence disease outcomes.

2.1 IMMUNE SYSTEM OF FISH

Fish represent the oldest group of vertebrates, having undergone extensive diversification, with more than 28,000 species colonizing nearly all aquatic environments. Their immune system can therefore be considered one of the most ancient forms of immune defense, playing a crucial protective role against viruses, bacteria, metazoan parasites, and toxic substances.

One of the key features of vertebrate immunity is its ability to distinguish self (host tissue) from non-self (pathogens or foreign substances). It is divided into innate (nonspecific) and adaptive (specific) immunity, which interact closely. The innate immune system is the first nonspecific line of defense, present at birth, while the adaptive immune system involves the production of T and B lymphocytes, which allow for more specific and efficient responses through cellular memory (McGuinness et al., 2003; Medzhitov, 2007). The innate immune response is advantageous due to its rapid deployment and the considerable number of effector cells it involves. However, unlike the adaptive immune system, it cannot form memory. The adaptive response, thanks to immunological memory, provides more targeted and efficient protection upon repeated exposure to the same pathogen.

Each system uses different receptors to recognize pathogens. The innate immune system uses pattern recognition receptors (PRRs) with low specificity that detect pathogen-associated molecular patterns (PAMPs) (Medzhitov & Janeway, 2002; Janeway & Medzhitov, 2002; Chen et al., 2025). The adaptive immune system, instead, utilizes highly specific antigen receptors (Pasare & Medzhitov, 2004). The main receptors of the innate immune system include Toll-like receptors (TLRs) (Rivera et al., 2016; Chen et al., 2025), as well as RIG-like receptors (RLRs), NOD-like receptors (NLRs), and C-type lectin receptors (CLRs). These recognize PAMPs and activate transcription factors regulating the inflammatory response (Dalmo & Bøgwald, 2008; Chen et al., 2025).

Unlike mammals, Gnathostomes lack bone marrow, but they possess mucosa-associated lymphoid tissue (MALT) made up of resident immune cells, including skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT), and gut-associated lymphoid tissue (GALT) (Salinas 2015, Salinas et al., 2022).

The thymus is a primary lymphoid organ located dorsally in the head region, above the gills

(Ge & Zaho, 2013; Rezzani et al., 2014) and is the site of lymphocyte maturation. It is a dynamic organ and, as in mammals, regresses with age. The kidney is the main immune organ in fish and is divided into an anterior portion (pronephros), with hematopoietic and immune functions analogous to mammalian bone marrow, and a middle/posterior portion (mesonephros/metanephros) with hematopoietic and excretory functions (Ellis & de Sousa, 1974; Zapata, 1979; Hu et al., 2024). The spleen, a secondary lymphoid organ, is involved in blood filtration, adaptive immune responses, and the removal of damaged or aged blood cells. Renal and splenic tissues are also characterized by melanomacrophage centers or, more correctly, macrophage aggregates (Zapata et al., 1996; Agius & Robert, 2003; Solakhiyah et al., 2023), which can retain antigens for extended periods and increase in size and number under stress conditions (De Vico et al., 2008; Suresh, 2009; Alesci et al., 2022a).

In elasmobranchs, in addition to the thymus and spleen, blood cells are generated in two organs associated with the gonads and esophagus, known as the epigonal organ and Leydig organ, respectively (Fänge & Mattisson, 1981; Bosi et al., 2025). Some studies suggest these organs are the primary lymphoid organs, almost analogous to bone marrow (Miracle et al., 2001; Rumfelt et al., 2002). In their record, Bosi et al. (2025) documented that many immune cells present in the Leydig organ are mast cells and macrophages, highlighting its immune function.

The blood cells involved in defense are white blood cells (leukocytes), divided into:

- Agranulocytes
- Granulocytes

Agranulocytes, including monocytes and lymphocytes, lack granules in their cytoplasm. Monocytes are the largest circulating leukocytes and differentiate into macrophages upon reaching tissues, where they exhibit intense phagocytic and secretory activity (Grayfer et al., 2014; Hodgkinson et al., 2015). They also produce soluble factors like lysozyme and interferons to modulate immune responses (Secombes, 1996; Alvarez-Pellitero, 2008). There are two main types of lymphocytes: T cells and B cells, both equipped with membrane-bound receptors that specifically recognize antigens. B cells are characterized by their ability to produce immunoglobulins (Ig) or antibodies, which bind to antigens with complementary structures (Adler et al., 2017; James, 2022). Elasmobranchs possess IgM, IgD/W, and IgNAR (Matz et al., 2021), in contrast, in bony fish, three immunoglobulin (Ig) isotypes have been identified: IgM, IgD, and IgT/IgZ. In both of them, the mammalian immunoglobulins (IgE, IgG, and IgA) are absent (Secombes & Wang, 2012). Despite this, fish immune responses remain effective due to the specialization of their immunoglobulins. IgT/IgZ, for instance, are mucosal antibodies expressed in the gut (Zhang et al., 2010) and gills, binding and neutralizing pathogens. These antibodies are key to mucosal immunity, protecting

exposed surfaces and helping regulate the microbiota.

Antibodies are produced through genetic recombination and clonal selection, each specific for one antigen. Upon antigen encounter, B cells proliferate and differentiate into plasma cells, which secrete antibodies in a humoral immune response. Antibodies bind to epitopes specific sites on pathogens leading to inactivation (Alvarez-Pellitero, 2008).

In mammals, T cells originate from bone marrow stem cells and mature in the thymus. In fish, they originate in the anterior kidney and migrate to the thymus for selection and maturation, where they express T-cell receptors (TCRs) and CD4/CD8 molecules for antigen recognition (Takama, 2006).

T cells are responsible for cell-mediated immunity and include:

- Tc (cytotoxic) cells
- Th (helper) cells
- Ts (suppressor) cells

Th cells, which express CD4, recognize antigens presented by MHC class II molecules, and help activate B cells and Tc cells via cytokine secretion. Tc cells, which express CD8, recognize MHC class I molecules on infected cells and induce their lysis (Abós et al., 2022). All nucleated cells express MHC I, whereas MHC II is expressed only on professional antigen-presenting cells (APCs) and interacts with Th cells. Granulocytes, on the other hand, contain cytoplasmic granules and are classified based on staining affinity into neutrophils, basophils, and eosinophils. Neutrophils and eosinophils are the most common, while basophils are often absent in many fish species (Secombes, 1996; Mokhtar et al., 2023).

Parasitic infections typically cause subacute or chronic inflammation, often leading to encapsulation of the pathogen. In cases where the parasite does not penetrate deeply (e.g., many trematodes), only mucous cells hyperplasia and mucus secretion are observed (Dezfuli et al, 2013; Reite, 2005). However, when parasites like *Pomphorhynchus laevis* or *Contracaecum rudolphii* penetrate deeper (beneath the epithelium or into the intestinal muscle layer), a fibroblast capsule may form around them, often involving epithelioid cells-transformed macrophages, collagen fibers, and mast cells (Secombes, 1996; Alvarez-Pellitero, 2008; Sayyaf Dezfuli et al., 2024a). In the following paragraphs, the different types of analyzed immune cells will be described.

2.1.1 Mucous cells

In their natural habitats, fish are constantly exposed to harmful agents such as viruses, bacteria, protozoa, fungi, and metazoan parasites, and their mucosal and epithelial barriers covering all external and internal surfaces, act as protective barriers and, at the same time play a crucial role in maintaining homeostasis and enabling interaction with the external environment (Aoki et al., 2008; Magnadottir, 2010; Salinas et al., 2022; Stosik et al., 2023). Mucus is one of the most important first lines of defense, which covers all mucosal surfaces in fish (Salinas, 2015; Cabillon & Lazado, 2019; Bosi et al., 2022a; Sayyaf Dezfuli et al., 2023a). This mucus is secreted by specialized cell, mucous cells, key components of the innate immune system (Magnadottir, 2010; Aoki et al., 2008; Mokhtar et al., 2023). The mucus is primarily composed of mucins (MUCs), a family of glycosylated proteins, that give it its viscoelastic and protective properties (Esteban, 2012; Ma et al., 2018; Bosi et al., 2022a).

Mucous cells are characterized by an elongated basal nucleus and spherical vacuoles containing mucus (Bosi et al., 2017), which has several physiological functions. In the intestine, the mucus promotes the transit of food, and at the same time plays an immune role, protecting the mucosa from mechanical damage (Sayyaf Dezfuli et al., 2016; Bosi et al., 2020). Through holocrine secretion, mucus is released onto the epithelial surfaces of the intestine, gills, and skin (Bosi et al., 2017). It is composed of various immune factors such as mucins, antimicrobial peptides (AMPs), lectins, and immunoglobulins (Ellis, 2001; Hasnian et al., 2013; Lazado & Caipang, 2014; Campoverde et al., 2017; Sharpe et al., 2018).

Few genes encoding mucins have been identified in teleosts, including Muc2, like that of mammals, which appear to be more expressed in the intestine and released by goblet cells, mucous cells present in the intestinal epithelium (Lang et al., 2004; van der Marel et al., 2012; Micallef et al., 2012). In mammals infected by intestinal helminths, an increased expression of Muc2 and Muc5AC is seen (Hasnain et al., 2013), which are also present in the gills and skin of some teleosts (Hosoya et al., 2013; Bai et al., 2020).

Mucous cells are capable of secreting acidic and neutral glycoconjugates, which can be characterized by Alcian Blue/PAS staining (Bosi et al., 2017). In stressful situations, such as the presence of pathogens/parasites, mucous cells undergo hyperplasia, hypertrophy, and the mucus changes its composition, with an increase in acidic components, associated with a greater viscosity of the secreted mucus (Diaz et al., 2008; Sayyaf Dezfuli et al., 2016; Bosi et al., 2017; 2020; 2022a; Souza et al., 2019; Sayyaf Dezfuli et al., 2023, 2024a). Similar qualitative changes have also been

observed in mammals, where mucus secretion appears to be functional in eliminating the parasite (McGukin et al., 2011; Rinaldi et al., 2011): the increased mucus viscosity limits the helminth movements and nutrient intake (Webb et al., 2007; Okumura et al., 2025). Histochemical studies conducted on the intestine of mullets infected with *Neochinorhynchus agilis* (Acanthocephala) have shown changes in mucus oligosaccharides compared to healthy individuals, resulting in increased mucus viscosity and enhanced resistance to the lytic enzymes of the pathogens (Bosi et al., 2020).

In several teleost-parasite systems, an increase in the number of mucous cells and the secretion-qualitative change of mucus has been observed (Bosi et al., 2005a,b, 2015, 2017, 2020; Dezfuli et al., 2010a; Bosi & Dezfuli 2015; Sayyaf Dezfuli et al., 2024a; Pérez-Sánchez et al., 2013; Marcos-Lopez et al., 2018), and in many cases it has been evident that the primary function of mucus is to protect the mucosa from the mechanical attack of the pathogen (Schroers et al., 2009; Bosi et al., 2015, 2020; Sayyaf Dezfuli et al., 2021a). However, information on the mucous cells of elasmobranchs is still scarce: in the gastrointestinal tract of *Galeus melastomus*, a greater number of neutral cells was observed in the spiral and proximal part of the intestine, while acidic mucous cells were observed in the distal part of the intestine, suggesting that the first part of the alimentary canal plays a key role in absorption, especially in the spiral intestine, while the distal part traps previously unabsorbed nutrients (Bosi et al., 2022a).

2.1.2 Mast cells

Mast cells (MCs) were first observed in 1863 by Friedrich von Recklinghausen, who described granular cells in the frog mesentery (von Recklinghausen, 1863; Blank et al., 2013). However, credit for their discovery was later given to Paul Ehrlich (1877–1879), who recognized their metachromatic staining with specific dyes. Ehrlich named them mastzellen, from the Greek “masto” (to nourish), initially believing that they were involved in tissue nourishment (Ehrlich, 1878). Later, they were called "mast cells" (MCs) or even "eosinophilic granular cells" (ECGs) (Roberts et al., 1971).

MCs have been observed in different vertebrate class: fish (Roberts et al., 1971; Reite, 1998), amphibians (Chiu & Lagunoff, 1972), reptiles (Sottovia-Filho & Taga, 1973), birds (Selye, 1966), and mammals (Galli, 1993; Chiarini-Garcia & Pereira, 1999; Baccari et al., 2011). These cells are distributed throughout all vascularized tissues and organs and are essential for the body's defense. MCs have an irregular shape, with an eccentric polar nucleus and numerous round/oval electron-dense granules, and are strategically positioned in connective tissues near blood vessels and nerves,

allowing them to play a crucial role in innate and adaptive immune defense (Shukla et al., 2006; Dezfuli et al., 2011a). They are sometimes described as basophilic and sometimes as eosinophilic (Mulero et al., 2007) due to their metachromatic properties and are therefore also called eosinophilic granular cells. Their granules stain with basic dyes such as toluidine blue and astra blue, or with acidic dyes such as eosin (Baccari et al., 2011). Some research has hypothesized the presence of two MC populations, one circulating and one resident, and that the presence of pathogens induces their recruitment to the site of infection (Vallejo & Ellis, 1989; Noya & Lamas, 1996; Murray et al., 2007; Dezfuli & Giari, 2008). MCs were initially considered cells exclusively involved in the innate immune response, but studies conducted on murine models have shown their involvement in the adaptive response as well. For example, they express the CD40-CD40L receptor on their surface, which allows for the activation of B cells (Gwain Ui et al., 2015; Valeri et al., 2020), and the release of IL-6 (interleukin-6) by MCs promotes B cell differentiation and IgA secretion (Merluzzi et al., 2010).

In the presence of inflammation, cytotoxic cells are recruited thanks to the release of chemokines from MCs such as CCL5 (Yana et al., 2021; Chen et al., 2023) and MCs are capable to present antigen through the major histocompatibility complex class I (MHC I) to CD8+ T cells, regulating their proliferation, secretion and cytotoxic activity (Wang et al., 2024). MCs can also present antigens to $CD4^+$ T lymphocytes by expressing major histocompatibility complex class II (MHCII) and OX40L molecules (Sahar et al., 2017). MCs are, therefore, cells capable of initiating the innate immune response and play a significant role in the adaptive immune response, in addition to the ability to suppress the inflammatory response through interaction with regulatory T cells (Tregs). The production of anti-inflammatory cytokines by MCs, such as interleukin-10 (IL-10) and Transforming Growth Factor-β (TGF-β), interacts with Tregs, which, through the secretion of mediators, suppress inflammation by acting directly on MCs (Gan et al., 2016; Reber et al., 2017). Functionally, MCs in fish are similar to those in mammals, and acute responses to pathogens or toxic substances are similar between the two classes, such as the release of granules contained in their cytoplasm, which are made up of inflammatory mediators (Reite & Evensen, 1994, 2006). Histochemical evidence has proved the presence of serotonin in mammalian MCs (Stefanov, 2024), and studies report the presence within fish MCs of serotonin, Tumor necrosis factor-α (TNF-α), and mucopolysaccharides with α-N-acetyl-galactosamine residues (Mulero et al., 2007; Dezfuli et al., 2008a, Galindo-Villegas et al., 2016; Sayyaf Dezfuli et al., 2018a; Serna-Duque et al., 2020). In some fish, like gilthead seabream, MCs store histamine but lack serotonin, which regulates the inflammatory response (Mulero et al., 2007; Galindo-Villegas et al., 2016). Mast cell granules could

contain piscidins (Silphaduang et al., 2006; Salger et al., 2016), that exhibit potent, broad-spectrum antimicrobial activity against viruses, bacteria, fungi, water molds, and metazoan parasites (Silphaduang & Noga, 2001; Dezfuli et al., 2011a, 2013). Furthermore, numerous proliferating cell nuclear antigen (PCNA)-positive MCs were observed at the site of helminth infection (Dezfuli et al., 2012a), indicating an increased cell division in tissues (Ortego et al., 1994).

The MCs' origin in fish is unclear and is still under study, but it is likely that they form in hematopoietic tissues as in mammals and subsequently reach their destination organs, as immature cells, through the circulatory system (Reite, 2005). Some studies have documented the migration of MCs from the connective tissue underlying the epithelial layer in the intestines of salmonids and wrasses (Reite & Evensen, 2006). MCs reach the infection site through the circulatory system (Reite, 1998, 2005; Dezfuli & Giari, 2008; Sayyaf Dezfuli et al., 2020) and respond to the parasites by releasing the granules contained within them, with a mechanism called degranulation. In the presence of pathogens, the number of MCs increases and consequently the release of granules also rises (Dezfuli & Giari, 2008; Andrews et al., 2010; Dezfuli et al., 2010b, 2011a). At the site of inflammation, the release of inflammatory mediators such as cytokines, piscidins (fish antimicrobial peptides)(Silphaduang & Noga, 2001; Salger et al., 2016), and arachidonic acid metabolites allows the dilation and increase in the permeability of blood vessels, which facilitates the passage of cells, including MCs, from the bloodstream to the surrounding tissue (Reite & Evensen, 1994; Dezfuli et al., 2008a, b).

Degranulation in fish occurs through diacytosis, whereby the granules are released to the outside. In contrast, in mammals, it occurs through exocytosis, i.e., the fusion of the granule membrane with the plasma membrane and the release of the substances contained within them to the outside (Schmale et al., 2004). The fish mast cells' degranulation process is characterized by a swelling of the granules, which may appear surrounded by clear halos and display a reticulated matrix; in some cases, fusion of the granules can also be observed. It is also common to detect the release of granules outside the cytoplasm, as indicated by the presence of free granules near the mast cells (Dezfuli & Giari, 2008). In quiescent conditions, mast cells display dense and compact cytoplasmic granules, with a regular and well-defined cytoplasm (Reite & Evensen, 2006), whereas upon activation, the granules swell and assume a less defined appearance, sometimes showing clear halos. During degranulation, they can release their contents either massively or gradually, leaving residual granules in the cytoplasm (Dezfuli & Giari, 2008). In an experimental study, the application of compound 48/80 induced mast cell degranulation, resulting in concentration-dependent intestinal contractions in rainbow trout (*Oncorhynchus mykiss*) (Manera et al., 2011).

Mast cells also act as sentinels and allow the involvement of other cells in the inflammatory process, such as neutrophils, especially in the early phase of inflammation (Matsuyama & Iida, 2002; Reite & Evensen, 2006; Dezfuli & Giari, 2008; Dezfuli et al., 2008a, b), in fact the co-presence of MCs with other leukocytes such as neutrophils, rodlet cells, mucous cells, fibroblasts, epithelioid cells and macrophage aggregates is often observed (Bosi et al., 2020, Sayyaf Dezfuli et al., 2024 a).

2.1.3 Rodlet cells

Rodlet cells (RCs), characteristic cells of teleosts, were first described by Theloan in 1892, believed to be sporocysts of an unknown sporozoan parasite, and their true origin and function have been debated for years. They are currently known as endogenous cells of the organism and involved in immune defense (Manera & Dezfuli 2004; Reite, 2005), and the first to hypothesize this nature was Plehn in 1906 (Plehn, 1906a, b). For a long time, there was a debate about their nature, thinking that they were parasites, but subsequent observations showed an increase in the number of RCs in infected fish at the site of inflammation (Dezfuli et al., 2003; Manera & Dezfuli, 2004; Bosi et al., 2018). Furthermore, analyses conducted on fish raised in the laboratory under sterile conditions highlighted how the fry already showed the presence of RCs in their organs (Kramer & Potter, 2003), pushing towards the theory of endogenous cells rather than the parasitic one. RCs are therefore considered to be cells of the innate immune system and associated with epithelia, mesothelia, and endothelia (Manera & Dezfuli, 2004; Reite, 2005; Sayyaf Dezfuli et al., 2022), present in several organs such as the swim bladder, intestine, gills, skin, heart, brain, thymus, gonads, and sensory organs (Sayyaf Dezfuli et al., 2022, 2023b).). In addition, a recent comparison between rodlet cells and apicomplexan cells, based on ultrastructural data, supports the notion that they are not parasites but rather endogenous cells with an immune function (DePasquale, 2024).

As for the MCs, the origin of RCs is unclear, but most likely they formed in hematopoietic organs and then migrate to their destination tissues through the circulatory system in response to stressful situations such as pathogens or toxic exposures (Giari et al., 2007; Sayyaf Dezfuli et al., 2016; Sayyaf Dezfuli et al., 2021a; Manera, 2024). They are easily recognizable by their distinctive morphology: they have an external fibrous capsule, a rodlet-rich cytoplasm, and an eccentric nucleus, and release their contents through holocrine secretion (Sayyaf Dezfuli et al., 2022). Once they reach the epithelium, they mature and migrate from the basement membrane to the upper region, where they release their cytoplasmic contents into the lumen (Bosi et al., 2018; Sayyaf Dezfuli et al., 2018b;

Kramer & Potter, 2002). RCs initially appear as mesenchymal progenitors, they subsequently enter the vesicular stage, characterized by intracellular vesicles associated with the rough endoplasmic reticulum, where rodlet precursors begin to form (Sarkar et al., 2020). A study on *Carassius auratus* (goldfish) characterized the different maturational stages of RCs in granular, transitional, mature, and "ruptured" (Alesci et al., 2022b). In the granular stage, rodlets become well-structured, followed by the transitional stage, during which a fibrillar fibrous cortex develops, conferring contractile capacity. In the mature stage, RCs are fully developed, with a basal nucleus, rodlets, and a well-defined fibrous structure, ready to release their cytoplasmic content. Finally, in the degenerative/ruptured stage, the cells have completed their secretory function; the cortex contracts, leading to cell deformation and death (Abd-Elhafeez & Soliman, 2016; Sayyaf Dezfuli et al., 2022).

Rodlet cells have microtubules, thin and thick filaments throughout the fibrous capsule, and a filament arrangement that is strikingly similar to that of smooth muscle cells (DePasquale, 2020). The actin and tropomyosin filaments allow the contraction of RCs and their discharge (DePasquale, 2020). The release of rodlet cell contents remains a topic of debate. Some authors suggest a holocrine mode, in which the release is mediated by contraction of the cellular cortex, allowing the apical portion to open and expel the contents, often accompanied by the loss of the cell itself (Sayyaf Dezfuli et al., 2022). In contrast, Hawkins (1984) documented the release of individual rodlets via a merocrine mechanism, which allows the cell to maintain the integrity of the plasma membrane through the fusion of the rodlet and plasma membranes. Additional ultrastructural observations have shown that RCs can also employ more complex forms of exocytosis, such as piecemeal and compound exocytosis, suggesting a finer regulation of the secretory process (Manera et al., 2023).

Recent immunohistochemical studies have detected in RCs the presence of TNF-α, iNOS (inducible nitric oxide synthase), lysozyme, and glucosidic residues of N-acetyl-galactosamine, sialic acid (Schroers et al., 2009; Bosi et al., 2018; Sayyaf Dezfuli et al., 2022), S100, tubulin, α-SMA, Piscidin, and, TLR-2 (Alesci et al., 2022b). Sialic acid residues in the carbohydrate backbone are involved in the protection of the mucosa from microorganisms (Schroers et al., 2009), while lysozyme is an antimicrobial enzyme; this highlights their involvement in inflammatory processes and in the innate immune response (Bosi et al., 2018; Sayyaf Dezfuli et al., 2022).

