Histopathological and ultrastructural assessment of two mugilid species infected with myxozoans and helminths

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Running head: intestinal cellular response against micro-macroparasites

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Abstract

The histopathology and ultrastructure of the intestine of mullets, *Liza ramada* and *L. saliens*, from Comacchio lagoons (Northern Italy) naturally infected with myxozoans and helminths were investigated and described. Sixty-two (80.5%) out of 77 mullets harboured one or more of the following parasites species: *Myxobolus mugchelo* (Myxozoa), *Neoechynorhynchus agilis* (Acanthocephala), *Haplosplanchnus pachysomus* and *Dicrogaster contractus* (Digenea). Co-occurrence of helminths with myxozoans was common. The main damage caused by digeneans was destruction of the mucosal epithelium of the villi, necrosis and degeneration of intestinal epithelial cells. More severe intestinal damage was caused by acanthocephalans which reach the submucosa layer with their proboscis. At the site of helminths infection, several mast cells (MCs), rodlet cells (RCs), mucous cells and few neutrophils and macrophages were observed in the epithelium. RCs and mucous cells exhibited discharge activity in close vicinity to the worm's tegument. *M. mugchelo* conspicuous plasmodia were encysted mainly in muscle and submucosa layers of the intestine. Indeed, spores of *M. mugchelo* were documented within the epithelial cells of hosts intestine and in proximity to MCs. Degranulation of the MCs near the myxozoans was very frequent.

Introduction

In fish and other vertebrates, alimentary canal is a primary route of infection (Ringo *et al.* 2007), most probably due to: ease of access for the pathogens; ready availability of attachment sites and access to nutrients; and relatively non-aggressive immune response of the host (Secombes & Chappell 1996). Numerous studies have documented histopathology of digestive tract in fish infected with helminths (Dezfuli *et al.* 1997, 2016, 2017; Mladineo 2006; Constenla *et al.* 2011; Kotb, Mahdy & Shaheed 2014; Bosi *et al.* 2017) and with myxozoans (Bartholomew *et al.* 2004; Dyková & Lom 2007; Hallett & Bartholomew 2012; Estensoro *et al.* 2013; Gomez, Bartholomew & Sunyer 2014; Sitjà-Bobadilla, Estensoro & Pérez-Sánchez 2016). Concurrent infection of fish by different pathogens/parasites is common in nature (Kotob *et al.* 2016), however, records on this topic are limited. Coexistence of myxozoans and helminths in fish was reported in Kristmundsson & Richter (2009) and Özer & Kirca (2013).

Inflammation has been recognized for more than 2000 years ago by a Roman physician as a biological phenomenon occurring in the host against invading organisms (Loria & Diegelmann 2016). In teleosts, mucosal surfaces (intestine, gill, skin and reproductive tissues) form the first line of defense against pathogen invasion (Parra *et al.* 2016). Such barriers are comprised of mucous cells, epithelial cells, neuroendocrine cells and different types of immune cells (Jutfelt 2011; Dezfuli *et al.* 2015a, 2016, 2017). Among immune cells, mast cells (MCs) (Silphaduang & Noga 2001; Buchmann 2012; Secombes & Ellis 2012; Dezfuli *et al.* 2015b, 2016, 2017; Galindo-Villegas, Garcia-Garcia & Mulero 2016), neutrophils (Havixbeck *et al.* 2016; Von Gersdorff Jørgensen 2016), macrophages (Agius & Roberts 2003; Hodgkinson, Grayfer & Belosevic 2015) and rodlet cells (RCs) (Manera & Dezfuli 2004; Reite 2005; Reite & Evensen 2006; Bosi *et al.* 2015; Dezfuli *et al.* 2015a, 2016) are active in immune response against parasites.

The Mugilidae, commonly known as grey mullets, are one of the most ubiquitous teleost families in coastal waters of the world, and have been considered an important food source in Mediterranean Europe since Roman times (Crosetti 2016). Mullet farming depends on fry or fingerlings from natural stocks. The adaptale physiology, which allows frequent migration from freshwater to seawater and *viceversa,* renders mugilids increasingly vulnerable to diseases and parasitic infection (Paperna & Overstreet 1981).

