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Enteromyxum leei (MYXOZOA: MYXOSPOREA) INFECTION IN GILTHEAD SEA BREAM (*Sparus aurata*): IMMUNE-RELATED STUDIES

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MEMORIA PRESENTADA PARA OPTAR AL GRADO DE DOCTORA EN CIENCIAS BIOLÓGICAS POR:

Itziar Estensoro Atienza

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–

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Informa: Que la presente Tesis Doctoral titulada *"Enteromyxum leei* (Myxozoa: Myxosporea) infection in gilthead sea bream (*Sparus aurata*): Immune-related studies", presentada por Doña Itziar Estensoro Atienza para optar al grado de Doctora en Biología, ha sido realizada dentro de la sublínea de investigación "Nutrición y Sanidad Animal" del Instituto de Acuicultura Torre de la Sal bajo mi dirección y, considerándola concluida, autorizo su presentación a fin de que pueda ser juzgada por el Tribunal correspondiente.

Castellón, 21 de junio de 2013

Fdo: **Ariadna Sitjà Bobadilla** Fdo: **Itziar Estensoro Atienza**

APOYO INSTITUCIONAL

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Just give that rhythm everything you've got!"
El gran duque… **Duke Ellington**

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01

GENERAL INTRODUCTION

1.1 FOREWORD: THE CONSTRAINS OF AQUACULTURE

According to FAO, aquaculture provides more than half of the global fish consumption, since fishery captures stagnate and world population grows. The expanding aquaculture sector has to face a growing demand not only of its production, but also of other key issues such as food safety and quality requirements, animal welfare and social and environmental concerns. Technological and scientific resources in research are already being directed towards sustainable development by, for example, incorporating "Integrated multi-trophic aquaculture" or identifying suitable species for farming depending on their feeding habits, which in turn will help to expand and diversify the sector.

With the intensification of aquaculture, the maintenance of high density fish stocks has increased the impact and importance of infectious pathological threats for fish. The introduction of new market species, as well as their worldwide transport also entails new risks for fish health. The study of the fish immune system and of its interaction with the pathogens has enabled the development of vaccines to keep control on bacterial infections. However, other etiologic agents, such as viruses, fungi and parasites, still deserve further attention.

The impact of parasites on fish cultures has increased in the last years. Their detrimental effect includes not only direct mortalities, but also arrested growth, reproductive failure, loss of quality of the product, reduced marketability and increased susceptibility to secondary infections and stress. In the case of parasites with direct life cycles, the potential to cause harm increases rapidly under culture conditions.

In any case, aquaculture activities should be conducted in a manner that optimizes the health and welfare of aquatic animals by minimizing stress, reducing aquatic animal disease risks and maintaining a healthy environment at all phases of the production cycle. To assure these basics the sector relies on the guidelines and standards established by the competent authorities, which in turn depend on the available knowledge on aquatic pathologies, including epidemiologic aspects and disease mechanisms of action. Accordingly, some fish diseases are included in European (Directive 2006/88/CE) and national (Royal Decrete 1614/2008) lists of mandatory declaration, but no fish parasite is included in them. However, if seafood is seriously damaged by parasites it has to be removed from market according to European Directive 93/149/EC and to the national Royal Decrete 1437/1992. In any case, arising minimum sanitary measures include quarantining of the stocks, routine monitoring of stocks and water quality, reduction of transmission chances or application of veterinary medicines in accordance with national legislation (FAO-COFI 2011). However, there are still many threatening pathogens for aquaculture species, which lack preventive or therapeutic treatments and entangle a challenge for scientific research.

The success of aquaculture relies on the control of production losses and species reproduction, on a better knowledge of their biology, on technological innovation and on the development of specific foods. Indeed, the main current limitations for the aquaculture sector are production losses by diseases and the cost of aquafeeds. Reduction of the costs of fish feeds through substitution of fish-based ingredients by vegetable ones is being weighted up. The ultimate goal of this thesis is to approach fish health and welfare management in aquaculture in the framework of a host-parasite model.