2.1.4 Neutrophils

Neutrophils are key participants in the innate immune response, being among the first granular cells to arrive at the site of injury/infection. They are characterized by an oval/round shape,

a lobed nucleus, and granule-containing cytoplasm (Sayyaf Dezfuli et al., 2016). In humans, they constitute 50% of the leukocyte population, while in fish, they constitute 5% to 15%–20% (Pettersen et al., 2003; 2005; Vázquez & Guerrero 2007; Havibex et al., 2016). The cephalic kidney in fish is the primary site of immune cell production, including neutrophils mobilized through the circulatory system (Havixbeck et al., 2016; Fingerhut et al., 2020). Like human neutrophils, those of teleosts migrate to the site of infection via chemotactic signals, and interleukin-8 (IL-8 or CXCL8) is the chemokine involved in their recruitment to the site of infection (Fingerhut et al., 2020). One of the main functions of neutrophils is the phagocytosis of pathogens (viruses, bacteria), but they are also able to release their granules, containing antimicrobial peptides, proteolytic enzymes, and cytokines that allow the recruitment of other immune cells (Teng et al., 2017; Othman et al., 2022). Furthermore, neutrophils can produce reactive oxygen species (ROS) and reactive nitrogen species (RNS), with a biocidal action against bacteria and parasites, and are involved in modulating cell apoptosis (Katzenback & Belosevic, 2012; Pérez-Figueroa et al., 2021). Once they reach the infection site, they recognize the pathogens through PRRs (including TLRs) and initiate netosis, i.e. they release extracellular traps (NETs), made up of smooth chromatin combined with histones and granules capable of immobilizing the pathogen and inhibiting it until its elimination (Brinkmann et al., 2004; Pijanowski et al., 2013; Fingerhut et al., 2020).

In humans and mice, neutrophils appear to be involved also in the initiation of the adaptive immune response, promoting the differentiation of Th cells by presenting antigen to MHC II (Takashima & Yao, 2015; Li et al., 2019). Immunohistochemical studies conducted on Atlantic salmon have highlighted the positivity of neutrophils exposed to bacteria, for MHC II, CD83, CD80/86, and IL-12p40, molecules essential for the activation of the adaptive response. Therefore, this study suggests the possibility that Atlantic salmon neutrophils may function as APCs (antigen-presenting cells) (Haugland et al., 2024), which are usually represented by dendritic cells (DCs), monocytes/macrophages, and B cells.

Several studies have documented the presence of numerous neutrophils in proximity to the site of helminth attachment (Nfon et al., 2006; Dezfuli et al., 2009; 2012a; Sayyaf Dezfuli et al., 2024b), thus playing a key role in innate immunity but at the same time acting as a bridge for adaptive immunity

2.1.5 Monocytes/macrophages

Macrophages are innate immune cells present in almost all organs of the body, such as the

kidney, spleen, intestine, liver, and gills, and are considered the main phagocytic cells along with neutrophils (Secombes & Ellis, 2012). They are large, irregularly shaped cells with undefinable structures inside, often containing pigments such as hemosiderin, lipofuscin, and melanin, and can organize into clusters called melanomacrophage centers or macrophage aggregates (Agius & Roberts 2003; Secombes & Ellis, 2012; Stosik et al., 2019). Melanomacrophage centres (MMCs) or macrophage aggregates (MAs) are found in various organs and tissues, mainly in the spleen, kidney, and liver. They can phagocytose pathogens, cellular debris, and toxic substances. The immune activity of MMCs/MAs is evidenced by the accumulation of pigments such as melanin, lipofuscin, and hemosiderin, which possess antioxidant and antimicrobial properties (Zaccone et al., 2017). In the presence of infection, exposure to toxicant or other immune stimuli, these structures significantly increase in size, reflecting a state of activation (Manera et al., 2018; Stosik et al., 2019; Alesci et al., 2022a). Functionally, MMCs are considered analogous to mammalian germinal centers (GCs) as they serve as sites where critical interactions between macrophages and lymphocytes occur and where both innate and adaptive immune responses are modulated (Victora & Nussenzweig, 2012). The MMCs/MAs characteristics are considered a sensitive indicator of chronic inflammatory processes and adverse environmental conditions, making them useful biomarkers of organismal health and stress (Broeg et al., 2003; Carreras-Colom et al., 2022).

Some studies have documented the presence of resident macrophages in tissues with diverse functions. For example, alveolar macrophages in the lungs ensure surfactant homeostasis (Trapnell & Whitsett, 2002), while in the zebrafish intestine, they participate in regulating the microbiota (Early et al., 2018). Nonetheless, their primary role appears to be that of defense against pathogens. In the presence of infection/tissue damage, macrophage precursors, monocytes, are recruited and undergo differentiation (Castro & Tafalla, 2015).

In the presence of pathogens, these cells adopt distinct differentiation systems, also known as polarization, switching from a resting phase (M0) to a pro-inflammatory phase (M1), characterized by the activation of inflammatory substances. M2 represents another alternative phase of macrophage activation, in which elevated levels of pro-inflammatory mediators are present (Shapouri-Moghaddam et al., 2018; Wiegertjes & Elks, 2022). M1 macrophages mediate ROS-induced tissue damage, have antitumor and antimicrobial activity (Shapouri-Moghaddam et al., 2018), and express proinflammatory markers such as MHCII, iNOS, CD80, TGF-α, IL-β, IL-8, and IL-12 (Shapouri-Moghaddam et al., 2018; Li et al., 2023). In contrast, M2 macrophages (further classified into M2a, M2b, M2c, and M2d) are involved in anti-inflammatory responses, tissue regeneration, and immunosuppression thanks to markers such as IL-10 and TGF-β (Shapouri-Moghaddam et al., 2018;

Bosco, 2019). The monocyte, presenting CD68 as a marker, is stimulated by CSF-1 and differentiates into an M0 macrophage. This macrophage, under stimulation by cytokines such as IFN-γ and TNF-α, then progresses to the M1 activation state. The M2 phase is instead stimulated by IL-4, IL-10, and TGF-β (Holness et al., 1993; Bosco, 2019). Macrophages exhibit remarkable plasticity, allowing them to assume different phenotypes in response to environmental conditions (Bosurgi et al., 2017). Their presence in fish infestation conditions has been reported, for example, in the liver of *Gymnotus inaequilabiatus* harboured nematode larvae (Sayyaf Dezfuli et al., 2025) or in the swim bladder and intestine of *Anguilla anguilla* infected with *Anguillicoloides crassus* (Sayyaf Dezfuli et al., 2023b) and *Contracaecum rudolphii* (Sayyaf Dezfuli et al., 2024a).

2.1.6 Epithelioid cells

Epithelioid cells are transformed macrophages in response to persistent stimulation, with the role of ingesting debris/foreign agents by phagocytosis (Cotran et al., 1999; Johnston, 1988; Ferguson, 2006), and possess an elongated-ovoid shape, reminiscent of the epithelial cells to which they owe their name (Cotran et al., 1999). These cells usually occur in the inner layer of granuloma, which encircle pathogens or parasite larvae in visceral organs like the gonad, heart, kidney, intestine, and liver (Sayyaf Dezfuli et al., 2024a; 2025; Kumas et al., 2025). The granuloma is a chronic inflammatory nodule-like structure formed by several concentric layers of different immune cells and collagen fibers (Sayyaf Dezfuli et al., 2016, 2024a). As mentioned above, the innermost layer is formed by epithelioid cells (Molnar 1995; Behrens et al., 2023; Sayyaf Dezfuli et al., 2024a, 2025; Kumas et al., 2025). This location most likely serves to isolate the pathogen or parasite, preventing further damage to the tissue. Alternatively, it may also provide protection for the pathogen from the host immune response (Cronan et al., 2016; Nathan, 2016).

3) MATERIALS AND METHODS

The fish used in this study belonged to the following species: *Dicentrarchus labrax* (Linnaeus, 1758), *Anguilla anguilla* (Linnaeus, 1758), *Salmo trutta* (Linnaeus, 1758), *Squalius tenellus* (Heckel, 1843), *Galeus melastomus* (Rafinesque, 1810), and *Raja clavata* (Linnaeus, 1758), and were collected from various geographical locations. Specifically:

• The seabass (*D. labrax)* specimens came from the Canneviè lagoon (Codigoro, FE), in the Po Delta area between 2022 and 2025;

• Eels (*A. anguilla*) were sampled in Lake Piediluco (Terni, Umbria Perugia, 42°31′57.36″N 12°45′48.42″E) between 2023 and 2025.

• Trouts (*S. trutta*) were sampled in the Nera Rive(Terni, Umbria) one of the outlets of Lake Piediluco (Perugia, 42°31′57.36″N 12°45′48.42″E) carried out over three years (2023-2025)

• The chub (*S. tenellus)* specimens were collected from Bosnia and Herzegovina in Lake Blidinje, in the Blidinje Natural Park, located in the Federation of Bosnia and Herzegovina (43°36′25″N, 17°29′48″E), between 2023 and 2024.

• The two elasmobranch species (*G. melastomus* and *R. clavata*) originate from the Gulf of Asinara (Sardinia, western Mediterranean Sea) between 2023-2024.

The selection of fish species, such as eels, trout, and sea bass, for the present study was based on their economic, nutritional, and ecological importance. The Bosnian chub was included due to its relevant ecological and scientific significance, being an endemic species of Blidinje Lake within the Blidinje Natural Park (Buj et al., 2020). *G. melastomus* and *R. clavata* were instead selected because they are two elasmobranch species common in the Mediterranean Sea and relatively easily accessible.

The teleost fish were transported live from the sampling sites to the laboratory, where they were sacrificed by spinalization following approved ethical protocols. Elasmobranchs, indeed, were provided via commercial trawl fishing during a haul at a 500–800 m depth in the Gulf of Asinara (Sardinia, western Mediterranean Sea). They were eviscerated, and the entire digestive tube of each was promptly fixed in 10 % neutral-buffered formalin while still on board. After landing, the samples were transported to the Department of Veterinary Medicine of the University of Sassari and processed within 24 h post-fixation. The spiral intestine of each specimen was sliced into small pieces, rinsed several times with chilled 70 % ethanol, and sent

to the University of Ferrara for the embedding process in paraffin wax. The following biometric parameters were recorded for each specimen: total and standard length, body mass, sex, and health status (presence or absence of ectoparasites and endoparasites). Subsequently, fragments of both healthy and parasitized organs (gills and intestine) were dissected and collected for histological and ultrastructural analyses.

Both healthy and infected organ fragments, intended for light microscopy, measuring up to 12 mm in size, were fixed in 10% buffered formalin for 24 hours. Subsequently, the samples were dehydrated through an increasing alcohol series (70%, 80%, 90%, 100%) and embedded in paraffin (Diawax, 56–58°C, Diapath) using an automatic paraffin embedder (Shandon Citadel 2000, London, UK). The paraffin blocks were sectioned with a rotary microtome (Leica RM 2145, Germany). Histological sections, 5 μm thick, were stained with Alcian Blue-PAS, Hematoxylin and Eosin, Masson's Trichrome, and Giemsa. Images were acquired using a transmitted light microscope with a Nikon Eclipse 80i imaging system (NIKON, Tokyo, Japan).

For ultrastructural analysis, fragments of infected and healthy tissues were selected, and 7 × 7 mm tissue fragments were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C for 3 hours and subsequently sent to the electron microscopy laboratory at the University of Ferrara. The fixed tissues were postfixed in 1% osmium tetroxide for 2 hours, rinsed, and stored in 0.1 M sodium cacodylate buffer with 6% sucrose for 12 hours. After dehydration in an increasing acetone series, the samples were embedded in epoxy resin (Durcupan ACM, Fluka, Buchs, Switzerland). Semi-thin sections (1.5 μm) were obtained with a Reichert Om U2 ultramicrotome and stained with toluidine blue. Ultra-thin sections (90 nm) were counterstained with 4% uranyl acetate in 50% ethanol and Reynolds lead citrate and analyzed with a Talos L120C transmission electron microscope.

To compare the number and type of mucous cells present in the intestines of infected and uninfected fish, sections stained with Alcian Blue 8GX pH 2.5 followed by Periodic Acid–Schiff (PAS) were analyzed, as described by Bosi et al. (2015, 2017).

Mucous cells were classified based on staining:

- blue = Alcian Blue (AB) positive,
- magenta = PAS positive,
- purple = AB/PAS double positivity.

To assess the differences in RCs and mucous cells densities between infected and uninfected

fish, five randomly selected areas of epithelium measuring 1000 μm^2 from two histological sections were analyzed for each specimen (n = 5 uninfected fish, n = 5 infected fish). Cell counting was performed using a Nikon ECLIPSE 80i microscope on images captured with an objective 40×. Since the data satisfied the assumptions of normality and homogeneity of variance, a parametric test, Student's two-tailed *t*-test was applied to compare cell density. The difference was considered statistically significant with a $P\ value < 0.05$.

4) RESULTS

4.1 Infected *Anguilla anguilla*

During a survey conducted at Lake Piediluco 44 European eel *Anguilla anguilla* (L.) were sampled to evaluate the presence of intestinal parasites and associated histopathological effects. The fish had a mean total length of 63.48 ± 1.95 cm (mean ± S.E.) and a mean weight of 577 ± 54.58 g (mean ± S.E.). The analysis revealed that 29 individuals (54%) were infected along the entire length of the intestine (Fig. 1) by the acanthocephalan parasite *Acanthocephalus rhinensis*, with intensity of infestation varying from 1 to 150 specimens per host (24.4 ± 1.7; mean ± S.E.). Destruction of the intestinal villi, detachment of epithelial portions, and disappearance of the typical intestinal folds were observed in the parasitized areas. In contrast, these structures were preserved in areas far from parasite attachment Fig. 2A). The action of the hooked proboscis (Fig. 2B), typical of acanthocephalans, caused severe damage to the intestinal mucosa. One of the most evident immune responses was mucosal cell hyperplasia at the parasite's attachment site. The hyperplasia of mucous cells is linked to intense secretory activity, evidenced by the presence of Alcian Blue (AB) positive mucus, indicative of an increase in the acid glycoprotein component of the intestinal mucosa (Fig. 2C). The mean number of mucous cells, counted on an epithelial area of 1000 μm², was significantly higher in infected individuals (4.92 ± 0.54; mean ± S.E.) than in healthy individuals (2.95 ± 0.18; mean ± S.E.) (t-test, $P < 0.05$) (Fig.3). Histological sections show the presence of numerous rodlet cells (RCs) located between the mucous and epithelial cells, particularly at the apex of the villi proximal to the parasite (Fig. 2D). Also the number of RCs per 1000 μm² of screened intestinal epithelium was significantly higher in parasitized individuals (2.14 ± 0.12; mean ± S.E.) than in uninfected ones (1.41 ± 0.19; mean ± S.E.) (t-test, $P < 0.05$) (Fig. 4). In most cases, the observed RCs showed the typical cellular cortex and were in a mature phase, ready for secretion. A further component of the observed immune response is represented by mast cells (MCs), located in the connective tissue layer of the submucosa, often in proximity to the parasite's proboscis (Fig. 2E). These cells showed marked degranulation, visible under the light microscope (positive PAS staining) (Fig. 2F).

By transmission electron microscopy (TEM) analysis, RCs showed an elongated shape, with a basal nucleus, an irregular cell border, and a spheroidal appearance (Fig. 5A). Characteristic club-shaped inclusions were present in the cytoplasm, oriented with their tails pointing toward the cell apex, where the opening for the release of their contents into the intestinal lumen is located (Fig.

5B). Mucous cells, recognizable by their basal nucleus and the presence of electron-lucent vesicles in the cytoplasm (Fig. 5A), were frequently observed close to RCs. Their secretory activity was particularly marked in the vicinity of the parasite body. At the ultrastructural level, MCs appeared elongated, with an eccentric nucleus, an irregular cell membrane, and electron-dense cytoplasmic granules (Fig. 5C).

4.2 Infected *Squalius tenellus*

Twenty-two chubs *S. tenellus* (10 males and 12 females) were subjected to parasitological analyses. Of these, 10 specimens (4 females and 6 males) were found to be hosts of the cestode *Caryophyllaeus brachycollis* (Janiszewska, 1953). The prevalence was 45%, the infestation intensity varying from 7 to 416 parasites per individual (88.27 ± 19.10; mean ± S.E.), 2913 parasites total. The individuals had a mean total length of 25.47 ± 1.01 cm (mean ± S.E.) and a mean weight of 235.60 ± 26.40 g (mean ± S.E.).

The presence of the parasite involved the entire intestinal tract but was particularly marked in the anterior and middle portions of the intestine, where abundant mucus secretion was observed especially around the worms (Fig. 6A). In three severely infected individuals, intestinal perforation was documented, with the cestode strobilus clearly visible through the macroscopic lesion (Figs. 6B, 6C); these cases represent serious and potentially lethal pathological conditions for the host.

Histological analyses showed that the scolex penetrates deeply (Fig. 7A), causing a marked inflammatory reaction of the intestinal mucosa, associated with high mucus production (Fig. 7B). Furthermore, AB/PAS staining highlighted the predominance of mucous cells in infected intestinal epithelium containing acidic glycoconjugates (3.62 ± 0.31; mean ± S.E.) compared to mixed/neutral ones (1.18 ± 0.09; mean ± S.E.), equal to 37% for AB^+ cells compared to 12% for magenta/purple cells (Fig. 8). In the areas proximal to the parasite attachment sites, mucous cells were present, while in the proximity of the scolex, they were almost absent, with a consequent reduction in secretory activity at that site (Fig. 7C).

Although the scolex of *C. brachycollis* lacks specialized anchoring structures, such as hooks or suckers, a remarkable adhesive capacity has been observed, probably due to the morphological modifiability of the scolex, which adapts to the epithelial surface to increase its adherence (Figs. 7D, 7E, 7F). This interaction has caused significant mechanical damage, including erosion and necrosis of the intestinal mucosa, atrophy of the intestinal villi, detachment of the epithelium from the underlying layers, and, in some cases, discontinuity of the muscular layer associated with intestinal

perforation (Figs. 10A, 10B).

The infected intestinal epithelium and the oesophageal epithelium of individuals with severe infection showed a high presence of RCs distributed in the intestine among intestinal enterocytes (Figs. 10C, 10D). The mean number of RCs, calculated on an area of 1000 μm², was significantly higher in infected intestine (3.24 ± 0.27; mean ± S.E.) than in healthy individuals (2.44 ± 0.14; mean ± S.E.) (t-test, $P < 0.05$) (Fig. 9). In the tunica propria and submucosa, few MCs and numerous neutrophils (Fig. 10E), both in intense degranulation, and macrophage and macrophage aggregates (MAs) were noticed (Fig. 10F).

TEM observations allowed to characterize in detail the morphology of the cells involved in the infection, confirming the presence of mucous cells, with packed electron-dense granules in the active phase of secretion into the intestinal lumen (Figs. 11A, 11B). The RCs presented a deformed cortex and a poor cytoplasm, containing few rodlets and a heterochromatic basal nucleus (Fig. 11A). The MCs showed an oval shape, with electron-dense granules in the cytoplasm (Fig. 11A, 11C). Some MCs were positioned close to neutrophils, which were distinguished by their irregular shape, the eccentric polar nucleus and the presence of numerous roundish electron-dense granules (Fig. 11C). Both cell populations frequently appeared in a state of activation and degranulation, especially in the areas closest to the parasite body.

4.3 *Salmo trutta*

During a series of monitoring campaigns 34 specimens of *Salmo trutta* (L.) were captured with a mean length of 23.34 ± 1.05 cm (mean ± S.E.) and a weight of 149.09 ± 22.16 g (mean ± S.E.). After ventral incision, the alimentary canal was removed, opened longitudinally, and subjected to observation under a stereomicroscope. *Dentitruncus truttae* (Acanthocephala) was found in 29 of 34 individuals (85%), the infestation intensity ranging from 1 to 60 acanthocephalans per individual (12.44 ± 2.79; mean ± S.E.). A total of 361 worms were counted. The parasites were located predominantly in the anterior portion of the intestine, corresponding to the pyloric caeca, and in the midgut, penetrating the compact layer of the intestinal wall.

D. truttae belongs to the Acanthocephala phylum and is characterized by a retractable proboscis covered in hooks, typical of acanthocephalans, but is distinguished by the presence of numerous triangular-shaped spines on the anterior portion of its trunk. These structures are responsible for significant mechanical lesions to the host's intestinal mucosa. Histological analysis conducted on parasitized intestines showed that not only does the proboscis damage the intestinal

epithelium by penetrating the lamina propria-stratum granulosum, but, at the same time, the spines (Fig. 12B) encounter the adjacent mucosal layer, inducing destruction of the mucosal layer, which no longer has the normal structure (Fig. 12A).

D. truttae infestation caused extensive laceration of the intestinal mucosa, involving the lamina propria and the granular layer (Fig. 12A). This damage prevented mucous cells from secreting mucus near the parasite's attachment site, compromising the intestinal epithelial defense barrier. Despite this, a marked local inflammatory response was observed, characterized by the presence of numerous mast cells (MCs) (Figs. 12C, 12D) and some macrophages in the intestinal submucosa (Fig. 12D), particularly in the portion close to the parasite's proboscis and trunk, where most were degranulating. Numerous mucous cells and RCs were present in the non-damaged intestinal epithelium, some of which were already exhausted (Figs. 12E, 12F).

TEM observations of parasitized intestines highlighted a high presence of RCs (Fig. 13A) located in the intestinal villi distant from the infection site. The observed RCs were mature and characterized by a cell cortex, a basal nucleus, a cytoplasm loaded with releasing rodlets, and a highly heterochromatic nucleus. Further observations confirmed the presence of MCs in the contact zone between the mucosa and the parasite, showing an irregular shape, an eccentric and polar nucleus, and a cytoplasm loaded with electron-dense granules, in different maturation stages. Many of them appeared in close contact with blood vessels, present in the muscular layer (Fig. 13B), encircled by fibroblast-like ensheathing cells. Some releasing granules showed evident swelling, indicative of an active degranulation phase, and some of the granules were adjacent to MCs (Fig. 13C).

4.4 *Dicentrarchus labrax*

In forty-one *D. labrax* (Linnaeus, 1758) four different helminths were detected, including ectoparasites and endoparasites. The seabass were weighted (815.09 ± 49.71 g, mean ± S.E.) and measured in total length (41.71 ± 0.83 cm, mean ± S.E.). Twenty-seven seabass (65%) harboured third-stage larvae (L3) of *Contracaecum rudolphii A (*Hartwich, 1964), the intensity of infection ranged from 5 to 50 parasites per fish (16.92 ± 2.19, mean ± S.E.). At the same time, the presence of larvae of *Southwellina hispida* (Van Cleave, 1925) was assessed on the external intestinal surface of twenty seabass (48%), with a range of infection from 2 to 15 parasites per host (8.05 ± 1.14, mean ± S.E.).

Further observations revealed the presence of metacercariae and adults of *Timoniella imbutiforme* (Molin, 1859) in the alimentary canal of twenty-two seabass (53%), ranging from 10 to

100 helminths for fish (20.68 ± 4.55, mean ± S.E.). In addition, the gills of thirty-two *D. labrax* (78%) hosted a high number of monogeneans *Diplectanum aequans* (Wagener, 1857) Diesing 1858 (Monogenea, Diplectanidae), with the intensity of infection ranging from 15 to 1600 per fish (221.87 ± 61.47 mean ± S.E.). Only 10 sea bass (24%) recorded the simultaneous presence of all four different parasite taxa.