Herein, in two mullet species *L. ramada* and *L. saliens* were studied for parasitic infestation. *M. mugchelo* (Myxozoa) co-occurred with three enteric helminth species: the acanthocephalan

Neoechinorhynchus agilis and the digeneans *Haplosplanchnus pachysomus* and *Dicrogaster contractus*. Histopathological examination of the alimentary tract revealed high numbers of MCs in intestinal epithelium and in submucosal layer in *L. ramada* and *L. saliens* in response to parasites. Based on this observation and previous studies, we hypothesized that mullet could be a promising candidate for mucosal immunity studies in teleosts. Furthermore, to the best of our knowledge, this study is the first detailed account on concurrent infection of myxozoans and enteric helminths resulting in new insights in understanding immune mechanism against micro-macro intestinal parasites in fish.

Material and Methods

Thirty-nine specimens of *L. ramada* and 38 of *L. saliens* were provided by the Po Delta Park Administration from the Comacchio lagoons (Northern Adriatic Sea, Italy, 44º 36′ N, 12º 10′ E) from April to December 2016. The fish were transported alive to the laboratory of the Department of Life Sciences and Biotechnology, University of Ferrara. Subsequently, mullets were anaesthetised with 125 mg/L MS222 (tricaine methanesulphonate, Sandoz), followed by severance of their spinal cords. Immediately after euthanasia, the mullets were weighed, measured (Table 1) and a complete necropsy was performed. Parasitological parameters such as prevalence, intensity and abundance were determined according to Bush *et al.* (1997). The 95% confidence limits of the mean (either mean intensity and mean abundance) were calculated according to Rozsa, Reiczigel & Majoros (2000) and Reiczigel *et al.* (2013).

Gills were screened for ectoparasites microscopically before each fish dissection. After dissection stomach, intestine, liver, heart, gonads, spleen, kidney were screened under stereomicroscope for endo-parasites. Several pieces of 15 x 15 mm from gills, stomach, liver, heart, gonads, spleen, kidney were excised, fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin wax and cut with routine techniques for presence of parasites. Histological sections confirmed that the above mentioned organs were parasite-free.

In the intestine, only acanthocephalans were visible with the naked eye during screening. For worms found still attached to the intestine, their position was registered and 15 × 15 mm pieces of the intestine were fixed in 10% neutral buffered formalin for 24 h, paraffin wax embedded (formalin fixed paraffin embedded = FFPE). Corresponding FFPE sections of uninfected fish intestine were similarly processed so that a direct comparison with the infected intestine could be made. Multiple 5 µm sections were

taken from each tissue block, stained with either Alcian Blue - Haematoxylin and Eosin (AB/HE) and Giemsa, examined and photographed using a Nikon Microscope ECLIPSE 80i (Nikon).

For transmission electron microscopy (TEM), 7 × 7 mm infected and uninfected intestinal tissues of both mullet species were fixed in cold 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 3 h. The fixed tissues were then post-fixed in 1 % osmium tetroxide for 2 h, rinsed and stored in 0.1 M sodium cacodylate buffer containing 6% sucrose for 12 h. Thereafter, tissue were dehydrated through a graded acetone series and embedded in epoxy resin (Durcupan ACM, Fluka). Semi thin sections (1.5 μm) were cut on a Reichert Om U2 ultra microtome and stained with toluidine blue. Ultra-thin sections (90 nm) were stained with 4% uranyl acetate solution in 50% ethanol and Reynold's lead citrate. Hitachi H-800 transmission electron microscope was used to examine stained sections.

Results

A total of 77 mullets (39 *L. ramada* and 38 *L. saliens*) were examined for presence of parasitic infection. Of these, 80.5 % harbored at the least one of the following intestinal parasites: *Myxobolus mugchelo* (Myxozoa), *Neoechinorhynchys agilis* (Acanthocephala), *Haplosplanchnus pachysomus* and *Dicrogaster contractus* (Digenea). Table 1 depicts the prevalence, abundance and intensity of infection. Often specimens of digeneans and acanthocephalans were found either free in the lumen or attached to the intestinal wall of the hosts. Co-occurrence of parasites was common: 25% of mullets were co-infected with all the above mentioned parasites, 17% with myxozoans and acanthocephalans, 32% with myxozoans and digeneans, 1% with the three helminth species. No parasites were encountered in stomach, liver, heart, gonads, spleen, kidney.