1.2 WORLD AQUACULTURE & GILTHEAD SEA BREAM PRODUCTION

Global aquaculture production has increased substantially during the past decades, as aquaculture has been and still is the fastest growing animal food-producing sector at an average annual rate of 8.8 % in the past three decades (1980-2010) (Fig. 1.1) (FAO 2012). In 2010 aquaculture accounted for 47 % of the world's food fish consumption, compared with 33.8 % in 2000, and is predicted to account for 65 % in 2030. In contrast, marine capture fisheries production remained relatively stable in the past decade (about 90 million tonnes). Moreover, 53 % of the marine fish stocks monitored by FAO are currently fully exploited, 28 % are overexploited and 3 % are depleted, thus being not able to produce more than their current catches. Therefore, aquaculture is set to overtake capture fisheries as a source of food fish. Global per capita supply from aquaculture products increased steadily from 0.7 kg in 1970 to 8.7 kg in 2010, year in which the total aquaculture production reached 79 million tonnes. More than half of the animal aquaculture production, 65.5 %, was finfish, though the production increase took place in all the cultured species groups. Marine fishes accounted in 2010 only for 3.1 % (1.8 million tonnes) of the global aquaculture production, while freshwater fishes dominated with 56.4 % (33.7 million tonnes) of the global production and diadromous fish meant 6 % (3.6 million tonnes) of the production. In 2010, the total amount of produced finfish accounted for 60.1 % of the total value of aquaculture products.

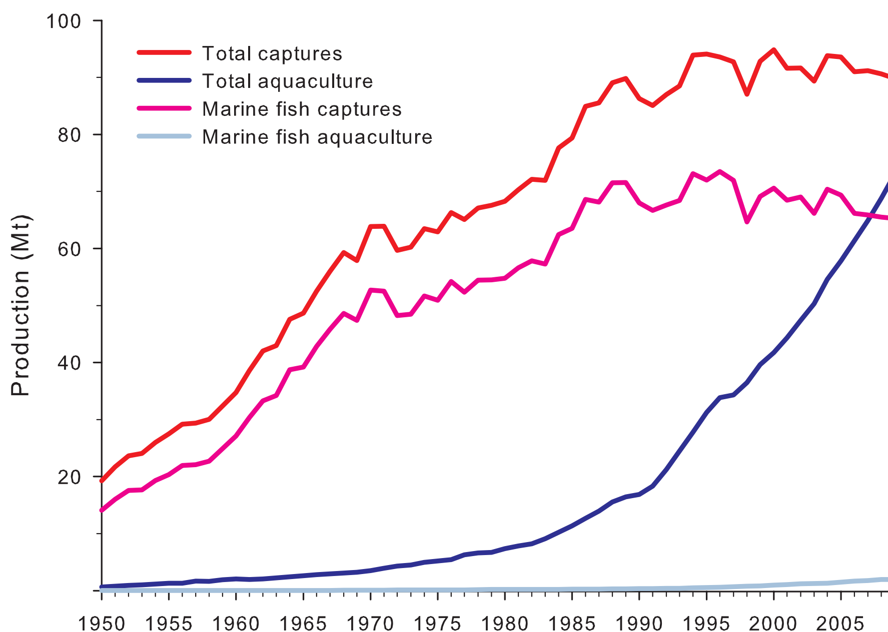

Figure 1.1. World fisheries production in million tonnes (Mt) by captures and aquaculture from 1950 to 2009. Marine fish production and total production (includes marine, fresh water and diadromous fish, mollusks, crustaceans and aquatic plants) are shown. Data by FAO-ISSCAAP (International Standard Statistical Classification of Aquatic Animals and Plants) divisions.