Examination of the gills during necropsy revealed that monogeneans were distributed mainly in the medial and apical areas of the primary lamellae, and in some cases, they were clustered (Fig. 14A). *D. aequans* specimens, as is typical for this species, were anchored to the gill by the dorsal and ventral spines of the squamodisc (opisthaptor) and by the amuli that penetrated deeply into the host tissue (Fig. 14C). Histological analysis of gill sections revealed that the movement of the anchoring organs caused erosion of the epithelium, penetrating deeply into the connective tissue, and resulting in the formation of cellular debris. The lamellae surrounding the opisthaptor showed intense cellular proliferation, which sometimes led to the fusion of adjacent lamellae (Fig. 14B), in some cases accompanied by small aneurysms of the blood vessels (telangiectasia). Mast cells (MCs) were free within the connective tissue outside the blood vessels (Fig. 14D)

At TEM the presence of mucous cells and RCs (Fig. 15A) near the *D. aequans* attachment site was documented; in the primary lamella, these cells were located close to the epithelial surface, presumably ready to release their contents. In both healthy and parasitized fish, MCs were documented in the primary lamella, basal to the mucous cells and RCs. The MCs had an irregular shape, a polar nucleus, and a cytoplasm rich in numerous electron-dense granules (Fig. 15B). In some parasitized sea bass, MCs were seen within blood vessels, and the degranulation was not observed in either healthy or parasitized specimens.

The digenean *T. imbutiforme* it has two suckers, one ventral (acetabulum) and one oral, with a crown of spines (Figs. 16A, 16B), a characteristic that distinguishes the species. The suckers allow the fluke to adhere firmly to the intestinal wall, causing direct mechanical damage to the host's tissues. In some histological sections, pieces of necrotic intestinal epithelium could be observed inside the suckers, confirming the active detachment of mucosal portions during adhesion (Fig. 16F). Histological analysis of parasitized intestines shows that, although the parasite does not penetrate deeply beyond the mucosa (Fig. 16C), the mechanical action of the suckers causes significant damage to the intestinal epithelium, resulting in necrosis and atrophy of the intestinal villi, and epithelial desquamation (Fig. 16D). The damage is not due to parasite penetration or migration but rather to the suction generated by the suckers, which creates localized destruction of the mucosa and compromises the continuity of the surface epithelium.

A protective tissue response is observed in the intestinal mucosa, with marked hyperplasia and hypertrophy of the mucous cells, increased mucus secretion, predominantly consisting of acidic glycoproteins (Fig. 16D), as evidenced by the positive AB staining. A strongly blue-stained mucus sheath can be observed around the fluke bodies, suggesting a physical isolation mechanism implemented by the host's intestinal epithelium (Fig. 16D). At the level of the lamina propria and in the immediate vicinity of the parasite, an inflammatory response characterized by the presence of MCs, often in degranulation, is observed (Fig. 16E).

The co-presence of larval forms of the nematode *C. rudolphii* (L3) and the acanthocephalan *S. hispida* was frequent in infected sea bass, consistent with the fact that both species use fish as paratenic hosts in their life cycle. During macroscopic analyses, most larvae were observed in the visceral peritoneal serosa (Figs. 17A, 17B, 17C), not fully encysted. Many of them (especially nematodes) were melanized, covered with dark brown spots. Histological analysis of intestinal sections confirmed the envelopment of the larvae within the peritoneal serosa (Fig. 17D) and revealed some encysted larvae within the muscular layer of the intestinal wall (Figs. 17E, 17F), particularly *C. rudolphii* larvae. Granuloma formation is observed around these larvae, a histopathological response aimed at isolating the parasite physically. However, the observed granuloma appears poorly developed, consisting of a single layer of connective tissue, without a marked fibrous encapsulation.

TEM revealed the presence of an immature granuloma surrounding the nematode cuticle, which appeared thick and clearly distinguishable (Fig. 18A). The granuloma was mainly composed of longitudinally arranged collagen fibers secreted by fibroblasts (Figs. 18B, 18C), characteristic of a chronic granulomatous response in which fibroblasts encircle the larva to isolate it from the surrounding tissue. In the innermost layer, adjacent to the nematode cuticle, several epithelioid cells and macrophages were observed (Figs. 18B, 18C).

4.5 Galeus melastomus

On two occasions (January and April 2023), fourteen specimens of *Galeus melastomus* (Rafinesque, 1810), five males and nine females. As in most elasmobranchs, the intestine of *G. melastomus* is composed of the spiral intestine, which is the longest intestinal region, placed between two short tracts, namely, the proximal and distal intestine. The spiral intestine is composed of the typical layers: mucosa (including epithelium and lamina propria), submucosa, muscularis, and serosa. The internal spiral fold itself, however, consists only of the epithelial layer and the lamina propria-submucosa (Fig. 19A). Analysis conducted on the black mouth shark intestine showed a

large type of granular cell in the intestinal epithelium displaying a goblet-like morphology (Fig. 19B). This granular cell was located both in the epithelium of the internal spiral fold and in the intestinal wall, extend from the basement membrane to the luminal surface, where they release their cytoplasmic contents (Fig. 19E). Hematoxylin/eosin (H&E) staining revealed that these granular cells are eosinophilic (Figs. 19B, 19F), whereas AB/PAS staining selectively highlighted the mucous cells, which appeared blue to blue-purple/magenta (Figs. 19C, 19D). Within the central region of the spiral fold (submucosal lamina propria), various immune cell types, including neutrophils, eosinophilic cells, and basophils, were observed, forming the intestinal lymphomyeloid population (Fig. 20A, 20B).

Macroscopic examination of the alimentary canal revealed no evidence of parasitic infection. However, histological analysis identified the presence of coccidian oocysts within the intestinal epithelium (Figs. 20C–E), many of which were located near the basement membrane. Coccidian infection was associated with necrosis of the intestinal epithelium.

Under transmission electron microscopy, the granular epithelial cells appeared ovoid and broad, containing a cytoplasm rich in swollen, electron-dense granules (Figs. 21A, 21C). In several sections, these cells were found at the apical surface of the epithelium, with the apical membrane open and releasing their cytoplasmic contents into the intestinal lumen (Figs. 21B, 21C). At this level, they formed junctional complexes with adjacent epithelial and mucous cells (Fig. 21C). Mucous cells were also visible at the apical portion of the epithelium, some of them actively secreting mucus into the lumen (Fig. 21D).

4.6 *Raja clavata*

Fifteen specimens of the thornback ray *R. clavata* (L.), ranging from 1 to 1.5 kg, were analyzed for this study. Their intestines display the typical subdivision (Fig. 22A) of most elasmobranchs, such as *G. melastomus* (Fig. 19A). No metazoan parasites were found during dissection, and histological analyses revealed no microscopic infections. Histological analysis showed the presence of three types of granular cells (I, II, and III), having different locations, morphology, and staining characteristics.

Type I granular cells were found at the base of the intestinal epithelium, closely adhering to the basement membrane (Fig. 22B). They were oval or slightly irregular in shape, with the cytoplasm completely occupied by fine, homogeneous granules. These granules were negative for PAS staining, indicating the absence of neutral mucopolysaccharides or glycoprotein complexes, while the

cytoplasm showed intense eosinophilic staining, suggesting a predominantly protein composition (Fig. 22D). By transmission electron microscopy, type I cells appeared large, oval in shape, and located within the folds of the intestinal mucosa close to the membrane basement (Fig. 23A). The nucleus was sometimes eccentric, with predominantly heterochromatic chromatin, indicating a low level of transcriptional activity (Figs. 23C). The cytoplasm contained numerous spherical/oval electron-dense granules (Fig. 23D) and several round or ovoid mitochondria, distributed near the nucleus and the cytoplasmic granules (Fig. 23D).

Type II granular cells were located in the lamina propria submucosa (Fig. 22C). They appeared oval in shape (Fig. 22D), with a cytoplasm rich in large, acidophilic granules that were negative for PAS staining. Clear degranulation of type II granular cells was observed in several sections (Fig. 22B, 22C), a phenomenon indicative of the release of granular contents into the surrounding microenvironment in response to inflammatory or immune stimuli. Ultrastructural analysis confirmed these observations: type II cells had an oval shape, with an eccentric, heterochromatic nucleus (Fig. 24A). The cytoplasm was entirely occupied by large, round, electron-dense granules. This abundance of granules and their compact morphology suggests a high secretory activity, consistent with the effector function of immune cells or support of the inflammatory response.

Type III granular cells were abundant in the lamina propria-submucosa (Figs. 22C, 22D) and, are distinguished by their elongated morphology. The granules were fine, weakly acidophilic and PAS-negative, uniformly distributed in the cytoplasm, which appeared clear or grayish-white in histological section (Fig. 22B, 22C). This coloration of the granules suggests a different granular composition compared to the other two types. Under the electron microscope, type III cells show an irregular shape (24B) and a nucleus with a non-uniform outline (Fig. 23B). The cytoplasm contains both large, round, electron-dense granules and elongated granules, some of which contain rod-shaped inclusions (Fig. 24C, 24D), potentially attributable to structures for the storage or secretion of bioactive substances.

5) DISCUSSION

5.1 Anguilla anguilla- Acanthocephalus rhinensis system

The survey conducted at Piediluco Lake highlighted the presence of intestinal infestation in eels (*A. anguilla*) by the acanthocephalan *A. rhinensis*. Over the last three decades, the European eel population has undergone a drastic decline due to several factors, such as habitat loss, pollution, climate and ocean changes, and parasites (Dekker, 2003; Jacoby et al., 2015).

A. rhinensis was first recorded in 2008 in the Rhine River in Germany (Amin et al., 2008) and for the second time in Italy in 2012 in Piediluco Lake (Dezfuli et al., 2012b). There is no communication between these two waterways, and there is no information on how the parasite arrived in Piediluco Lake; maybe eels from the Rhine River were introduced into the lake for food aims, hiding the parasite.

Acanthocephalans, during their life cycle, require the presence of an intermediate host, usually an invertebrate. The gammarid *Echinogammarus tibaldii*, a small invertebrate at the base of the food chain of several fish species inhabiting the lake, was identified as the intermediate host of *A. rhinensis* (Dezfuli et al., 2012b). This, moreover, is also an intermediate host for other acanthocephalans such as *D. truttae, E. truttae, P. minutus* (Dezfuli et al., 2008a), and the cestode *Cyathocephalus truncatus* (Dezfuli et al., 2010a). Although the gammarid is at the base of the food chain, no other species except eels showed infestation by this acanthocephalan during dissection and histological analyses. This suggests that *A. rhinensis* shows specificity for its definitive host, a topic described in Poulin (2007). From an evolutionary perspective, specificity is the result of a complex balance between physiological constraints, adaptation, and ecological opportunism (Adamson & Caira, 1994). The mechanisms that determine it include immunological compatibility, parasite localization within the host, and reciprocal coevolution between the two species.

Despite the inability to penetrate beyond the lamina propria, *A. rhinensis*, through its hooked proboscis, caused considerable damage to the intestinal mucosa, including destruction of villi and loss of epithelial architecture in colonized areas. The pathogenicity of intestinal helminths is attributed to two factors: the first is density, the number of worms present in the organ, and the second is the worm's penetration capacity (Mackiewicz et al., 1972; Bosi et al., 2022b). The intestinal canal represents an excellent environment for helminths as it is rich in nutrients and offers them protection (Buchmann, 2014), and the damage they cause in it could affect intestinal physiological functions, such as nutrient absorption, compromising the host's health. The alimentary canal of vertebrates is

protected by a layer of mucus secreted by mucous cells, which acts as a physical barrier and has the function of lubricating, hydrating, aiding digestion, and trapping pathogens (Corfield et al., 2000; Grondin et al., 2020). In fish, mucosal membranes are constantly exposed to numerous pathogens, and mucin secretion increases under pathological conditions. Indeed, in the eels examined, hyperplasia and hypertrophy of mucous cells were present in the infected intestines, which aim to strengthen the intestinal mucosal barrier. Most of the mucous cells observed were positive for Alcian blue staining, highlighting that the mucous cells had a higher content of acidic glycoconjugates compared to mixed conjugates, and this finding is consistent with observations reported in previous studies (Dama & Pathan, 2019; Bosi et al., 2020; Sayyaf Dezfuli et al., 2024b). The increase in acidic mucins is associated with a higher viscosity of the secreted mucus, allowing for greater protection of the mucosa (Bosi et al., 2017).

In addition to mucous cells, other immune cells, such as RCs and MCs, also increase in number at *A. rhinensis* attachment sites. RCs are immune cells unique to teleost fish and have been described in several studies as pear-shaped cells, characterized by a distinctive cortex, a basal nucleus, and typical club-shaped inclusions called rodlets (Reite, 2005; Reite & Evensen, 2006; Dezfuli et al., 2008a). In the presence of pathogens or foreign bodies, RCs increase in number, and mature RCs, observed in intestinal sections of infected eels by TEM, show a morphology and positioning consistent with a role in releasing substances with antimicrobial or pro-inflammatory activity into the intestinal lumen, potentially aimed at modulating the presence of the parasite. An immunohistochemical study conducted on eels from Piediluco highlighted the presence of antimicrobial peptides such as lysozyme and i-NOS (inducible nitric oxide synthase) (Bosi et al., 2018), underlining their involvement in the immune response.

Another noteworthy element is the presence of MCs in the submucosa, with evident degranulation activity at the proboscis attachment sites. MCs are present in all vertebrates and possess the same characteristics and functions (Mulero et al., 2007; Baccari et al., 2011), playing an active role in attracting and activating other immune cells by releasing their contents through degranulation. The granules contain various inflammatory mediators (Silphaduang et al., 2006; Dezfuli et al., 2010b; Salger et al., 2016), serotonin (Dezfuli et al., 2000, da Silva et al., 2017), and histamine (Mulero et al., 2007; Salim et al., 2012; Galindo-Villegas et al., 2016). The present study has documented the histological changes caused by *A. rhinensis* in the intestine of *A. anguilla*. The high intensity of infection and the penetration of the worm's proboscis induced severe damage to the intestinal wall.

5.2 Squalius tenellus- Caryophyllaeus brachycollis system

The investigation conducted on the chub *S. tenellus* is part of a Bosnia and Herzegovina project under European environmental protection directives; the species was introduced into the park more than 100 years ago (Kottelat & Freyhof, 2007). The results obtained in the two sampling campaigns in Blidinje Lake reveal a high prevalence of intestinal infection by *C. brachycollis*, reaching unexpectedly extreme numbers in some individuals (416 worms per host). There are scarce information on the parasite fauna of that environment, but these data suggest that the parasite is well established in the local fish population. *C. brachycollis* has been reported in the literature in *S. cephalus* in Turkey (Yigit & Ozturk, 2016), but the first record of this cestode in *S. tenellus* in Blidinje Lake is reported by Sayyaf Dezfuli et al. (2024b), describing the molecular analysis conducted on individual worms to confirm their species.

Infection in the examined chub affected the entire intestinal tract but was more marked in the anterior and middle portions. Mucus accumulation was macroscopically visible around the parasites, an attempt by the host to isolate the worms. Most tapeworms do not cause considerable damage to the host's intestine; the main damage is the destruction of the epithelium covering the villi, causing necrosis and mild inflammation (Santos et al., 2017; Barčák et al., 2021). However, some tapeworm species, such as *M. wageneri* (Dezfuli et al., 2011b), can reach the intestinal muscular layer, causing more intense inflammation. Similarly, in three individuals, *C. brachycollis* was able to perforate the intestinal wall, with its strobilus visible in the body cavity. This represents a severe pathological condition and indicates the worm's ability to cause structural and potentially lethal damage to the host. Another peculiarity of this cestode is the ability to modify its scolex, assuming different shapes that allow greater adaptability to the epithelial surface.

Intestinal parasites can cause various mechanical damages, such as tissue erosion and necrosis, intestinal villous atrophy, epithelial detachment, and, in severe cases, muscle lesions. These events lead to the onset of intestinal inflammation, confirmed by the presence of numerous immune cells. Mucous cells represent a component of the innate immune system (Johansson & Hansson, 2014; Salinas, 2015; da Silva et al., 2017; Cabillon & Lazado, 2019; Bosi et al., 2022a; Sayyaf Dezfuli et al., 2023a), and play a fundamental role in host defense through the secretion of mucus, which has multiple protective functions (Bosi et al., 2017, 2022a; Schröeder, 2019; Souza et al., 2019; Sayyaf Dezfuli et al., 2021a, 2024b). Today, mucus is not considered only a passive barrier but a dynamic and biologically active medium, rich in molecules with immune functions, such as lectins, antimicrobial peptides, immunoglobulins, lysozymes, and mucins (Ellis, 2001; Sharpe et al.,

2018). Mucins contained in mucous cells are important for initial protection against intestinal parasites (Sharpe et al., 2018; Bosi et al., 2020; Sayyaf Dezfuli et al., 2021a; 2023a). In *S. tenellus*, parasite-induced tissue damage increased the number of mucous cells, and histological examination of sections from infected chub revealed that most mucous cells contained acidic glycoconjugates compared to neutral glycoconjugates. However, a depletion of these cells was observed at sites adjacent to the scolex, suggesting a potential local immunosuppressive effect exerted by the *C. brachycollis*. In fact, this tapeworm possesses a scolex glandular complex capable of producing secretory granules; the presence of this substance inhibits the mucus layer covering the intestinal folds, suggesting a possible antagonistic interaction between the secretion of the parasite's frontal glands and the host's intestinal mucus (Sayyaf Dezfuli et al., 2024b). In addition, the adhesive substance enables *C. brachycollis* to attach firmly to the intestinal wall of the chub (Sayyaf Dezfuli et al., 2024c).

RCs were abundant in the intestinal epithelium, and in some cases, even in the esophageal epithelium of severely infected individuals. RCs are secretory cells and proliferate in response to tissue damage or in the presence of pathogens or other stressors (Leino et al., 1996; Bosi et al., 2018; Sayyaf Dezfuli et al., 2022). These cells are recruited and mobilized in response to microparasites such as viruses (Sulimanović et al., 1996), bacteria (Bosi et al., 2018; Salinas et al., 2008), protozoa (Sitjà-Bobadilla et al., 2016; Sayyaf Dezfuli et al., 2022, 2023b), and in the presence of macroparasites (Sayyaf Dezfuli et al., 2021b, 2024a). Their increased presence at the site of infection provides further evidence of their role in the immune system; their peri-parasitic distribution and their altered morphology observed by TEM (poor cytoplasm, deformed cortex) suggest intense secretory activity and direct involvement in the local immune response in the intestine of *S. tenellus*. RCs are often associated with the presence of other immune cells such as MCs, neutrophils, and macrophages.

MCs play a key role in both innate and adaptive immune defense (Jain et al., 2019; Katsoulis-Dimitriou et al., 2020; Wang et al., 2024). In fish, they are characterized by an irregular shape and an eccentric nucleus with cytoplasmic granules (Jain et al., 2019; Sayyaf Dezfuli et al., 2024b). They are in connective tissue and often near blood vessels (Abraham & John, 2010), a strategic location for the defense role these cells play (Reite & Evensen, 2006; Sayyaf Dezfuli et al., 2021a, 2023a). In the present study, a high number of MCs was observed in the lamina propria-submucosa, especially in sites adjacent to the parasite, where numerous neutrophils were also co-present, and both cell populations were often in a state of degranulation. Neutrophils represent the first immune cell population to arrive at the site of injury (Havixbeck & Barreda, 2015; Bader et al., 2021)

and play an essential role in the innate defense against various microorganisms (Buchmann, 2022). However, parasites are larger than viruses, and therefore neutrophils cannot phagocytose them but can release their proinflammatory granules.

Monocytes are the precursors of macrophages, which undergo differentiation upon recruitment to the site of infection. They play a significant role in linking the innate and adaptive immune responses (Grayfer et al., 2018). These cells can regulate the inflammatory response, promote the repair of damaged tissues, and maintain immune system homeostasis (Cao et al., 2015; Oishi & Manabe, 2018). Macrophages sometimes contain melanin, lipofuscin, or hemosiderin pigments and can form clusters called melano-macrophage centers or macrophage aggregates (MAs) (Agius & Roberts, 2003; Sitjà-Bobadilla et al., 2015; Sayyaf Dezfuli et al., 2016, 2021a; Stosik et al., 2019), which have also been observed in the intestinal submucosa of *S. tenellus*. *C. brachycollis* infection poses a significant threat to the health of the chub *S. tenellus* in Blidinje Lake. The combination of high prevalence, high intensity of infection, and severe tissue damage suggests that this cestode may have negative effects on host survival and physiological performance. Furthermore, the presence of sophisticated adhesion mechanisms, local immunosuppressive effects, and a complex immune response indicates that the host-parasite relationship is highly dynamic and warrants further investigation.

5.3 Salmo trutta- Dentitruncus truttae system

In trout (*S. trutta*) infected by *D. truttae*, the pathogenic action is primarily mechanical; in fact, the spines present in the anterior portion of the trunk had a significant damaging impact, demonstrated by deep lacerations in the intestinal mucosa. This, combined with the hooks present on the proboscis, has led to a loss of the protective mucus layer, involving the deeper layers, such as the lamina propria and the granular layer, reducing the absorptive capacity and mucus production of the mucous cells. The inability of the mucous cells to secrete mucus near the parasite exposes the epithelium to further mechanical aggression. However, a local inflammatory response is present, and the submucosa shows a significant infiltration of mast cells, many of which were in degranulation, and some macrophages.

MCs are essential components of the innate immune system and have a secretory function (da Silva et al., 2017; Sayyaf Dezfuli et al., 2021a, 2023a). MCs in infected trout were located near the parasite's anchorage site, many in the degranulation phase, and close to blood vessels and collagen fibers in the compact layer. Electron and light microscopic observations confirmed MCs

activation, with morphologies typical of the degranulation phase (swollen granules, release of mediators), suggesting a key role in regulating the local immune response and tissue repair. Fibroblasts have often been observed in proximity to MCs in infected *S. trutta*, able to influence their motility and their function, suggesting that they are involved in tissue remodeling processes (Temkin & McMillan, 1986; Rocha & Garcia; 2007). The close association between MCs and capillary endothelial cells increases the suggestion that these cells migrate across the endothelium and are involved in immune cells recruitment (Dezfuli & Giari, 2008). MCs react to parasite invasion by releasing their granular contents (Sayyaf Dezfuli et al., 2021a,b) activated by tissue damage (Flaño et al., 1996), and attracting other immune cells.

Micrographs show the presence of mature RCs in intestinal villi distant from the infection site. These RCs have a morphology compatible with active cells: their cytoplasm is loaded with rodlets, highlighting an active role in defense. It has been shown that they can express immune molecular markers such as lysozyme, which strengthen the defense system (Bosi et al., 2018). RCs are often considered an indicator of physiological stress in fish, and their presence reflects the systemic response to infection, the presence of RCs has been observed in various tissues such as the gills, intestine, heart, gonads, brain, thymus, liver, spleen, and kidneys (Sayyaf Dezfuli et al., 2022; Sayed et al., 2025). In fish, they are often associated with other inflammatory cells such as MCs and are recruited in response to helminthic parasites or other pathogens (Matisz et al., 2010; Sayyaf Dezfuli et al., 2023a,b, 2024a).