Histology

Table 2 summarized the main histopathological findings for each of the parasites found in the intestines of mullets. Histological examination of infected intestines of *L. ramada* and *L. saliens* showed that plasmodia of *M. mugchelo* were present in all the intestinal layers, but most of them were located within the muscle layer and in submucosal layer (respectively Fig. 1a, 1b). The presence of histozoic irregular plasmodia with variable dimensions containing developmental stages of the parasite and mature spores were noticed (Fig. 1a, 1b). In mullets infected with *M. mugchelo*, free mature spores of the myxozoan were observed in the intestinal epithelium, in close vicinity to the mucous cells (Fig. 1c) and epithelial MCs (Fig. 1d). Free spores were common in submucosal layer in proximity to the MCs (Fig. 1d). No apparent pathological changes due to myxozoans were observed. However, in severe

infections, histological sections revealed several areas of the intestinal wall occupied by plasmodia (Fig. 1a). The cellular immune response of the host was variable depending on the location of the cyst. In intestinal muscle layer (Fig. 1a), a mild inflammatory reaction was elicited by myxozoans with few MCs near the parasite (data not shown), whereas, in submucosa layer, a high number of MCs occurred (Fig. 1b, 1d). These MCs were in close proximity to the plasmodia and scattered in the connective tissue of mucosa layer (Fig. 1b, 1d). Intense degranulation of the MCs, especially in connective tissues of mucosal and submucosal layers as well as in epithelium, was documented (Fig. 1d).

With regard to the three enteric helminth species, *Neoechinorhynchus agilis* trunk in both mullets species was in contact with the mucosal folds (Fig. 2a) and damaged the apex of the villi with detachment of cells and their release into intestinal lumen (Fig. 2b). However, folds more distant from the worm body remained intact. In some instances, a thick mucus blanket separated acanthocephalan trunk from apex of intestinal folds (Fig. 2b). At the site of proboscis's attachment, numerous MCs were seen in mucosal and submucosal layers (Fig. 2a). Co-presence of helminths either alone (Fig. 2c) or with myxozoans were frequent and in co-proximity in the intestine in both mullet species.

Digeneans *Haplosplanchnus pachysomus* and *Dicrogaster contractus* remain between two adjacent folds or attach to the apex of intestinal fold with their sucker and detach portion of the host tissue (respectively Fig. 2c, 2d). Frequently, within the sucker, presence of fragment of the fold with cells in different stages of degeneration was noticed (Fig. 2d). In both species of mullets, *H. pachysomus* and *D. contractus* do not penetrate deep into the intestinal wall and do not go over the epithelium of the mucosal layer (Fig. 2c). Near the point of sucker attachment, presence and recruitment of the MCs was common (Fig. 2d). The architecture of the intestinal layers and the morphology of intestinal cells (mucous cells, MCs, neutrophils, RCs) of uninfected mullets presented the same characteristics of the fish harboring parasites, but the occurrence of immune cells was lower. Furthermore, degranulation of MCs was rarely seen in uninfected intestines.

Transmission electron microscopy

TEM revealed the interface region between parasites and mullets intestine. Figure 3a depicts part of a plasmodium within the submucosal layer in contact with the surrounding host cells. The plasmodium presents a sinuous and irregular outline, formed by numerous pseudopodia-like structures and projected towards the external region of the cyst wall (Fig. 3b). Plasmodium containing mature spores was surrounded by several MCs (Fig. 3a). The spores showed symmetrical and smooth valves and their anterior end contained two equal pyriform polar capsules and the wall of the polar capsule was filled

with a hyaline substance (Fig. 3b) contrasting with the very dense internal matrix. In many instances, in mucosal layer, spores made close contact with the MCs (Fig. 3b).