Gilthead sea bream (*Sparus aurata* Linnaeus, 1758) is mainly cultured in the Mediterranean basin, though it is also present in the Black Sea, the Red Sea, the Persian Gulf and the Arabian Sea. Greece is the largest producer (39.6 %), followed by Turkey (22.5 %) and Spain (11.2 %) (Table 1.1). In addition, considerable production occurs in Italy, Croatia, Cyprus, Egypt, France, Malta, Portugal, Tunisia and Morocco, and minor producers are Albania, United Arab Emirates, Bosnia, Libya, Kuwait and Oman. According to FAO, global aquaculture production of gilthead sea bream reached in 2011 almost 151,346 tonnes, lower than the peak of 178,554 tonnes produced in 2008 (Fig. 1.2). As for the global fishery production (Fig. 1), fishery captures of gilthead sea bream in Mediterranean and Atlantic waters remained relatively stable between 5,000 tonnes and 8,000 tonnes during the past 20 years, while aquaculture production raised steadily and accounted in 2010 for the 94.4 % of the total gilthead sea bream production (APROMAR 2012).

Table 1.1. Gilthead sea bream aquaculture production in tonnes (t) by major producer countries from 2002 to 2010. (FAO Fishery Statistic).

COUNTRY	YEAR								
	2002	2003	2004	2005	2006	2007	2008	2009	2010
Croatia	700	1,000	1,000	1,200	1,500	1,500	1,500	2,200	2,200
Cyprus	1,266	1,181	1,356	1,465	1,879	1,404	1,600	2,572	2,799
France	1,361	1,100	1,600	1,900	2,200	1,392	1,636	1,648	1,377
Greece	37,944	55,000	48,000	50,000	66,000	79,000	94,000	90,000	74,000
Italy	8,000	9,000	9,050	9,500	6,500	11,650	9,600	9,600	9,800
Malta	1,066	827	784	645	894	1,097	1,574	1,984	2,000
Portugal	1,855	1,449	1,685	1,519	1,623	1,600	1,611	1,600	1,600
Spain	11,335	12,442	13,034	15,577	20,220	22,320	23,930	23,690	20,360
Turkey	11,681	16,735	20,435	27,634	28,463	33,500	34,000	25,000	22,000
TOTAL	**75,208**	**98,734**	**96,944**	**109,440**	**132,279**	**153,463**	**169,451**	**158,294**	**136,136**