The data collected suggest the activation of a robust immune response by trout against the parasite, but it is insufficient to contain tissue damage, especially in cases of massive occurrences of parasites. *S. trutta* infection due to *D. truttae* in the Nera River represents an emblematic case of host-parasite interaction with significant pathological and immunological implications. Histological and ultrastructural observations highlight the activation of several components of the innate immune system, particularly MCs and RCs, demonstrating the complexity of defense responses in teleosts.

5.4 Polyparasitism in *Dicentrarchus labrax*

D. labrax (L.) is one of the most important marine species for fish farming in Italy and in the countries bordering the Mediterranean Sea and Europe (Lotfy et al., 2023). In 2020, according to the FAO report, its production was 243,900 metric tons (FAO, 2022; Abdel-Rahim et al., 2023). The genus Diplectanum is the largest of the Diplectanidae family (Hayward, 1996) and is widely distributed in the Mediterranean and in the Atlantic areas that coincide with the host's range. *D.*

aequans is the most common parasite species in both farmed and wild sea bass. Monogeneans are widespread in the Mediterranean and the mortality of several farmed species has been attributed to them: examples are *Microcotyle* (Van Beneden & Hesse 1863) in *Sparus aurata* L. (Alvarez-Pellitero & Crespo, 1995; Padrós & Crespo, 1995) and *Zeuxapta seriolae* (Meserve 1938) in *Seriola dumerili*, Risso (Grau et al., 2003; Montero et al., 2004). Monogeneans possess an anchoring and feeding system that induces various histopathological changes in the epithelium, sometimes generating secondary infections caused by bacteria and/or viruses, which have a greater impact on the health of the host (Stoskopf, 1993; Cone, 1995). Many studies report the histological damage caused by monogeneans (Hayward et al., 2001; Padrós et al., 2001; Ogawa, 2002; Buchmann et al., 2004; Montero et al., 2004; Mansell et al., 2005; Dezfuli, 2007).

The gills in fish constitute one of the first lines of contact with the external environment as they are the site where gas exchange, ammonium excretion, and ionic regulation occur, and the presence of monogeneans stimulates the release of mucus from the gills, sometimes inducing hypoxia (Chaves et al., 2006). Analysis of the parasitized gills during dissection revealed that they were covered with a whitish mucous exudate, and histological sections confirmed excessive mucus secretion due to hyperplasia and hypertrophy of mucous cells. Some studies have reported that fish are able to generate a protective response against monogeneans and sometimes even eliminate them (Buchmann & Lindenstrøm, 2002; Lindenstrøm et al., 2003). When *D. labrax* is infected with *Diplectanum* species, histological analyses show severe damage to the primary and secondary lamellae, accompanied by an infiltration of mononuclear cells (Eissa et al., 2017). As consequence, monogeneans affect the growth range, health, and economic performance of the fish population (Ogut & Uzun, 2014).

MCs, present in all teleosts in various tissues and organs, have been documented in the primary lamella of infected sea bass (Murray et al., 2007; Dezfuli & Giari, 2008; Sayyaf Dezfuli et al., 2023a). They are motile cells often observed in the vicinity of capillaries (Vallejo & Ellis, 1989; Reite & Evensen, 2006). There is evidence of their migratory capacity (Dezfuli & Giari, 2008), which enables them to cross the endothelium of the vessels and reach the destination tissue (Murray et al., 2007). Several studies have shown that MCs release their contents upon degranulation, following exposure to various stimulants and pathogens (Ellis, 1985; Sharp et al., 1989; Vallejo & Ellis, 1989; Flaño et al., 1996; Murray et al., 2007; Dezfuli et al., 2008a; Manera et al., 2011). In our study, no release of MC granules was observed in either healthy or parasitized gills.

Mucous cells and some RCs have been observed far from the monogenean attachment site; these are present in epithelia of different organs (Morrison & Odense 1978; Manera & Dezfuli 2004).

A high number of RCs have been recorded in hosts in response to a wide range of parasite infections (Leino 1996; Sayyaf Dezfuli et al., 2021a,b,2024a). The monogenean can generate erosion at the gill level and fusion of the lamellae, which could compromise oxygenation and increase the risk of mortality.

Histological analyses of the sea bass intestine have revealed the presence of a digenean, *T. imbutiforme*. Its life cycle includes different definitive and secondary intermediate hosts, with *D. labrax* as the main definitive host. Although *T. imbutiforme* does not penetrate beyond the intestinal mucosa, the adhesion mechanism through the suckers causes direct mechanical damage, such as epithelial desquamation. The histopathological reactions observed are hyperplasia of the mucous cells with the production of mucus that envelops the parasite to isolate it. As previously mentioned, mucus secretion represents one of the first lines of defense for the host. Mucus is secreted continuously and has not only an immune but also a physiological function (Lamont 1992; Hansson 2012; Dupont et al. 2014). Abundant mucin secretion has been described in several helminth-host systems (Bosi et al. 2005a,b, 2015; Bosi & Dezfuli 2015; Sayyaf Dezfuli 2016; current study). The role of this increased secretion is not clear. In mammals, it is thought to help expel worms (Peterson & Artis 2014; Zaph et al., 2014), but it is not sufficient when the parasite is an acanthocephalan (e.g., *Pomphorhynchus laevis*) or a cestode (e.g., *Monobotrium wageneri*), which can adhere firmly to the host's intestine. Therefore, mucus is considered to act as a protective layer of the intestinal mucosa from mechanical or chemical damage due to parasite invasion (Dezfuli et al. 2010a; Bosi & Dezfuli 2015; Castro & Tafalla 2015; Bosi et al. 2017). Several digeneans are enteric parasites of fish; their site of infection is almost entirely restricted to the paramucosal lumen, mucosa, or epithelial tissues (Dezfuli et al., 1997; Paperna & Dzikowski, 2006). The main pathology caused by digeneans is destruction of the epithelium covering the villi, with necrosis and degeneration of the epithelial cells (Dezfuli et al., 1997; Mladineo, 2006; current study).

Co-infection with larvae of *C. rudolphii* A and of *S. hispida* affected 65% and 48% of the sea bass analyzed, respectively. Both species, using sea bass as paratenic host, cause tissue damage and trigger evident immune responses, such as melanization, observed macroscopically in many nematode larvae. This melanization is an innate immune response of fish aimed at isolating and neutralizing foreign bodies through the accumulation of melanin around the parasite, associated with larval death.

The granulomas observed around the encysted larvae (especially *C. rudolphii*) were poorly developed and lacked fibrous encapsulation, suggesting a moderate chronic response, not effective enough to eliminate the parasite but useful for containing its systemic effect. The absence of massive

mast cell (MC) degranulation could indicate an immune tolerance mechanism or an advanced stage of infection in which the acute response has subsided. In contrast, a massive encapsulation was observed in eels from the Canneviè lagoon infected with *C. rudolphii* larvae (Sayyaf Dezfuli et al., 2024a). The substantial difference between eels and sea bass is the formation of the granuloma; in the eels, the granuloma is composed of several layers of immune cells (Sayyaf Dezfuli et al., 2024a), while in the sea bass, such stratification is not observed in fact, the granuloma is poorly developed, indicating a moderate response to the presence of nematode and acanthocephala larvae. The granuloma is considered a chronic inflammatory lesion that appears as a nodule in the host organ (Ferguson, 2006). The granuloma production in hosts infected by extraintestinal helminths has already been documented (Weber et al., 2022; Sayyaf Dezfuli et al., 2024a, 2025; Kumas et al., 2025). Many studies report the ability of helminths to manipulate or evade the host immune response (Secombes & Chappell, 1996; Buchmann, 2012; Sayyaf Dezfuli et al., 2021a, 2023a), and parasite encapsulation is seen as an adaptive method of the parasite to evade the immune response, that allows the survival of both so the parasite can reach the definitive host. Furthermore, encapsulation allows the host to prevent the parasite from migrating to other visceral organs (Buchmann, 2012).

Epithelioid cells, fibroblasts, and macrophages are present in the *D. labrax* granuloma. Epithelioid cells constitute the innermost layer of the lesion, in direct contact with the parasite; most macrophages present in the granuloma appear differentiated into epithelioid cells, which form a compact barrier that delimits and isolates the parasite from the surrounding host tissue. Monocytes/macrophages execute phagocytosis during inflammation, a process mediated by membrane receptors and target particles (Grayfer et al., 2018). These cells are essential for epithelial renewal and for maintaining mucosal homeostasis, regulating inflammatory responses (Estensoro et al., 2014). Fibroblasts are also present within the granuloma and play a crucial role in initiating tissue repair and regeneration processes (Sayyaf Dezfuli et al., 2023a, 2025).

D. labrax represents an excellent model for studying polyparasitism in the site of study, hosting several ectoparasites and endoparasites, playing a fundamental ecological role, supporting the cycle, and ensuring the stability of these four parasite species at the study site.

5.5 Immune cells in *Galeus melastomus*

Histological and ultrastructural analysis of the intestine of *G. melastomus* has provided information on the organization and cellular composition of the digestive tract of this elasmobranch. The general structure of the intestine, divided into the duodenum, spiral intestine, and colon, follows

the typical pattern described for most chondrichthyans (Holmgren & Nilsson, 1999). The spiral intestine, a morpho-functional adaptation that allows for an enlarged absorptive surface while maintaining a short intestinal length, is composed of mucosa, submucosa, muscular layer, and serosa, while the internal spiral fold is composed exclusively of epithelium and lamina propria.

Three main cell types have been identified within the epithelium: mucous cells, enterocytes, and granular cells. The latter, dome-shaped and with a cytoplasm rich in eosinophilic granules, display secretory characteristics, as indicated by their extension from the basement membrane to the intestinal lumen and by the observation of open apical portions ready to release the cytoplasmic content. Immunohistochemical analyses have shown that epithelial granular cells are positive for TNF-α but not for i-NOS, IL-6, IL-1β, lysozyme, and serotonin (Sayyaf Dezfuli et al., 2018a). Based on these observations, epithelial granular cells are epithelial in nature and play a defensive role in the blackmouth shark. It is therefore plausible that these cells participate in both digestive processes and mucosal defense mechanisms through the secretion of bioactive substances. The presence of a lymphomyeloid population consisting of neutrophils, eosinophils, basophils, lymphocytes, and thrombocytes has been observed in the lamina propria of the spiral valve. This observation confirms the role of the intestine as an important site of local immune defense, consistent with studies describing gut-associated lymphoid tissue (GALT) in chondrichthyes, considered an essential component of the immune system (Zapata et al., 2006).

Elasmobranchs are, in fact, the most primitive vertebrates with an adaptive immune response and true lymphoid organs, such as the thymus, spleen, and GALT (Zapata et al., 1996). In the spiral intestine of *G. melastomus*, the GALT is organized as a lympho-myeloid aggregate, like that described in other elasmobranchs (Lauriano et al., 2019). Histological and ultrastructural analyses have highlighted the presence of granulocytes with distinct types of cytoplasmic granules in the GALT. Granulocytes have been classified into three types based on their ultrastructural characteristics and staining properties: heterophilic or fine eosinophilic granulocytes, granulocytes like mammalian neutrophils and coarse eosinophilic granulocytes (Smith et al., 2019). All three types have been recognized in the GALT of *G. melastomus*, along with basophils, lymphocytes, and thrombocytes. Precise identification of immune cells in elasmobranchs, however, remains challenging due to species-specific variations and dye differences (Hine & Wain, 1987).

Immunohistochemical reactions have also highlighted cells positive for the biogenic amines histamine and serotonin, localized in proximity to blood vessels and the epithelial basement membrane (Bosi et al., 2024). These substances play a key role in the intestinal inflammatory response in the presence of parasitic infections (Sayyaf Dezfuli et al., 2018b, 2021a, 2023a). In

mammals, the release of histamine and serotonin is induced by the binding of the FCεRIγ receptor to the antigen-IgE complex (Kraft & Kinet, 2007). Also, in the GALT of *G. melastomus*, cells immunoreactive for FCεRIγ, histamine, and serotonin were found, suggesting that the amine release mechanism is evolutionarily conserved, and together with these, further markers such as TLR2, CD4, lysozyme, IL-6 and TNF-α were revealed (Bosi et al., 2024). The microbial pattern recognition receptor (TLR2) is involved in the activation of macrophages and dendritic cells (Anandhakumar et al., 2012; Korenaga et al., 2013). Lysozyme is an enzyme with bactericidal activity also found in other cells, such as RCs in eels (Bosi et al., 2018). CD4-positive cells, likely mature macrophages, participate in antigen presentation and regulation of the immune response (Schridde et al., 2017; Shaw et al., 2018). The cytokines IL-6 and TNF-α, detected in both macrophages and epithelial granule cells, play roles in mediating inflammation and regulating the immune response (Pérez-Cordón et al., 2014; Sayyaf Dezfuli et al., 2018a).

Although no pathogens were observed during dissection, histological examination revealed the presence of coccidian oocysts localized in the intestinal epithelium, often near the basement membrane, associated with necrosis. These infections, known in various marine fish (Paperna, 1995; Alvarez-Pellitero et al., 2008), can cause epithelial damage of varying degrees depending on the host's immune response. The necrosis detected in *G. melastomus* indicates an active pathogenic effect of coccidia and highlights the importance of microscopic examinations to identify infections that are not macroscopically evident.

Overall, the results confirm the structural and functional complexity of the *G. melastomus* intestine, highlighting its dual digestive and immunological function. The identification of granular cells with potential secretory activity, together with the presence of a complex lymphomyeloid compartment, demonstrates the high degree of specialization of mucosal defenses in elasmobranchs.

5.6 Granular cells in *Raja clavata*

Mucosal surfaces of fish, including the gut, constitute the largest area of the organism and play a primary role in the defense against pathogens, being highly active sites from an immune point of view (Parra et al., 2015). Mucosal barriers are constituted by different cell types, including epithelial cells, neuroendocrine cells (Holmgren et al.,1992; Bosi et al., 2015), mucous cells (Bosi et al., 2015, 2017), and various categories of immune cells (Secombes & Ellis, 2012; Rombout et al., 2014), among the latter, mast cells (Buchmann, 2012; Galindo-villegas et al., 2016) and neutrophils (Havixbeck & Barreda, 2015; Pijanowski et al., 2015; Havixbeck et al., 2016). The immune cells

present in the spiral intestine of cartilaginous fish have been the subject of limited studies, and knowledge regarding the mechanisms of innate immunity in the intestinal tract of elasmobranchs remains scarce. Histological and ultrastructural analysis of the intestine of *R. clavata* revealed the presence of three diverse types of granular cells (I, II, and III), characterized by specific locations, morphologies, and staining properties. These cells, distributed between the epithelium and lamina propria, represent key components of this elasmobranch's mucosal immune system, contributing to the protection of the intestinal tract against potential pathogens and modulating local inflammatory responses.

Type I granule cells of *R. clavata* can be considered epithelial granule cells, as they are located exclusively in the epithelium and intimately associated with the basement membrane. Morphological and ultrastructural observations clearly demonstrate a secretory function: the cytoplasm is completely occupied by electron-dense, homogeneous, and intensely eosinophilic granules, indicative of a high concentration of protein substances. The absence of a positive PAS stain excludes the presence of neutral mucopolysaccharides or glycoproteins, suggesting instead the presence of enzymatic or antimicrobial protein components. These cells resemble mast cells or Paneth-like cells, described in the intestinal tract of other chondrichthyans (Sayyaf Dezfuli et al., 2018a; Lauriano et al., 2019), and suggest their possible involvement in the secretion of substances with antimicrobial or immunomodulatory action, such as lysosomal enzymes or the cytokine TNF-α. In *R. clavata*, epithelial granular cells express lysozyme and show immunoreactivity for mast cell anti-tryptase and anti-TNF-α (Sayyaf Dezfuli et al., 2018a). Similar or homologous molecules are also present in mammalian Paneth cells, which are known to represent a first line of defense in the intestine, thanks to the production of lysozyme and defensins with antibacterial activity (Bel et al., 2017). The presence of TNF-α in type I granular cells further strengthens the hypothesis of their role in the regulation of the immune response. TNF-α is a multifunctional cytokine, implicated in numerous physiological and pathological processes (Chu, 2013), including epithelial proliferation and modulation of the inflammatory response. It participates in the regulation of the production of other cytokines and the activity of immune cells. In mammals, Paneth cells have also been identified as a source of TNF-α, as demonstrated in humans (Tan et al., 1993), mice (Schmauder-ChockStephen et al., 1994) and rats (Seno et al., 2002). Overall, the evidence indicates that type I granule cells actively participate in the innate defense mechanisms of the spiral intestine of *R. clavata*. Numerous similarities in terms of location, morphology, and expression of lysozyme and TNF-α suggest a functional convergence between Paneth cells and type I granule cells.

Type II granule cells, prevalent in the submucosa-lamina propria and near the capillaries of

the spiral intestine of *R. clavata*, have large, electron-dense, PAS-negative granules and are often observed in the degranulation phase. This latter phenomenon is particularly significant, as it indicates secretory activity induced by immune or inflammatory stimuli. Their perivascular location, along with their oval morphology and eccentric heterochromatic nucleus, suggests that these cells may be functionally like activated granulocytes or tissue MCs. These cells show morphological similarities to intestinal MCs described in some teleosts, such as *Salmo trutta* (Dezfuli et al., 2008). Common features include the ovoid shape of the cell, the presence of an eccentric heterochromatic nucleus, and numerous large, electron-dense, round cytoplasmic granules. These cells did not test positive for any of the antibodies used in another study (Sayyaf Dezfuli et al., 2019); therefore, they cannot be considered analogous to MCs (Sayyaf Dezfuli et al., 2019).

Type III granule cells represent the predominant population among the granule cells present in the spiral intestine of *R. clavata*. Morphologically, they show characteristics like those of bony fish neutrophils (Flerova & Balabanova, 2013), with similarities in cell shape, nuclear arrangement, and ultrastructural characteristics of the cytoplasmic granules. The presence of elongated, axial, rod-shaped inclusions within the granules is an important diagnostic element supporting a possible identification as neutrophils. All type III granule cells contain acidophilic granules, which are abundant in the lamina propria-submucosa. They show an elongated shape and a cytoplasm rich in granules of different electron density, some of which contain rod-shaped inclusions, indicating a possible secretory activity. This morphological complexity could reflect a functional differentiation compared to the other two types of granular cells. Immunohistochemical analyses have highlighted a positivity for the anti-IL6 antibody in some type III cells (Sayyaf Dezfuli et al., 2019). Interleukin 6 (IL-6) is a multifunctional cytokine that plays a key role in the regulation of innate and adaptive immunity (Naka et al., 2002). IL-6 is not normally synthesized under physiological conditions, but its expression rapidly increases in response to inflammatory stimuli, viral or bacterial infections, or the action of other pro-inflammatory cytokines (Castellana et al., 2008). In mammals, IL-6 is produced by numerous cell types, including neutrophils (Van Snick, 1990; Hirano, 1998; Joubert et al., 2001). This dual function of IL-6 suggests that the weak expression found in approximately half of the type III granule cells of *R. clavata* may represent a local immune regulatory response aimed at maintaining homeostatic balance (Sayyaf Dezfuli et al., 2019).

The absence of both macroscopic and microscopic parasites in the intestinal sections analysed suggests that the observed granule cells primarily perform an immune surveillance function and maintain mucosal homeostasis, rather than a response to an ongoing infection; the intestine of *R. clavata* represents an immunologically dynamic region, capable of reacting promptly to potential

antigenic stimuli. In summary, the data obtained strengthen the hypothesis that the spiral intestine of elasmobranchs is not only a digestive organ but also a highly specialized immune system, where granule cells with distinct functions cooperate in mucosal defense. Further investigation of the biochemical nature of the granules and immunohistochemical analysis of their contents may contribute, in future studies, to further clarify the role of these cells in the immune physiology of chondrichthyans.

6) CONCLUSIONS

The comparative analysis conducted on the different fish species examined (*Anguilla anguilla, Salmo trutta, Squalius tenellus, Dicentrarchus labrax, Raja clavata,* and *Galeus melastomus*) allowed us to outline a complex and multifaceted picture of the tissue and cellular immune responses activated following parasitic infections of different nature and location, and in an infection-free system. Despite the taxonomic and environmental differences between the fish studied, common elements emerge that reflect the high degree of evolutionary conservation of defense mechanisms in aquatic vertebrates. In all the species analyzed, the presence of parasites induces a clear localized inflammatory response, characterized by the infiltration of immunocompetent cells.

Overall, the results highlight how, despite their phylogenetic and environmental diversity, fish share common immune strategies based on granule cells and highly conserved chemical mediators. These mechanisms, which include the production of antimicrobial enzymes, the secretion of cytokines, and the modulation of local inflammation, constitute an efficient defense network against intestinal and systemic parasites. From an evolutionary perspective, the coexistence of cells with characteristics intermediate between those of teleosts and mammals, observed especially in elasmobranchs, suggests that many of the innate immune mechanisms of vertebrates have ancient origins and have been maintained throughout evolution with specific functional adaptations.

In conclusion, the collected observations confirm that fish mucosal immunity, far from being a rudimentary system, represents an extremely efficient and complex model, capable of integrating cellular, humoral, and morphological responses to ensure the survival of the organism in an environment constantly exposed to microbial and parasitic challenges.

7) ACKNOWLEDGMENTS

I want to express my sincere gratitude to Prof. Massimo Lorenzoni and Dr. Antonella Carosi of the University of Perugia for their valuable collaboration and continuous scientific support.

I am also deeply grateful to Mr. Luca Bellini for kindly providing the materials necessary for the sampling activities conducted at the Canneviè lagoons.

My sincere thanks go to Dr. Giampaolo Bosi for his constant help, availability, and collaboration throughout my three years of PhD studies.

Finally, I would like to thank Dr. Paolo Merella for providing the essential materials for the sampling and study of the two elasmobranch species investigated in this research.

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Figures and graphics

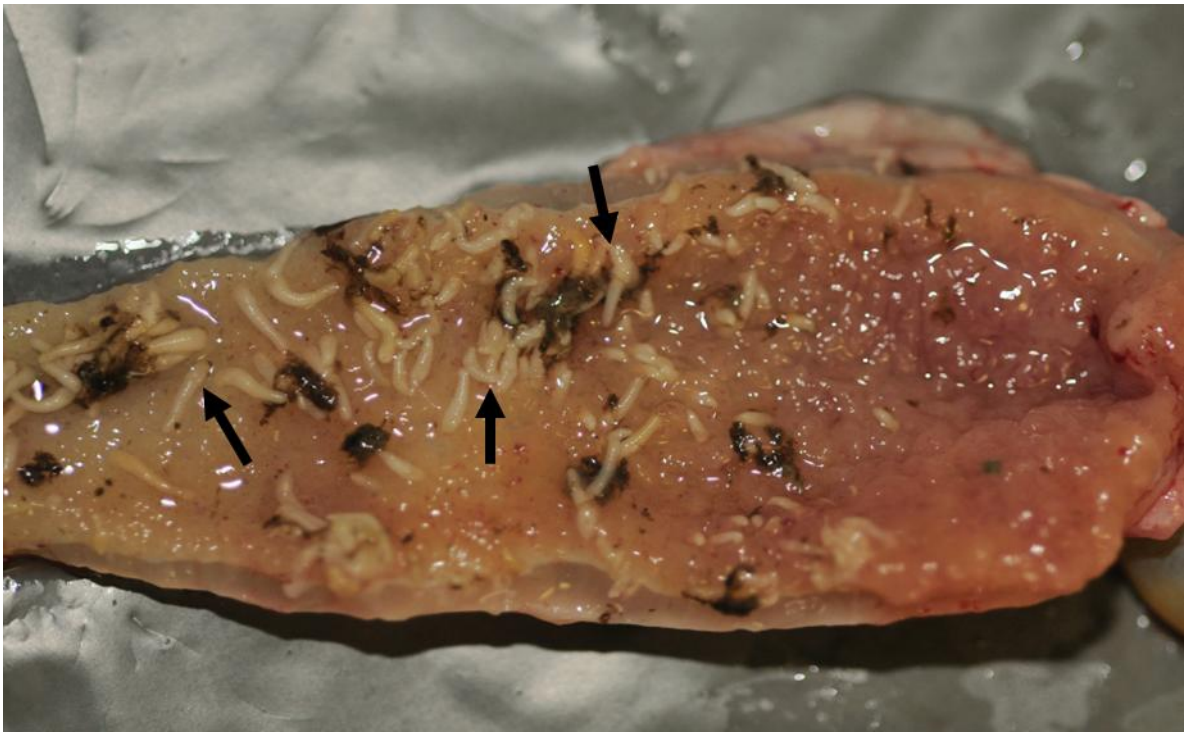

Figure 1. Photograph taken during the necropsy of *Anguilla anguilla* specimen: a severe intestinal infection with *Acanthocephalus rhinensis* (arrows) and mucus around the worms can be seen.