In interface region between helminths (digeneans and acanthocephalan) and intestinal epithelium, presence of numerous mucous cells and several RCs very close to parasite's tegument was documented (Fig. 3c, 3d). In several cases, mucous cells and RCs discharge their contents in interface region and near the parasite body (respectively Fig 3c, 3d). Moreover, in intestinal epithelium, co-occurrence of different types of host immune cell (*i.e.* mucous cells, RCs and MCs, Fig. 4a) was frequent. Figure 4b shows apical part of the fold in which mucous cell, macrophage and a RC are near brush border of the epithelium. In few cases, within the epithelium, neutrophil was in proximity to the mucous cell (Fig. 4c). Nevertheless, in submucosal layer of mullets infected with myxozoan and/or helminths, numerous MCs and neutrophils were noticed and often close contact between them was observed (Fig. 4d).

The ultrastructural features of mucous cells, MCs, neutrophils and RCs in the infected and uninfected intestines of mullets were the same described previously in other fish species (see Vallejo & Ellis 1989; Dezfuli *et al.* 2015a).

Discussion

Myxozoans are among the most abundant parasites in nature. There are approximately 2000-2500 species (Paladini *et al.* 2017) and the histozoic myxozoans make for most dangerous form of infection (Gomez *et al.* 2014), causing outbreaks of mortality in fish (Lom & Dyková 2006; Gomez *et al.* 2014; Sitja-Bobdilla *et al.* 2015). Detailed information on species of myxozoans infecting mullets was published by Bahri and collaborators (Bahri & Marques 1996; Bahri, Andree & Hedrick 2003) and recently reviewed by Ovcharenko (2015). Many myxozoans infect gut of freshwater and marine fish (Bahri *et al.* 2003; Gomez *et al.* 2014). *Ceratomyxa* and *Enteromyxum* species are most studied and several investigations have enumerated hosts' immune response to them (Fleurance *et al.* 2008; Hallett & Bartholomew 2012; Estensoro *et al.* 2013; Bjork *et al.* 2014; Gomez *et al.* 2014). The first account of presence of *Myxobolus mugchelo* (Myxozoa) in intestine of *Liza ramada* from North Adriatic Sea was reported by Ovcharenko *et al.* (2017).

The immune response elicited by myxozoans depends on several factors including host and parasite species, target organ and the individual resistance (Gomez *et al.* 2014). *Enteromyxum leei* and *E. scophthalmi* occur in intestinal tissue of some host species and are accounted for loss of important Mediterranean fish species (Gomez *et al*. 2014). In the current study, plasmodia of *M. mugchelo* were

encountered in all intestinal layers of both mullet species. In the intestinal muscle layer very few MCs were noticed near the parasite. Whereas, numerous MCs were observed in close proximity to plasmodia encysted in submucosal layer and in some instances, neutrophils and macrophages also co-occurred with MCs. Free mature spores of *M. mugchelo* in intestinal epithelium in vicinity to mucous cells and MCs commonly have been noticed in this study. Our findings on contact between MCs and myxozoan plasmodia and spores are in agreement with previously published studies (Katharios, Rigos & Divanach 2011; Estensoro *et al.* 2013; Sitja-Bobadilla *et al.* 2016; Ovcharenko *et al.* 2017) (Table 2). However, to the best of our knowledge, this is the first account of intense degranulation of the MCs near plasmodia and free spores, in submucosal layer and epithelium respectively.

Occurrence of enteric helminth species in mullets in Mediterranean countries were reported in Merella & Garippa (2001), Ragias *et al.* (2005), Kotb *et al.* (2014). In *L. saliens* and *L. ramada* of the area studied, digeneans *H. pachysomus*, *D. contractus* and acanthocephalan *N. agilis* co-occurred with *M. mugchelo*. Several digeneans are enteric parasite of fish, their site of infection is almost entirely restricted to the paramucosal lumen, mucosa or epithelial tissues (Dezfuli *et al.* 1997, 2009a; Paperna & Dzikowski 2006). Intestinal digeneans are considered to be parasites that do not induce evident clinical disease, regardless of their abundance in host intestine (Paperna & Dzikowski 2006). Frequently the main pathology caused by digeneans is destruction of the mucosal epithelium covering the villi, with subsequent necrosis and degeneration of the cells (Dezfuli *et al.* 1997; Mladineo 2006; Constenla *et al.* 2011; current study see Table 2).