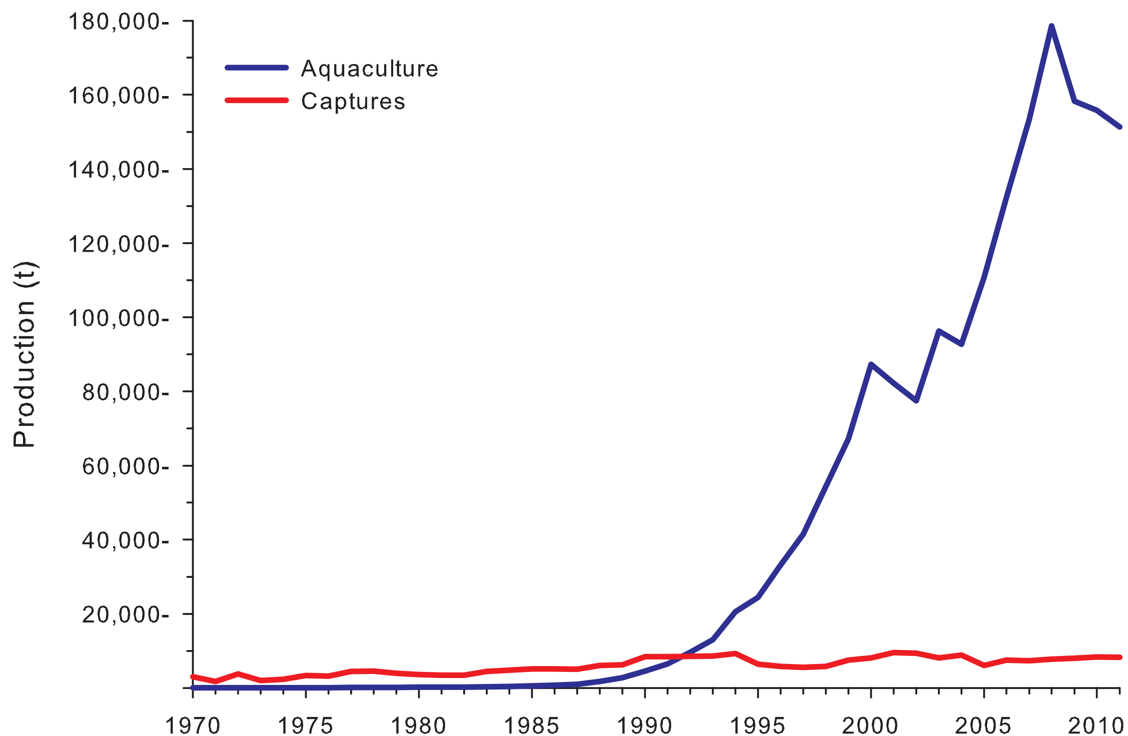

Figure 1.2. Global production in tonnes (t) of gilthead sea bream by captures and aquaculture from 1970 to 2011. (FAO Fishery Statistics).

1.3 GILTHEAD SEA BREAM: GENERAL OVERVIEW

Gilthead sea bream belongs to the Sparidae family (Teleostei: Perciformes), which comprises 35 genera and 112 different marine fish species. The main distinctive features of the species are the silvery grey color with a large black blotch at the origin of the lateral line extending over the upper margin of the opercle and the golden frontal band between the eyes (Fig. 1.3). Gilthead sea bream is found in temperate, subtropical and tropical littoral waters and in brackish inshore waters, commonly in seagrass beds and sandy or rocky bottoms. This species is eurythermal (tolerance range 5°-33° C) and euryhaline (tolerance range 3-70 ‰), thought its optimum temperature and salinity ranges for reproduction are 14°-18° C and 37-38 ‰. Juveniles migrate towards shallower protected coastal areas in spring, living in waters of 30 m depth, while adults return to deeper waters during autumn, being found in depths up to 100-150 m. They are sedentary fish, rather solitary, though also forming aggregations during reproductive migrations. Gilthead sea bream is mainly a carnivorous fish, feeding preferably on bivalve molluscs and small fish, cephalopods or crustaceans, though also occasionally herbivorous. It is a protandrous hermaphrodite fish developing sexual maturity as male at 2 years of age (20-30 cm) and as female at 2-3 years (33-40 cm).

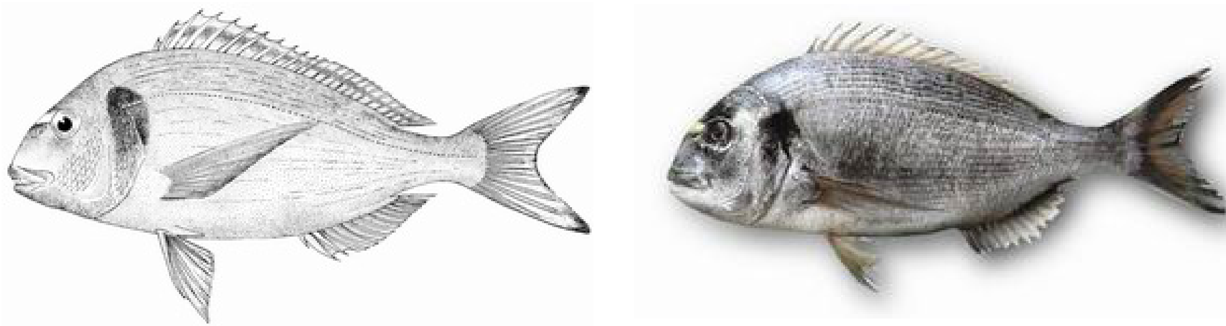

Figure 1.3. Gilthead sea bream (*Sparus aurata*) (drawing by FAO).