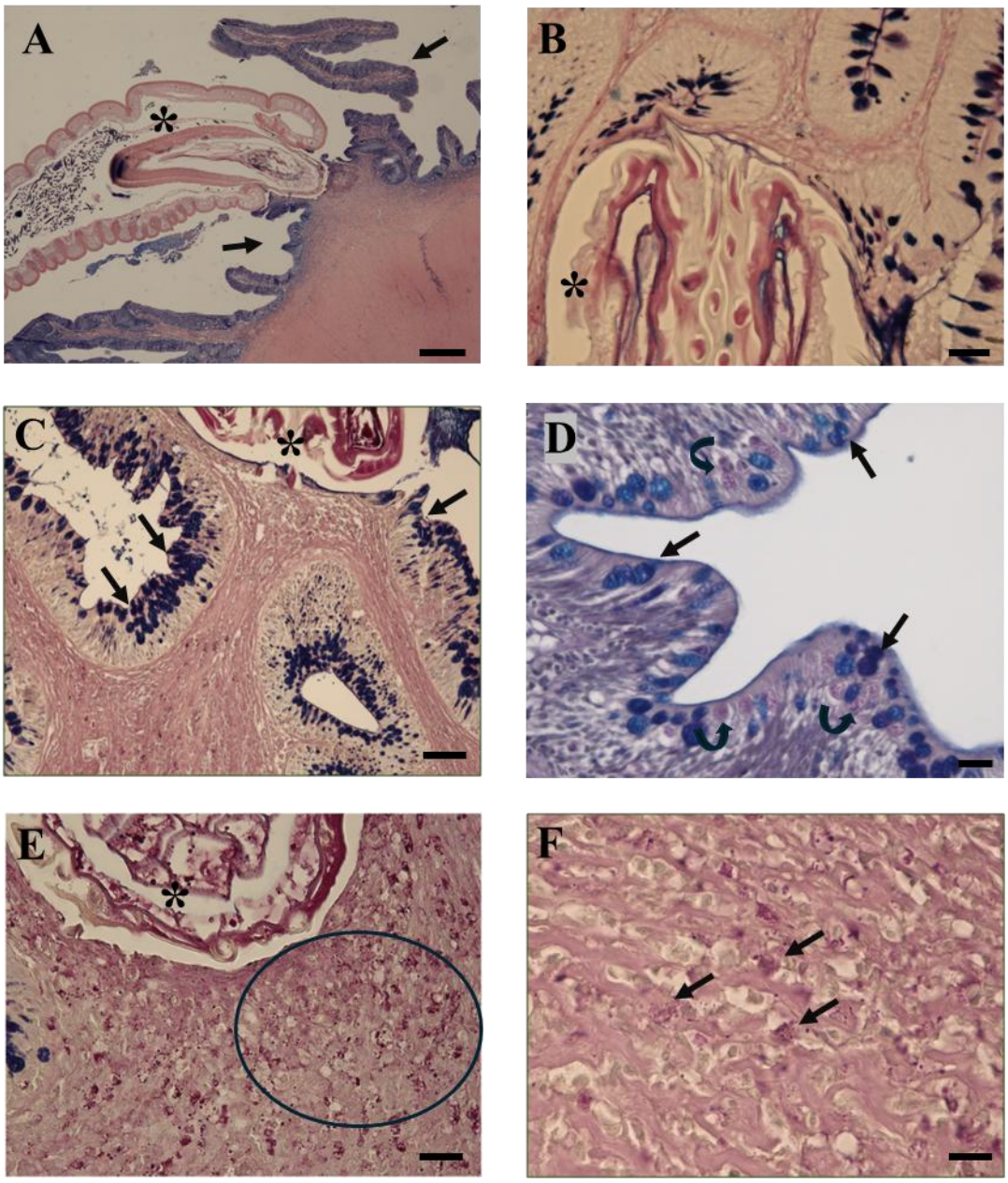

Figure 2. Histological sections of the infected intestine of *A. anguilla*. (A) Transverse section of the intestine infected by an acanthocephalan (asterisk), showing detachment of the intestinal epithelium (arrows); scale bar = 100 µm, Giemsa. (B) Section of the worm proboscis (asterisk) in contact with the intestinal mucosa; scale bar = 20, AB/PAS. (C) Numerous AB-positive mucous cells (arrows) observed near the worm attachment site (asterisk); scale bar = 25 µm, AB/PAS. (D) Epithelium of an infected intestine showing numerous rodlet cells (curved arrows) adjacent to mucous cells (arrows); scale bar = 20 µm, AB/PAS. (E) Micrograph showing mast cells (circle) in the lamina propria–submucosa near the parasite proboscis (asterisk); scale bar = 25 µm, AB/PAS. (F) High magnification of mast cells (arrows), some of which are in degranulation; scale bar = 10 µm, AB/PAS.

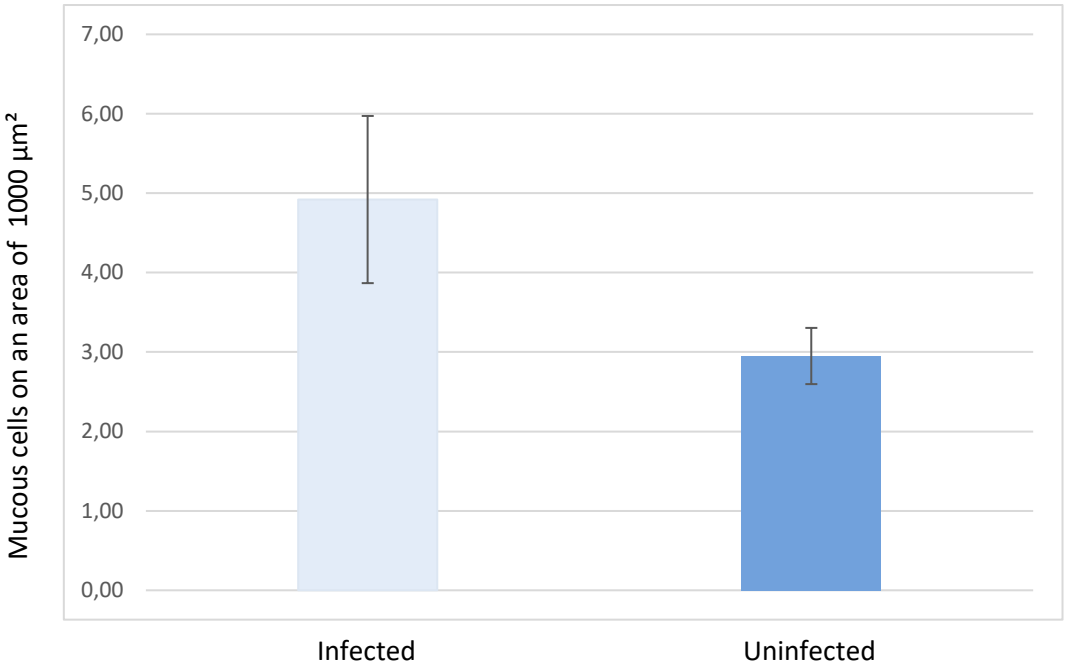

Figure 3. Number of mucous cells in the intestinal epithelium of healthy and infected *A. anguilla*, measured over an area of 1000 µm². The bar plot represents the mean, and the notches represent the 95% confidence interval of the mean.

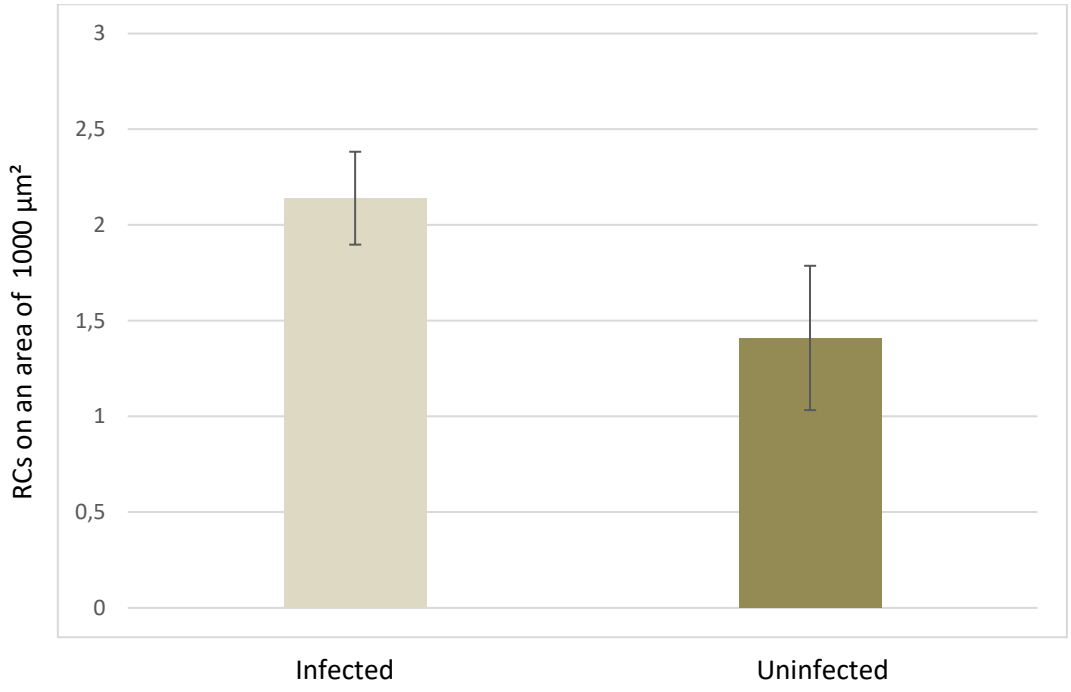

Figure 4. Number of RCs in the intestinal epithelium of healthy and infected *A. anguilla*, measured over an area of 1000 µm². The bar plot represents the mean, and the notches represent the 95% confidence interval of the mean.

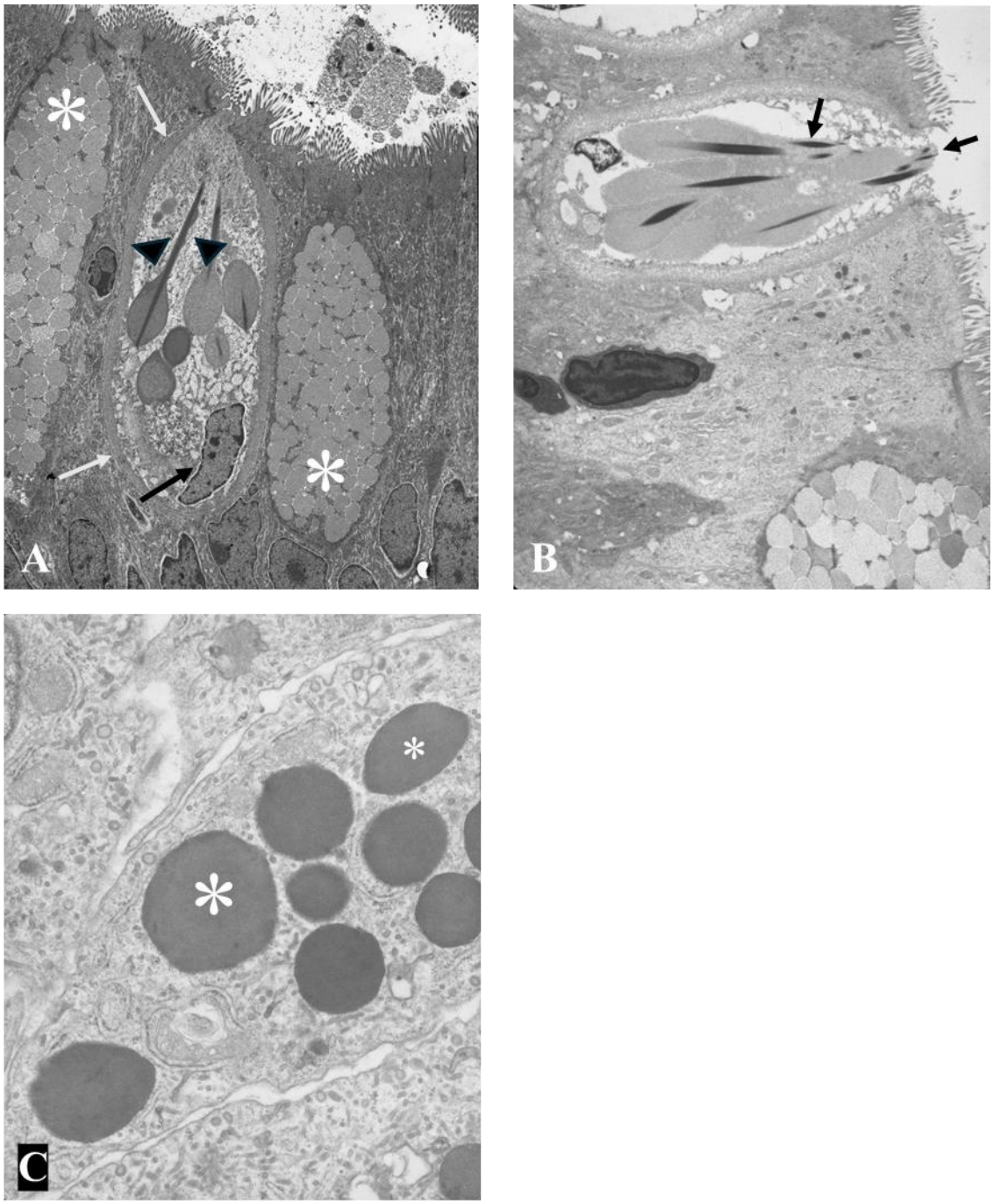

Figure 5. Transmission electron micrographs of intestinal cells in infected *A. anguilla*. (A) Mature rodlet cell showing a basal nucleus (thin arrow), cortex (white arrow), and the characteristic rodlets (arrowheads). The rodlet cell is flanked by mucous cells (white asterisks) containing electron-lucent vesicles; scale bar = 3.3 µm. (B) A rodlet cell discharging its contents (arrows) into the intestinal lumen; scale bar = 2.5 µm. (C) Mast cells with electron-dense granules (white asterisks) located in the lamina propria–submucosa; scale bar = 0.7 µm.

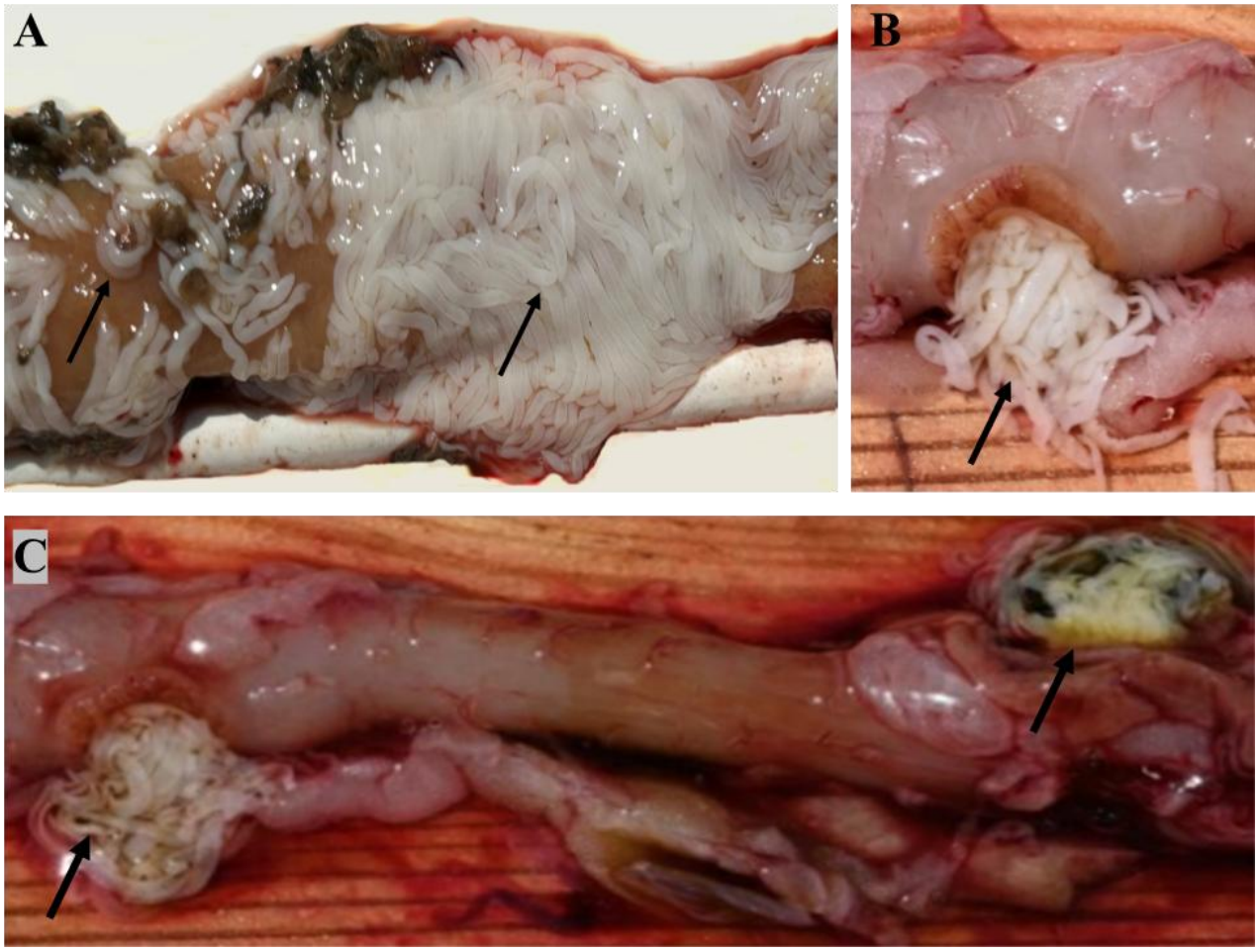

Figure 6. Post-mortem photographs of *Squalius tenellus*. (A) Heavy intestinal infection caused by *Caryophyllaeus brachycollis*: parasites form dense clusters (arrows). (B) Extrusion of several strobilae from the intestine (arrow). (C) Severely infected chub showing clusters of worms perforating the intestinal wall at two points (arrows).

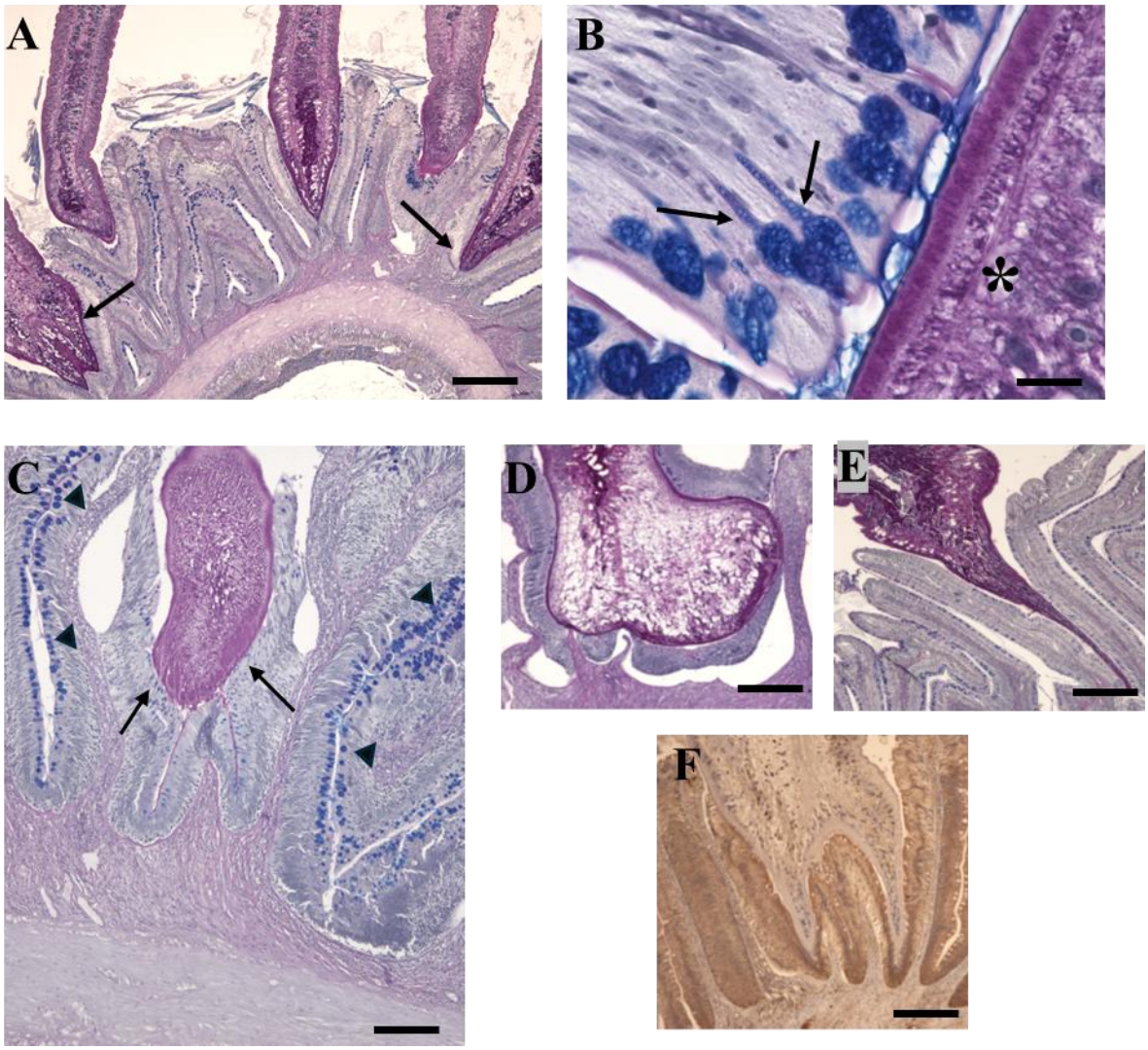

Figure 7. Histological sections of infected intestines of *S. tenellus*. (A) Transverse section of the chub intestine showing *Caryophyllaeus brachycollis* deeply embedded in the intestinal wall (arrows); scale bar = 200 µm, AB/PAS. (B) Mucous cells (arrows) containing acidic glycoconjugate products, and a continuous mucus layer between the parasite tegument (asterisk) and the intestinal epithelium; scale bar = 10 µm, AB/PAS. (C) A few mucous cells are present in the intestinal epithelium near the scolex attachment (arrows), but numerous mucous cells in the adjacent villi without parasites (arrowheads); scale bar = 100 µm, AB/PAS.