The acanthocephalan *N. agilis* is a widely spread parasite of grey mullets in the Atlantic and Pacific Oceans (Tkach, Sarabeev & Shvetsova 2014). This study showed that *N. agilis* penetrates with the proboscis in mucosal and submucosal layers and most likely induce an intense response of mucosal immunity in *L. saliens* and *L. ramada* (Table 2). However, due to the co-presence of the acanthocephalan with *M. mugchelo* encysted in above intestinal layers (Fig. 2a), it is difficult to attribute the host reaction to a single parasite.

Herein, in intestinal epithelium, mucous cells were in close proximity to innate immune cells, namely RCs, MCs and neutrophils. RCs are unique cells to teleost fish, were found in different tissues of many fish families and have an important role in host defense against parasites (Manera & Dezfuli 2004; Reite & Evensen 2006; Mazon *et al.* 2007; Vigliano *et al.* 2009; Matisz, Goater & Bray 2010; Palenzuela *et al.* 2014; Dezfuli *et al.* 2016). In infected intestine of different fish-parasite systems, RCs frequently were in close proximity to the mucous cells and discharge of both cell types in interface

region and/or toward the parasite body was well documented in Dezfuli *et al.* (2015a) and in the current study.

The presence of parasitic worm activates host innate immunity with recruitment and/or formation of the different type of immune cells like neutrophils and MCs (Reite & Evensen 2006; Häger, Cowland & Borregaard 2010; Buchmann 2012; Secombes & Ellis 2012; Dezfuli *et al.* 2013, 2016). Both neutrophils and MCs have important role in intestine mucosal immunity and can co-occur in epithelium but most frequently in submucosal layer (Dezfuli *et al.* 2013, 2016, 2017) and the same finding was observed herein. MCs are present in many teleost species and are key regulatory cells active in coordination of many functions of the innate immunity (Secombes & Ellis 2012; Dezfuli *et al.* 2015b, 2016). In mammals host reaction against parasites relies mainly on eosinophils, which represent the hallmark of parasite inflammatory reaction, whereas, most fish react against parasites by recruitment of the MCs. This reaction is well known and apparently is common in likely all vertebrate classes. In fact, even in mammals, in spite of the aforementioned typical recruitment of eosinophils, MCs are involved against parasite (Chieffi Baccari *et al.* 2011).

Records on contents of granules of fish MCs revealed that they have a panel of inflammatory mediators (Silphaduang & Noga 2001; Dezfuli *et al*. 2009b; Galindo-Villegas *et al.* 2016; Da Silva *et al.* 2017). MCs degranulation was observed after inoculation of *Aeromonas salmonicida* exotoxins (Ellis 1985; Vallejo & Ellis 1989), although, degranulation of MCs was induced *ex vivo* by use of Compound 48/80 on intestinal tissue of rainbow trout (Manera *et al.* 2011). In present survey, in *L. saliens* and *L. ramada* infected intestines, intense MCs degranulation was documented in close proximity to *M. mugchelo* plasmodia and its free mature spores. The degranulation of MCs in fish in response to parasite has been reported (e.g. Dezfuli & Giari 2008; Dezfuli *et al.* 2013, 2015b) but only very few studies have investigated this phenomenon in intestinal epithelium of infected fish (Reite 2005; Dezfuli *et al.* 2015a, current study). It seem that, MCs degranulation may have a role in attracting other types of granulocytes such as neutrophils (Dezfuli *et al.* 2013). In a recent paper, the penetration of MC granules into the tegument of a helminth was reported (Dezfuli *et al.* 2015b). The MC granules, which contain piscidins, have been shown to be involved in the permeabilization of bacterial membranes by toroidal pore formation (Campagna *et al.* 2007). Thus, it is presumable that the release of the MCs granules noticed in the intestine of two mullet species may have the same mechanism against *M. mugchelo*.

It is of great interest that, both mullet species possess high number of MCs in intestinal epithelium and in submucosal layer, although, in epithelium, frequently MCs have been found to

adhere to the mucous cells or were in their close vicinity. Therefore, it is reasonable to promote *L. saliens* and *L. ramada* species as a good model for mucosal immunity investigations in fish-myxozoan and fish-helminth systems. Furthermore, better understanding of fish mucosal immunity against micro/macroparasites might provide new insights about the evolution of fish immunity and control mechanisms for inflammation elicited by pathogens/parasites.