Morphological variations of *C. brachycollis* scolex: (D) Spatulate form; scale bar = 100 µm, AB/PAS. (E) Elongated form; scale bar = 100 µm, AB/PAS. (F) Scolex open between villi; Grimelius stain with hematoxylin counterstain; scale bar = 100 µm.

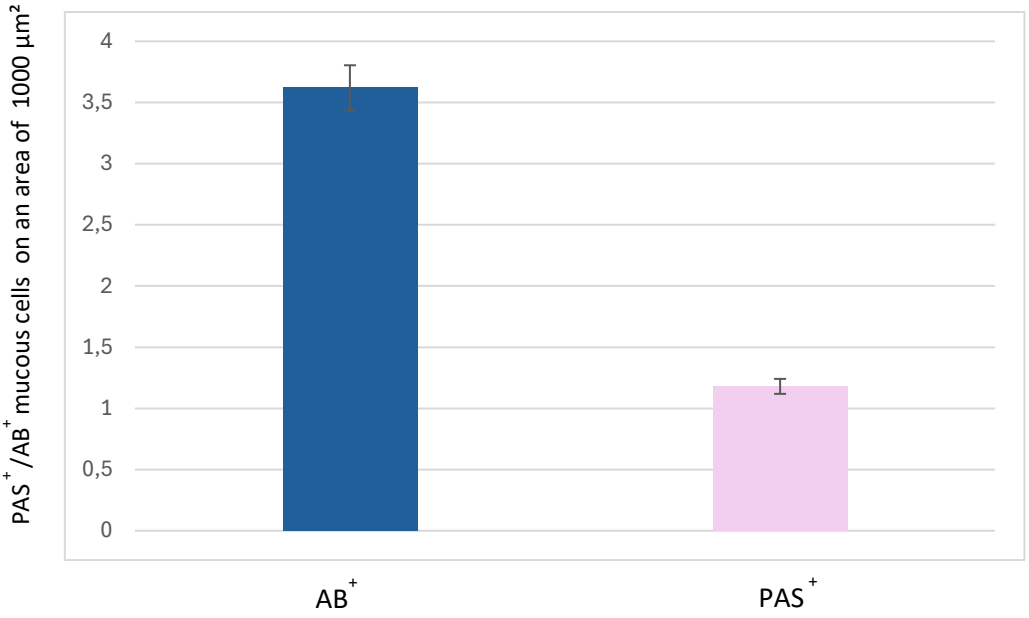

Figure 8. Number of mucous cells AB^+ in the intestinal epithelium of infected *S. tenellus*. The bar plot represents the mean, and the notches represent the 95% confidence interval of the mean.

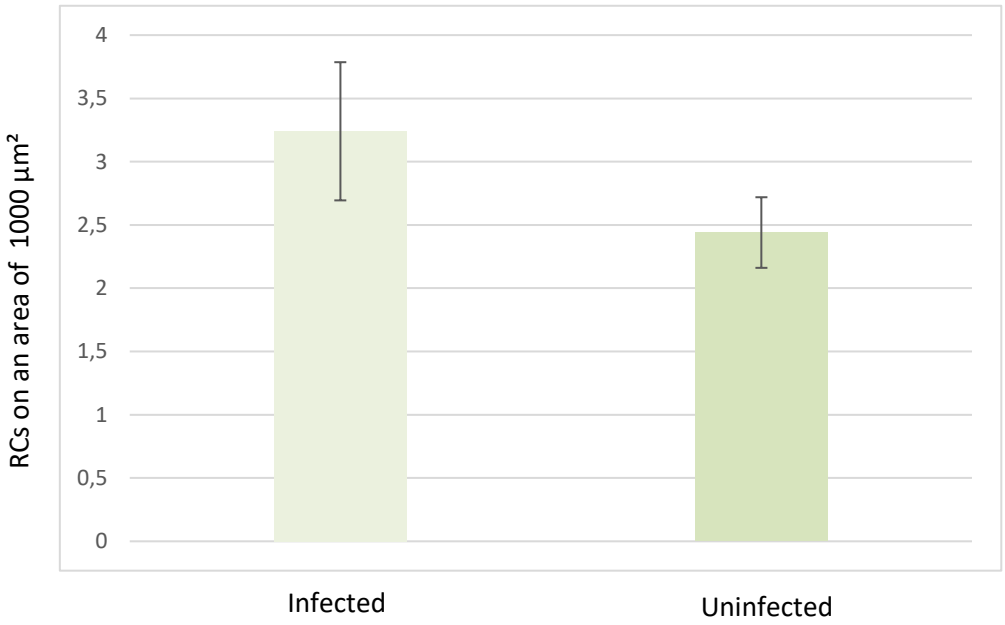

Figure 9. Number of RCs in the intestinal epithelium of healthy and infected *S. tenellus*, measured over an area of 1000 µm². The bar plot represents the mean, and the notches represent the 95% confidence interval of the mean.

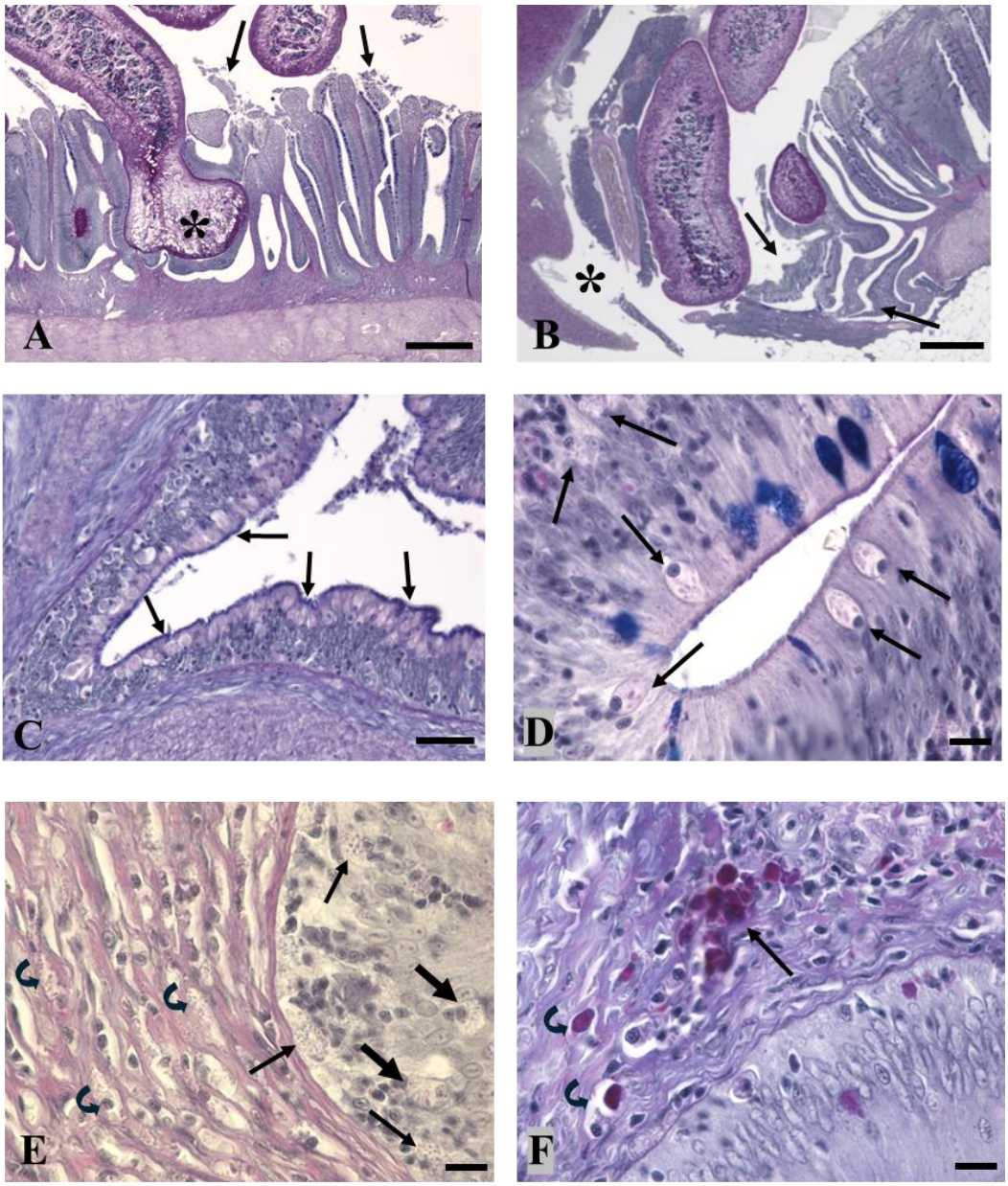

Figure 10. Histological section of the infected intestine of *S. tenellus* stained with Alcian blue/periodic acid Schiff, AB/PAS. A) Penetration of tapeworm within the depth of the fold; note spatulate shape of scolex (asterisk) and its intimate contact with the epithelium. Erosion and desquamation (arrows) of the epithelia are evident; scale bar = 200 µm. B) Micrograph shows interruption of the intestinal muscle layer (asterisk) and disorganization of the epithelium (arrows); scale bar = 200 µm. C) Epithelium of the infected esophagus is tapered with numerous RCs (arrows); scale bar = 50 µm. (D) High magnification of the parasitized esophagus; note the presence of the RCs (arrows) in different levels of the epithelium; scale bar = 10 µm. (E) Micrograph shows MCs (arrows) near the RC (thick arrows) within the epithelium; note neutrophils (curved arrows) in lamina propria-submucosa; scale bar = 10 µm. (F) A MA (arrow) and single macrophages (curved arrows) in lamina propria of an infected intestine; scale bar = 10 µm.

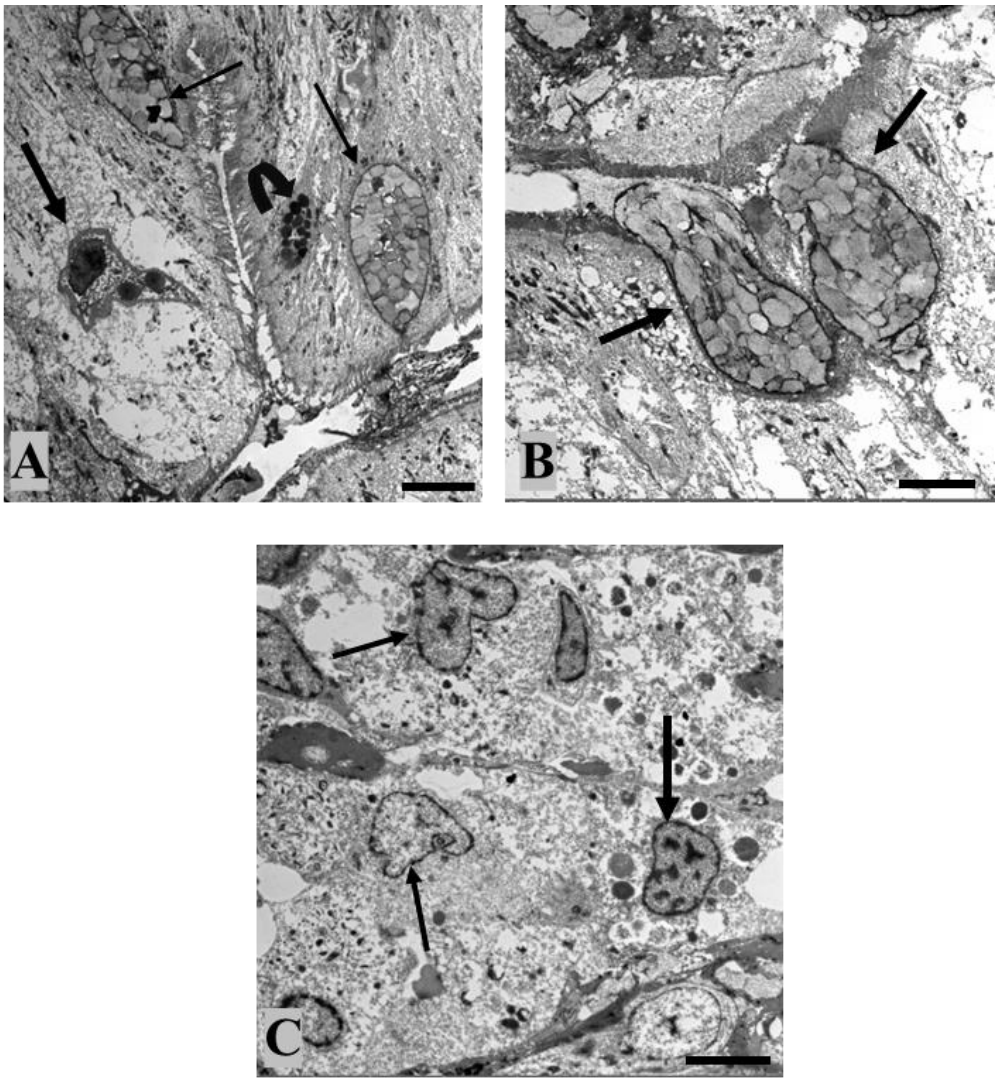

Figure 11. Transmission electron micrographs of the interface region between the infected intestine of *S. tenellus* and *C. brachycollis* tegument (asterisk). (A) Upper part of the epithelium; deformed RC (thick arrow) with basal heterochromatic nucleus; mucous cells (arrows) with numerous mucous granules and a MC (curved arrow) are evident; vacuolation of the enterocytes around the RC is appreciable; scale bar = 5 μm. (B) Two mucous cells (arrows) released the contents in the lumen; different electron density of mucous granules is visible; vacuolation of the enterocytes around the mucous cells is evident; scale bar = 5 μm. (C) Submucosal layer of an infected intestine; numerous neutrophils (arrows) and a MC (thick arrow); both types of cells are in degranulation; scale bar = 5 μm.

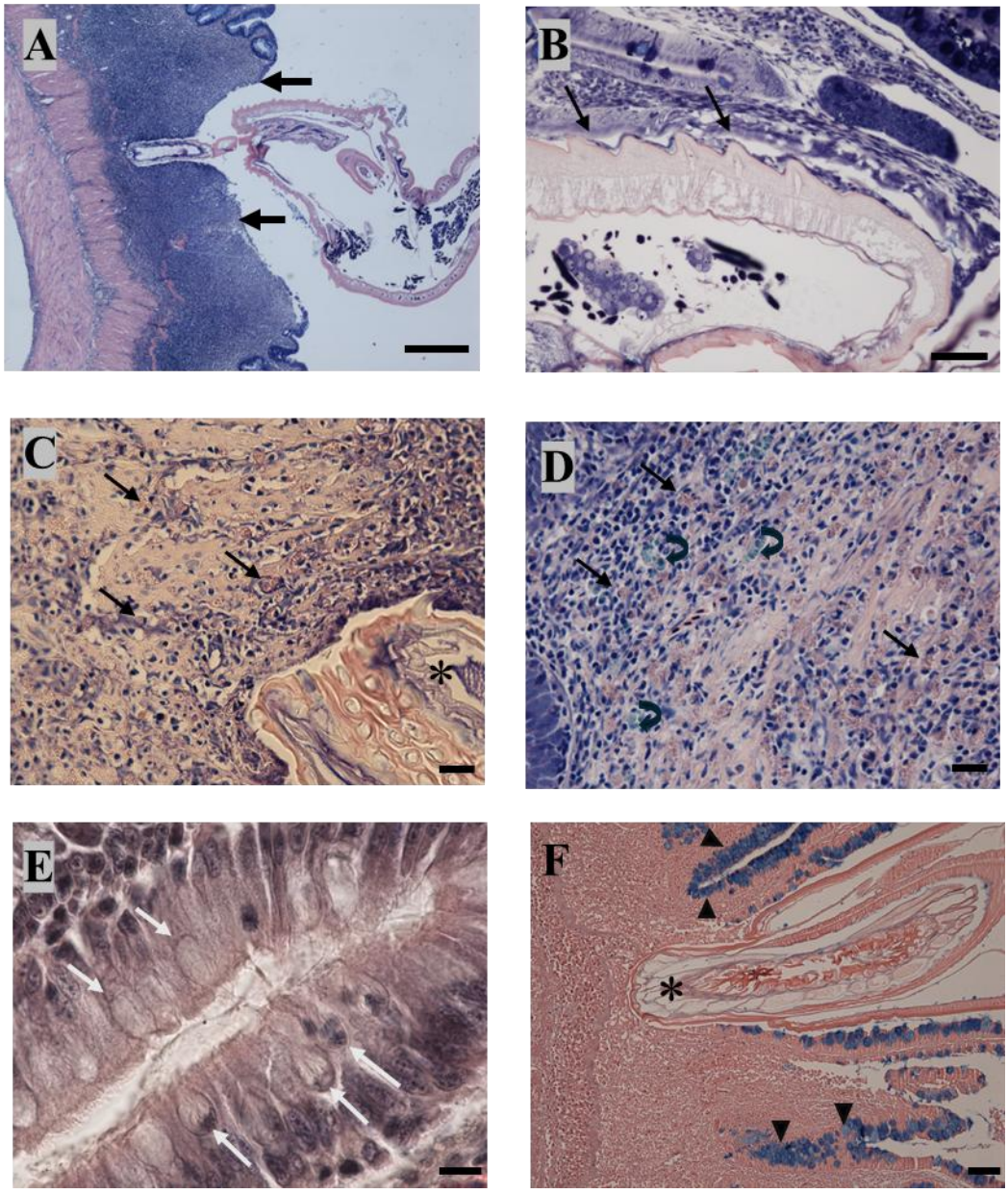

Figure 12. Histological sections of the parasitized intestine of *Salmo trutta*. (A) Sagittal section of the brown trout intestine showing *Dentitruncus truttae* attached to the mucosa; intestinal folds are absent in the attachment area (arrows); scale bar = 200 µm, Giemsa. (B) Close contact between the parasite trunk spines (arrows) and the intestinal mucosa; scale bar = 50 µm, Giemsa. (C) Mast cells (arrows) located near the parasite proboscis (asterisk), some exhibiting degranulation; scale bar = 25 µm, Giemsa. (D) Macrophages (curved arrows) observed in the submucosal layer near mast cells (arrows); scale bar = 25 µm, Giemsa. (E) Rodlet cells (white arrows) within the intestinal epithelium; scale bar = 10 µm, Masson's trichrome stain. (F) Intact intestinal epithelium distant from the parasite attachment site (asterisk), showing numerous mucous cells (arrowheads); scale bar = 100 µm, AB/H&E

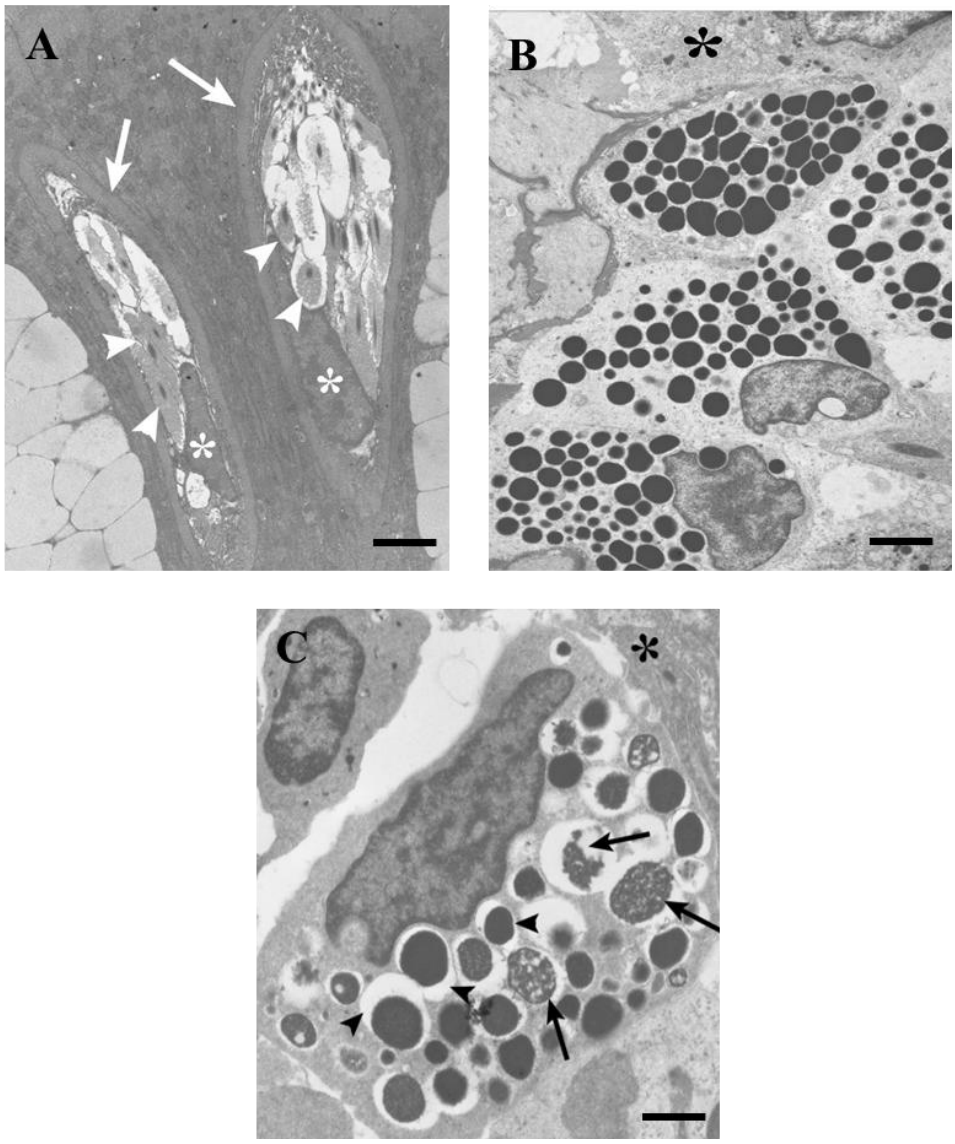

Figure 13. Electron micrographs of *S. trutta* infected intestine. (A) Two rodlet cells in intestinal epithelium: cell cortex (white arrows), basal nuclei (asterisks), and club-shaped rodlets (arrow heads); scale bar = 3.45 μm. (B) Mast cells inside the muscular layer encircled by fibroblast-like ensheathing cells (asterisk); scale bar = 3.43 μm. (C) A mast cell degranulating, reticulated aspects of some granules (arrows), electron-lucent halos around several granules (arrow heads) and collagen fibres (asterisk); bar = 1.78 μm.

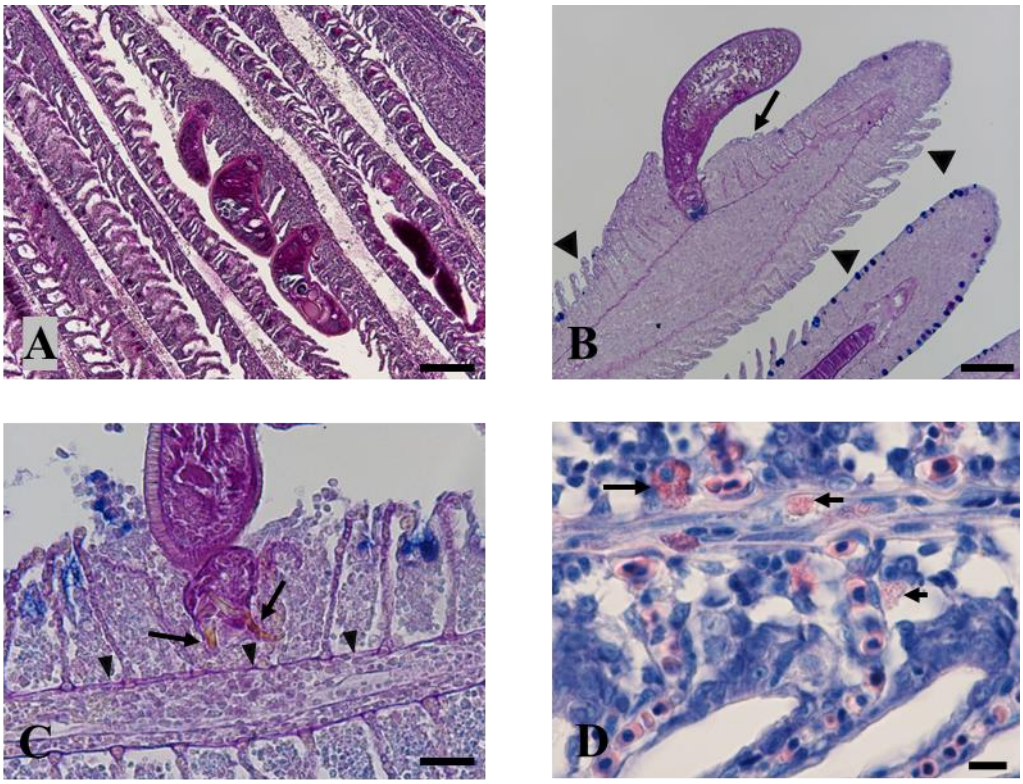

Figura 14. Histological sections of gills of *Dicentrarchus labrax* infected by *Diplectanum aequans* (Monogenea). (A) Three *D. aequans* anchored to the gill lamellae; their hamuli (attachment organs) penetrate deeply into the tissue; scale bar = 50 µm, AB/PAS. (B) Fusion of the secondary lamellae (arrow) near the site of parasite attachment. Intact secondary lamellae (arrowheads) are visible in adjacent filaments; scale bar = 50 µm, AB/PAS. (C) Parasite attachment point: the opisthaptor (arrows) lies close to the basal membrane (arrowheads); note the cellular debris near the parasite body; scale bar = 100 µm. (D) Mast cells in the connective tissue of infected gills (arrows); scale bar = 25 µm, Giemsa.

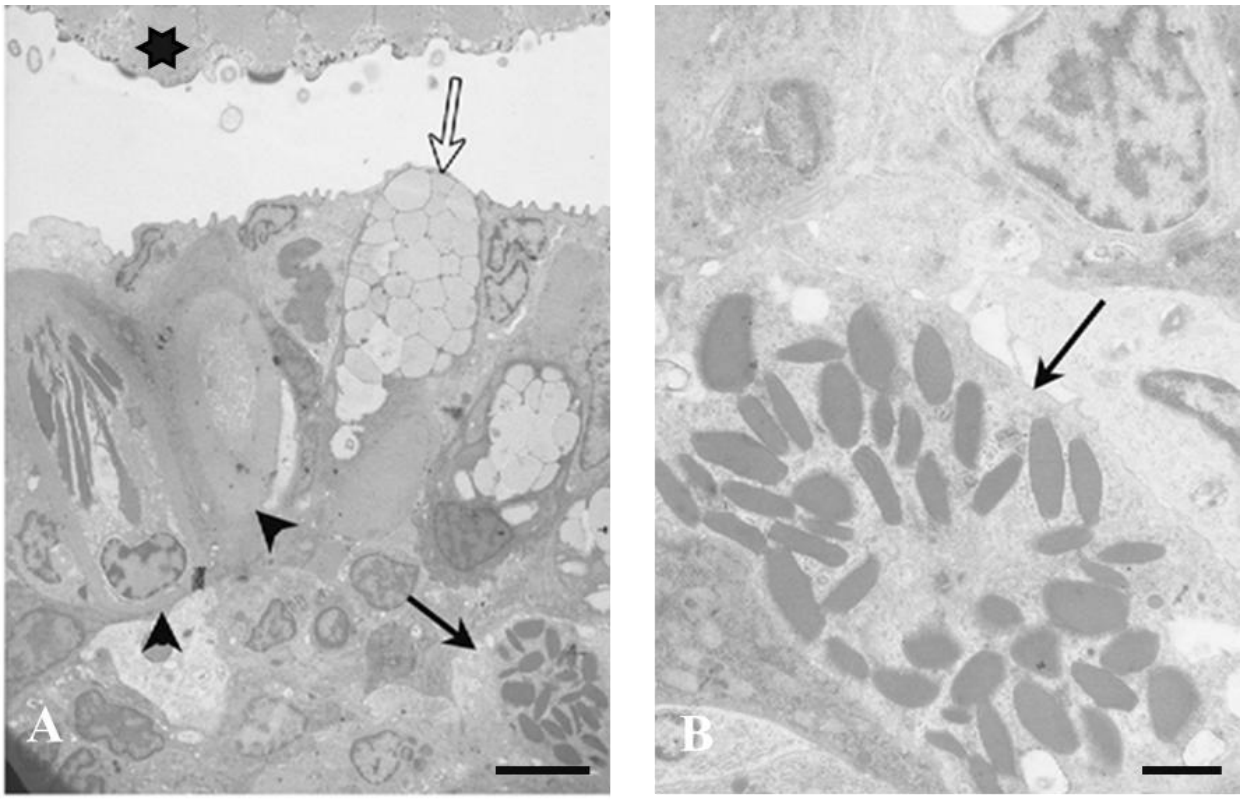

Figura 15. Gill sections of *Dicentrarchus labrax* infected by *Diplectanum aequans*. (A) Parasitized gill section showing *D. aequans* (star), mucous cells (empty arrows), rodlet cells (arrowheads), and a mast cell (arrow); scale bar = 4.5 µm. (B) Mast cell (arrow) appear oval in shape and rich in electron-dense granules; scale bar = 0.48 µm.

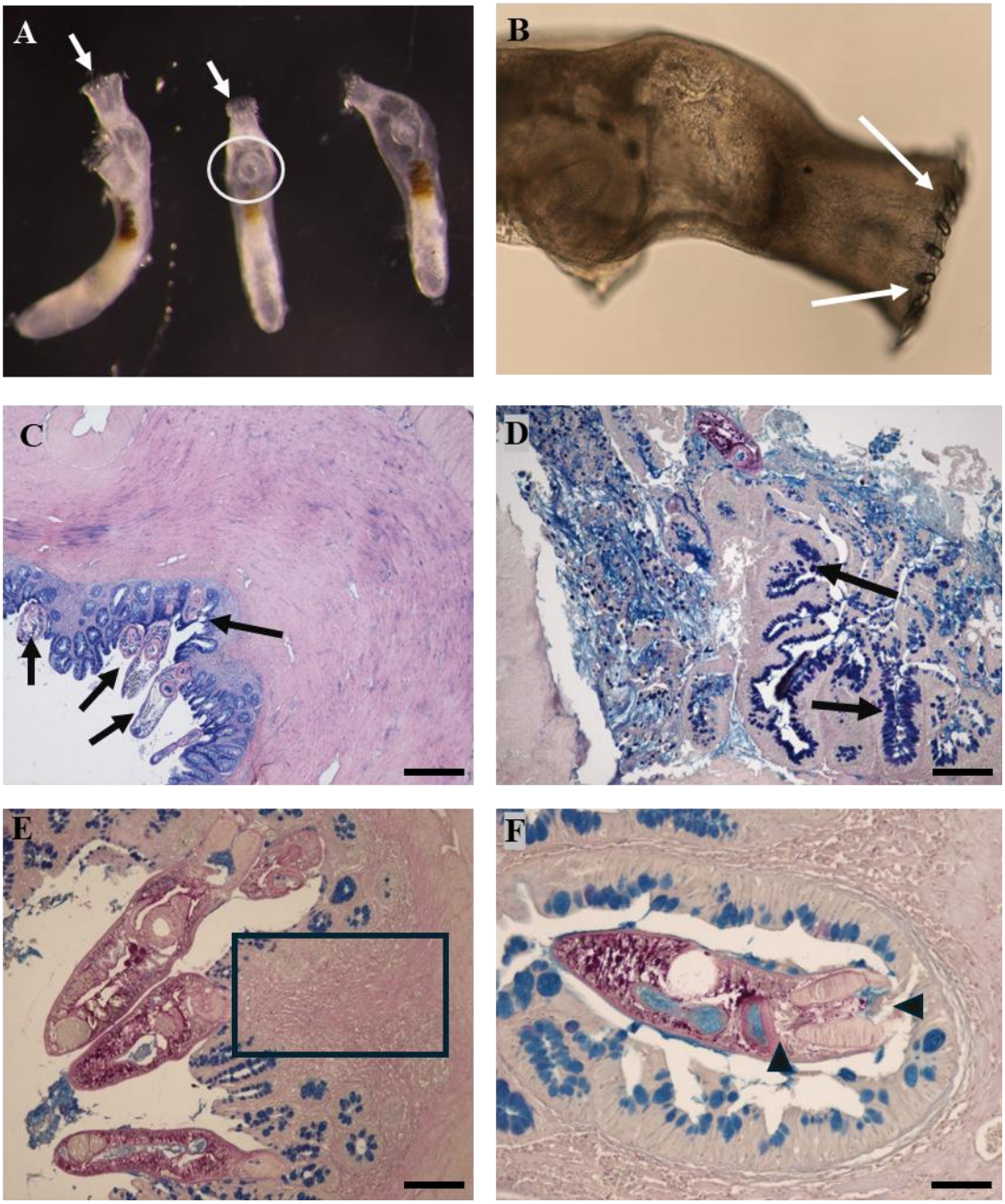

Figura 16. Histological section of seabass intestine infected by *Timoniella imbutiforme.* A) Photo of three *T. imbutiforme* in lactic acid, note the ventral sucker (circle) and the crown around the oral sucker (white arrows). B) Particular of the crown around the oral sucker (white arrows). C) The trematodes (arrows) don't penetrate deeply in the intestinal wall, remaining attached to the mucosa layer; scale bar= 25 µm, Giemsa. D) Hyperplasia of the mucous cells (arrows) is evident near the site of *T. imbutiformis* attachment; note the mucus sheath that encircles the trematode; scale bar= 25 µm, AB/PAS. E) Note inflammation of lamina propria and the presence of some MCs (rectangle); scale bar= 25 µm; AB/PAS. F) Inside the oral and ventral suckers portions of intestinal tissue (arrow heads) are appreciable ; scale bar=20 µm; AB/PAS.

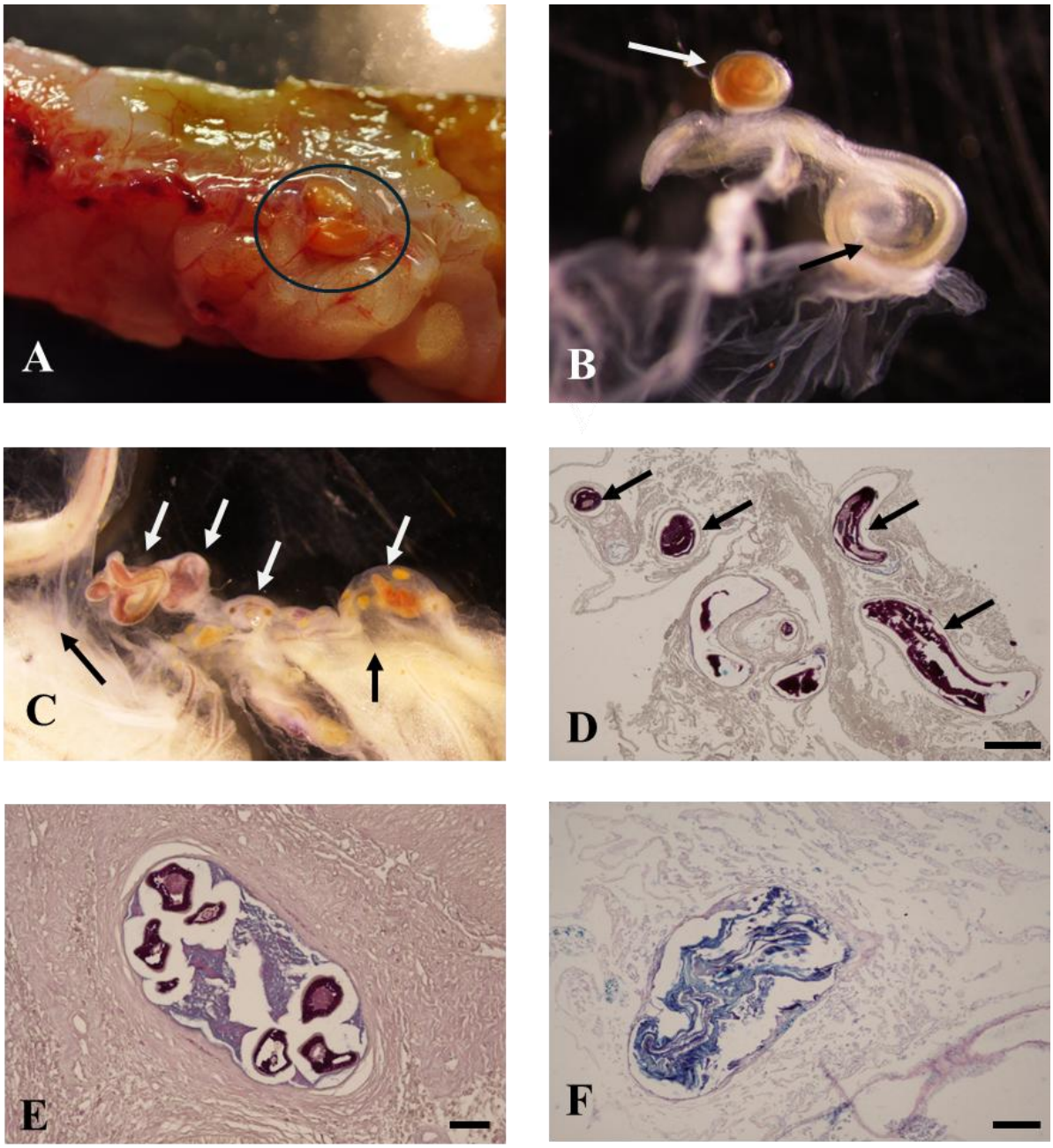

Figura 17. Intestine of seabass *D. labrax* infected by the nematode *C. rudolphii* A or the acanthocephalan *S. hispida* A) Acanthocephalan larvae (circle) in the external surface of the intestine. B) Co-presence of *C. rudolphii A* larvae (arrow) and *S. hispida* (white arrow) larva embedded in the serosa. C) Extraintestinal *C. rudolphii A* larvae (white arrows) surrounded by serosa (black arrows). D) Section of *C. rudolphii A* larvae (arrows) within the serosa; scale bar= 200 µm, AB/PAS E) *Contracaecum* larva encysted in the muscular layer; scale bar=25 µm; AB/PAS. F) *S. hispida* larva within the intestinal wall thickness, scale bar=25 µm, Giemsa.

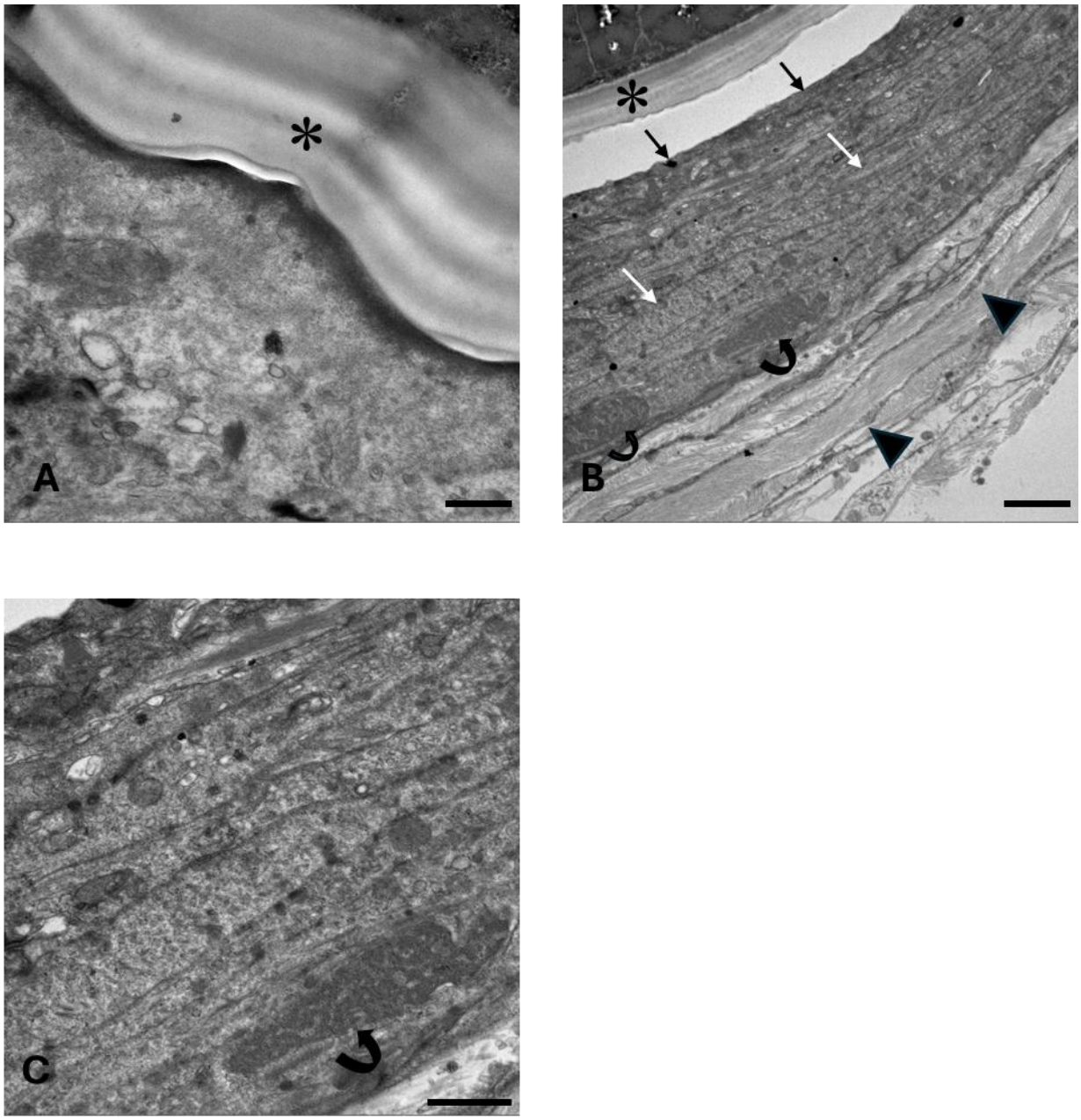

Figura 18. Micrograph of the granuloma around *C. rudolphii. A*) Nematode cuticle (asterisk) in proximity to the granuloma; scale bar = 5 μm. B) Layers of epithelioid cells (arrows), macrophages (curved arrows), fibroblasts (white arrows), and collagen fibers (arrowheads) compose the granuloma near the worm (asterisk); scale bar = 2 μm. C) Higher magnification of the granuloma layers showing a single macrophage (curved arrow); scale bar = 1 μm.

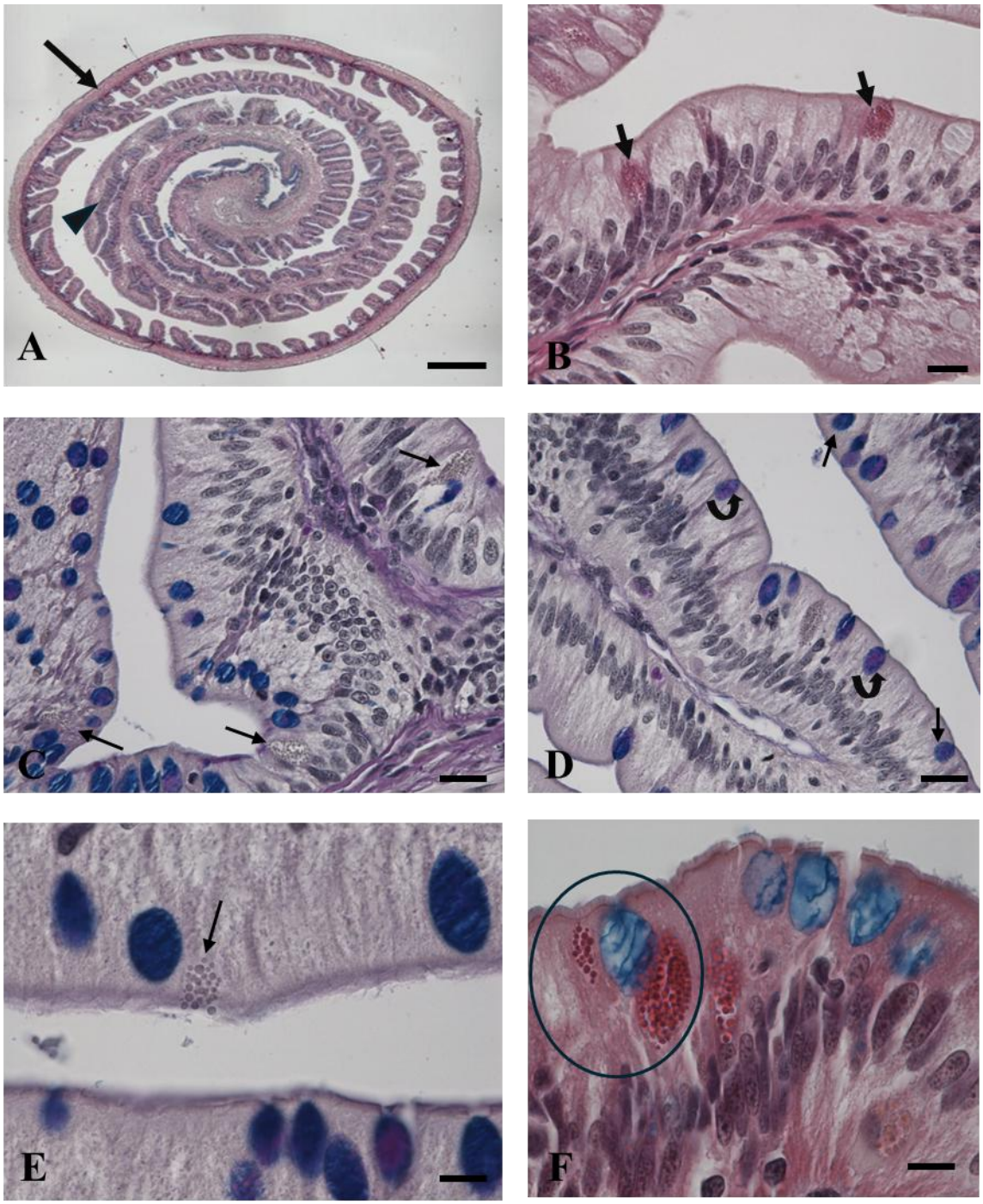

Figure 19. *Galeus melastomus*, histological sections of the spiral intestine region. A) Transverse section demonstrates the aspect of the intestine: The arrow points to the intestinal wall and the arrowhead shows the spiral internal ring composed only of epithelium and lamina propria-submucosa; scale bar = 200 µm, Alcian blue/Hematoxylin-eosin (AB/H&E). B) Presence of granular cells (arrows) in the epithelium; scale bar = 20 µm, (AB/H&E). C) Section stained with AB/PAS, note the unstained granular cells, AB/PAS negative, near the mucous cells; scale bar=25 µm. D) Mucous cells are AB positive (arrow) or AB/PAS positive (curved arrows); scale bar = 10 µm, (AB/PAS). E) An epithelial granular cell discharges its content (arrow) in the intestinal lumen, scale bar = 10 µm; (AB/PAS). F) Contact (circle) between a mucous cell and granular cells in the intestinal epithelium; scale bar = 10 µm, (AB/H&E).