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Figure captions

Fig 1. Histological sections of intestine of *Liza ramada* (a and b) and intestine of *L. saliens* (c and d) infected by *Myxobolus mugchelo* (Giemsa stain). **(a)** Spindle like plasmodia developing in the muscle layer (arrow heads) and in submucosal layer (arrows), scale bar = 100 µm; **(b)** Plasmodium (asterisk) encysted in submucosal layer, several mast cells (arrows) are in close vicinity to the plasmodium, scale bar = 20 µm; **(c)** Micrograph shows intestinal epithelium of *L. saliens*, numerous free spores of *M. mugchelo* are in proximity to mucous cells (circles), some mast cells (arrows) are visible within the epithelium, scale bar = 10 µm; **(d)** Free spores in epithelium and in connective tissue in close vicinity to the mast cells (circles), intense degranulation of the mast cells (arrows) is evident, scale bar = 10 µm.

Fig. 2. Histological section of intestine of *Liza ramada* (a and c) and intestine of *L. saliens* (b and d). **(a)** Occurrence of *Neoechinorhynchus agilis*: contact (arrows) between mucosal folds and *N. agilis* trunk is shown, at the site of proboscis attachment numerous mast cells are present (arrow heads) (Alcian Blue and Haematoxylin & Eosin stain), scale bar = 100 µm; **(b)** Detachment of apex of villi (arrow heads) near *N. agilis* trunk (asterisk) is visible, note presence of blanket mucus (arrows) between acanthocephalan trunk and mucosal layer, (Alcian Blue and Haematoxylin & Eosin stain) scale bar = 100 µm; **(c)** Co-presence of *Haplosplanchus pachysomus* (black asterisk) and *N. agilis* (white asterisk), note detachment of intestinal fold by digenean sucker (arrow) (Alcian Blue and Haematoxylin & Eosin stain), scale bar = 100 µm; **(d)** Sucker of *Dicrogaster contractus* (black arrow) damaged the epithelial cells by their detachment from the fold (asterisk), numerous mast cells (white arrows) are visible near the sucker site of attachment, (Giemsa stain), scale bar = 10 µm.

Fig. 3. Transmission electron microscopy of intestine of *Liza ramada* (a and d) and intestine of *L. saliens* (b and c). **(a)** Micrograph shows periphery of the plasmodia of *M. mugchelo* (asterisk) encysted in submucosal layer, some mature spores (arrow heads) are evident, few mast cells (arrows) are in proximity to the plasmodium, scale bar = 4.2 µm; **(b)** High magnification of interface region between a mast cell (black asterisk) and a mature spore (white asterisk), note hyaline substance (arrows) encircling the spore and numerous pseudopodia-like structures (arrow heads), scale bar = 0.8 µm; **(c)** Interface region between *Haplosplanchus pachysomus* (asterisk) and *L. saliens* intestine: a mucous cell

(arrow) in discharge activity and two rodlet cells (arrow heads) near the apex of the fold are visible, scale bar = 3.3 µm; **(d)** *Dicrogaster contractus* (curved arrow) near the surface of *L. ramada* intestinal fold: a rodlet cell (arrow head) released the contents in interface region, two mucous cells (arrows) are near the fold surface, scale bar = 2.9 µm.

Fig. 4. Transmission electron microscopy of immune cells in intestine of *L. saliens* (a and d) and *L. ramada* (b and c). **(a)** Portion of the intestinal epithelium showing mast cell (curved arrow), rodlet cell (arrow head) and mucous cell (arrow), scale bar = 4.3 µm. **(b)** Apical part of *L. ramada* intestinal fold: rodlet cell (arrow head), macrophage (thick arrow) and mucous cell (arrow) are near the brush border, scale bar = 3.3 µm. **(c)** Portion of intestinal epithelium of *L. ramada*, a neutrophil (curved arrow) is in proximity to a mucous cell (arrow), scale bar = 1.7 µm. **(d)** Micrograph shows submucosal layer of *L. saliens*: numerous mast cells (arrows) encircling two neutrophils (curved arrows), scale bar = 3.6 µm.