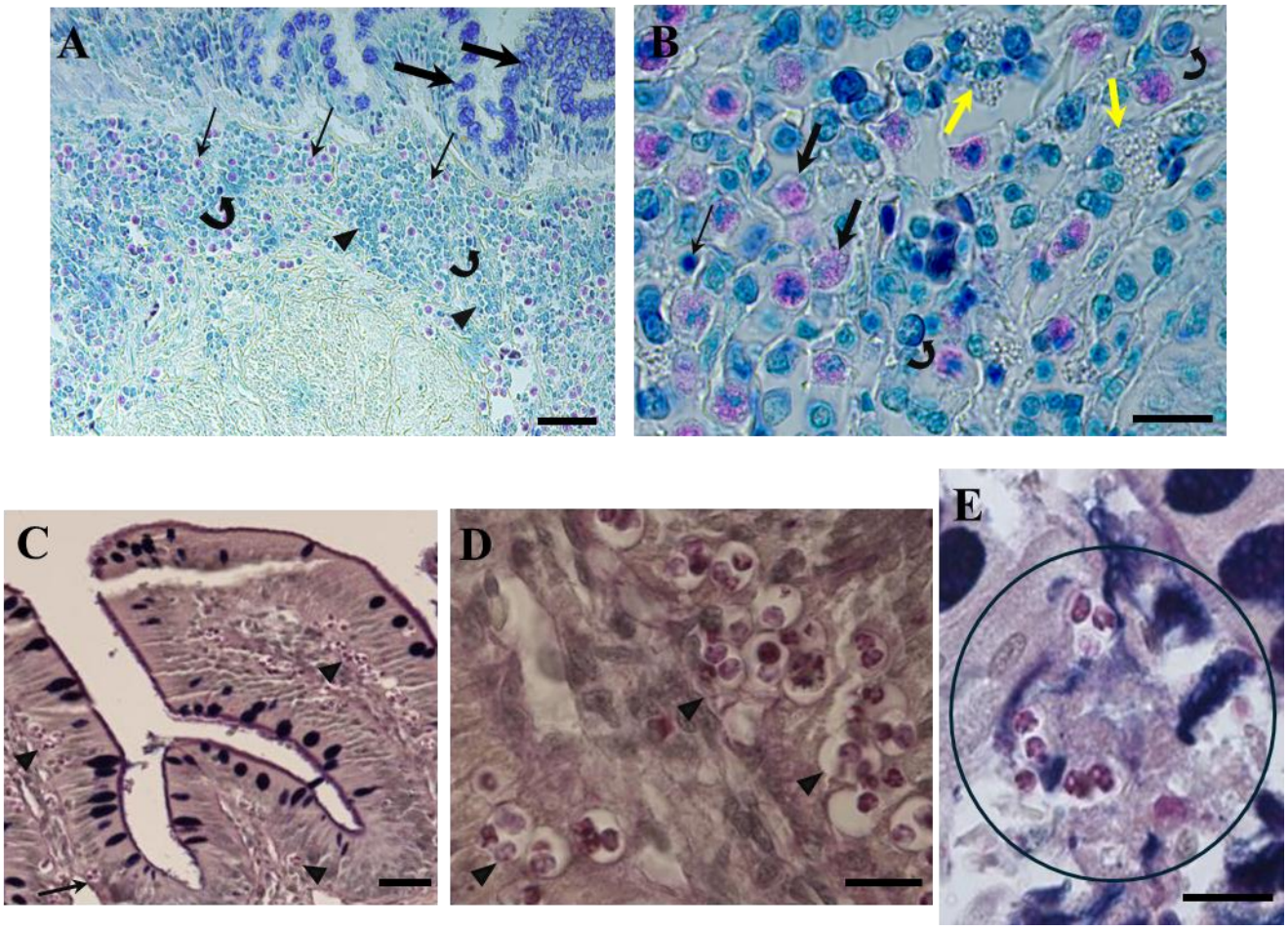

Figure 20. *Galeus melastomus*, histological sections of the spiral intestine region. A) Several immune cells, eosinophilic cells (arrows), basophilic cells (arrowheads), neutrophils (curved arrows), and mucous cells (thick arrows); scale bar: 50 µm, Giemsa. B) High magnification of the lympho-myeloid cell aggregate with eosinophilic cells (arrows), neutrophils (curved arrows), unstained cells (yellow arrows) with coarse cytoplasmic granulation, lymphocytes (thin arrow); scale bar: 20 µm, Giemsa. C) Oocysts (arrowheads) in the intestinal epithelium in proximity of the membrane basement (arrow); scale bar=25, AB/PAS. D) High magnification of oocysts, scale bar= 200 µm; AB/PAS. E) Oocysts in the intestinal epithelium (circle), note the discharge of mucus from the mucous cells, scale bar= 200 µm.

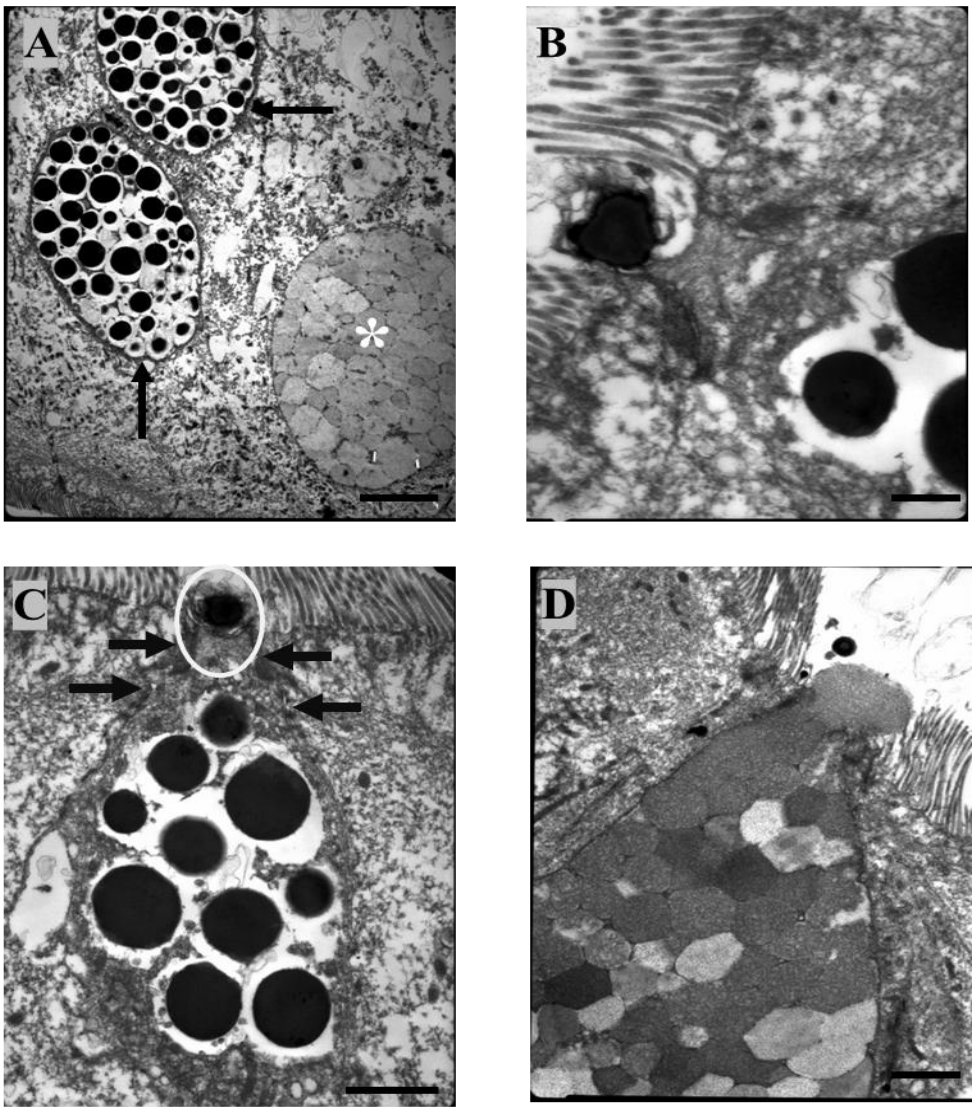

Figure 21. Transmission electron micrographs of the spiral intestine of *Galeus melastomus*. A) Epithelial granular cells (arrows) and a mucous cell (asterisk) at the apex of the epithelium; scale bar = 5.00 μm. B) Round electron-dense granules within the cytoplasm of an epithelial granular cell can be observed; note the release of a granule from the cell; scale bar = 3.33 μm. C) Micrograph shows the junctional complexes between epithelial granular cell and adjacent cells (arrows); note the opening of the apical part of the epithelial granular cell with release of a granule in the intestinal lumen (circle); scale bar = 1.11 μm. D) High magnification of a mucous cell during the release of the mucus in the intestinal lumen, scale bar = 0.67 μm.

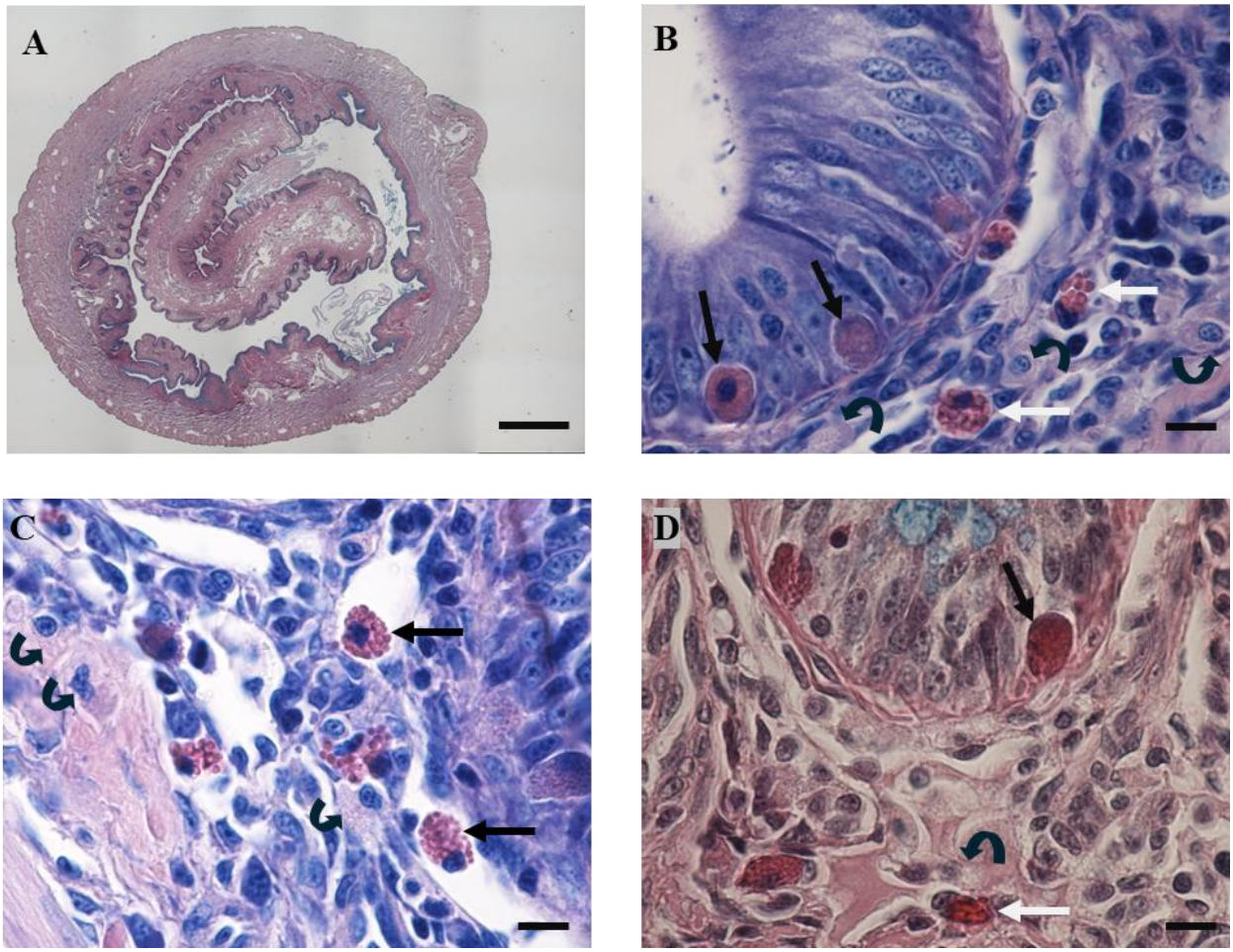

Figure 22. Histological sections through the spiral intestine of *R. clavata.* A) Transverse section shows the screw-like aspect of the intestine. The intestinal wall is composed of four layers: mucosa, submucosa, muscular layer, and serosa. The internal ring is composed only of epithelium and lamina propria-submucosa, Alcian-Blue and Hematoxylin & Eosin (AB/H&E), scale bar = 200 mm. B) The type I granular cells (arrows) in the epithelium in proximity to the basement membrane, in the lamina propria-submucosa, are visible, along with the type II (white arrows) and III (curved arrows) granular cells, Giemsa, scale bar = 20 μm. C) The micrograph shows type II granular cells (arrows), and type III granular cells (curved arrows) in the lamina propria-submucosa. Giemsa, scale bar = 20 μm. D) Several type II (white arrow), and type III granular cells (curved arrows) (H&E). Note the presence of type I granular cells (black arrow) at the epithelial base, scale bar = 10 μm.

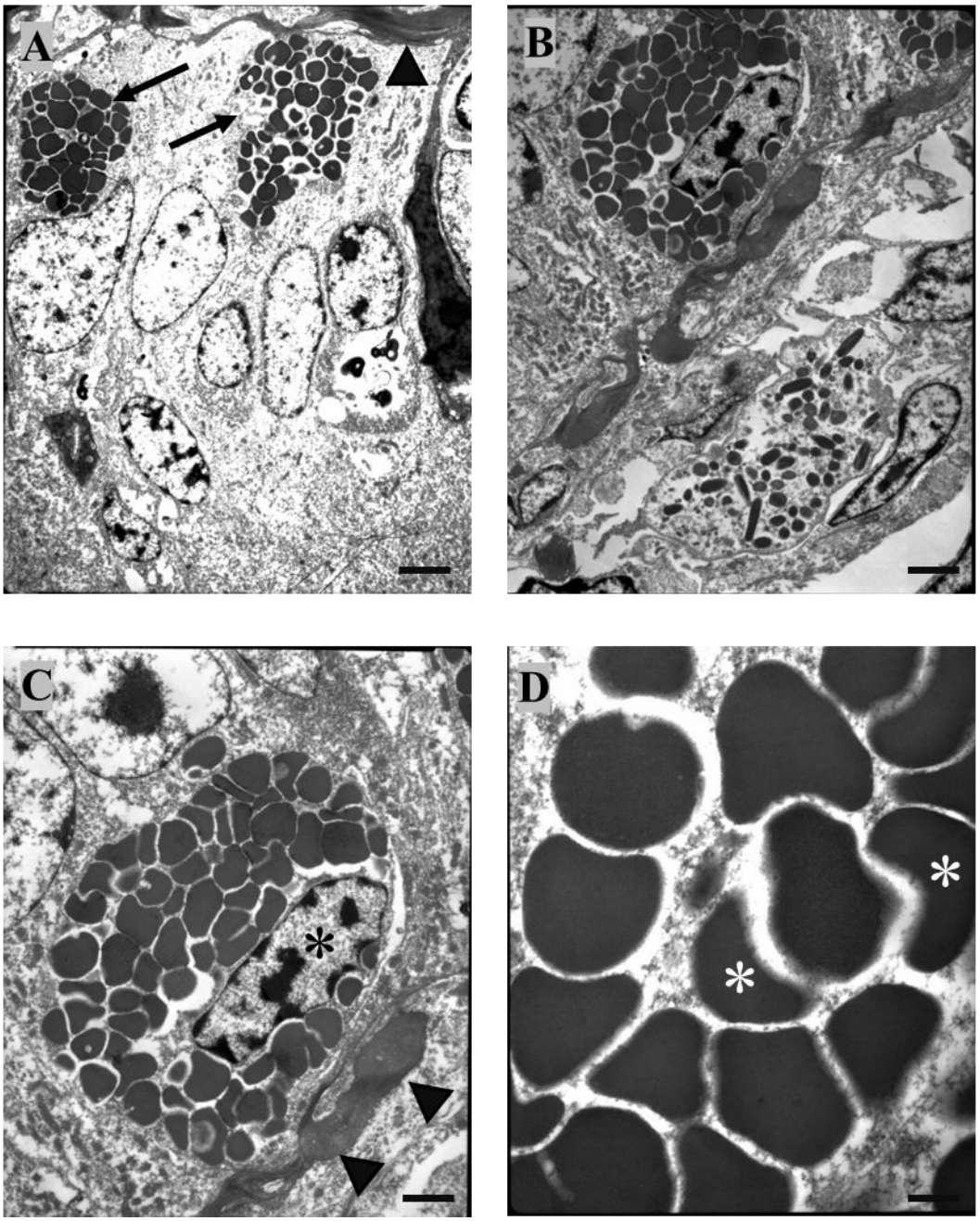

Figure 23. Transmission electron micrographs of the spiral intestine of *R. clavata*. A) Type I granular cells (arrows) in contact with the membrane basement (arrowhead); scale bar = 3.33 μm. B) Occurrence of epithelial granular cell type I and type III on opposite sides of the basement membrane can be seen, scale bar = 2.50 μm. C) A type I granular cell with an eccentric and heterochromatic nucleus (asterisk) in contact with the basement membrane (arrowheads), scale bar = 1.43 μm. D) High magnification of type I granular cell: the granules contain finely granular electron-dense material. Note scroll pattern of some granules (asterisks), scale bar = 0.67 μm.

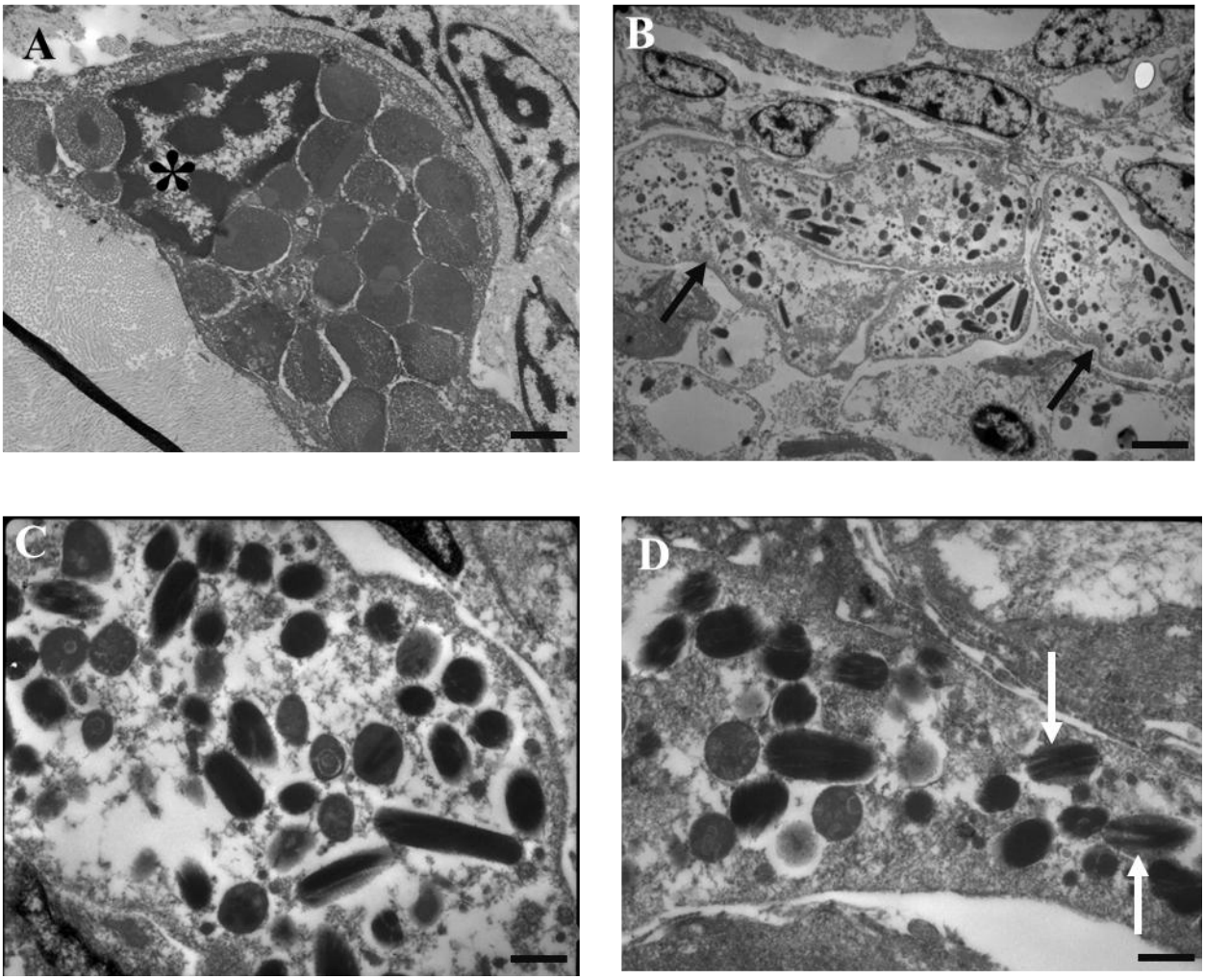

Figure 24. Granular cells in the spiral intestine of *R. clavata*. A) High magnification of type II granular cell in lamina propria-submucosa, note the eccentric heterochromatic nucleus (asterisk). scale bar = 1.83 μm. B) Numerous type III granular cells (arrows) in lamina propria-submucosa, scale bar = 5.0 μm. C) Two types of electron-dense granules in type III granular cells: small round and large elongated electron-dense granules filled the cell cytoplasm, scale bar = 2.0 μm. D) Micrograph shows elongated granules (arrows) with axial rod-like inclusions inside the cytoplasm of a type III granular cell, scale bar = 0.25 μm.