Gilthead sea bream is an excellent food fish and has been traditionally cultured extensively in coastal lagoons and saltwater ponds of the Mediterranean. During the early 80's artificial breeding was achieved and in turn large-scale production of juveniles started. The species adapted quickly to intense rearing conditions and is nowadays cultured in cages, ponds and tanks. From the hatching, it takes between 18 and 24 months for gilthead sea breams to reach a 400 g size and commercial sizes range from 250 g to 1,500 g.

1.4 PARASITOSES IN CULTURED GILTHEAD SEA BREAM

Parasitic pathologies have acquired an increasing importance among the factors affecting animal welfare and limiting aquaculture productivity. Intensification of marine fish aquaculture has favoured the development and severity of parasitic infections which can be further worsened by an immunodepressed status of the fish due to environmental or dietary factors. The main detrimental consequences caused by parasites on cultured fish are high mortalities during outbreaks or chronic mortalities throughout life cycle, decreased growth performance and increased susceptibility to secondary infections.

Several parasites have been reported in gilthead sea bream, though their pathogenicity is variable. This sparid is susceptible to ectoparasites, affecting skin or gills, which include ciliates, flagellates, crustaceans and helminths as well as to endoparasites, affecting internal organs and tissues, which include Myxozoa, Microsporea, Apicomplexa and Digenea (Table 1.3).

Table 1.2. Main parasites in cultured gilthead sea bream.

	PARASITE GROUP	SPECIES	AFFECTED TISSUE	REFERENCE
ECTOPARASITES	Ciliophora	*Cryptocaryon irritans*	Gi, Sk, Eye	Colorni (1985)
		Trichodina spp. (peritrich ciliate)	Gi, Sk	Álvarez-Pellitero et al. (1995)
		Scuticociliates	Wounds, Gi, MC (Syst)	Fioravanti et al. (2006)
		Porpostoma notatum (scuticociliate)	Sk	Paperna (1984)
		Brooklynella hostilis	Gi, Sk	Diamant (1998a)
	Flagellata	Mastigophora: *Amylodinium ocellatum*	Gi, Sk	Paperna (1980)
		Kinetoplastida: *Ichthyobodo sp.*	Sk, Gi	Álvarez-Pellitero et al. (1995)
		Cryptobia spp.	Gi	Blanc et al. (1989) Diamant (1990)
	Platyhelmintha	Monogenea: *Sparicotyle chrysophrii*	Gi	Faisal and Imam (1990)
		Furnestinia echeneis	Gi	Reversat et al. (1992)
		Gyrodactylus sp.	Body surface	Paladini et al. (2009) Paladini et al. (2011)
		Neobenedenia melleni	Body surface	Colorni (1994)
	Crustacea	Copepoda: *Caligus minimus*	MC	Paperna (1984)
		Ergasilus spp.	Gi	Fioravanti et al. (2006) Dezfuli et al. (2011)
		Isopoda: *Ceratothoa oestroides*	MC, Gi	Mladineo (2003a)
		Ceratothoa parallela	MC, Gi	Papapanagiotou and Trilles (2001)
ENDOPARASITES	Platyhelmintha	Digenea: *Cardicola aurata*	Blood vessels, Gi, Kd	Holzer et al. (2008) Padrós et al. (2001b)
	Myxozoa	*Ceratomyxa sparusaurati*	GB, Int	Sitjà-Bobadilla et al. (1995)
		Enteromyxum leei	Int	Diamant et al. (1994)
		Henneguya sp.	Gi, Heart	Caffara et al. (2003)
		Kudoa iwatai	Mu (Syst)	Diamant et al. (2005)
		Leptotheca sparidarum	Kd	Sitjà-Bobadilla and Álvarez-Pellitero (2001)
		Polysporoplasma sparis	Kd	Sitjà-Bobadilla and Álvarez-Pellitero (1995)
	Microsporea	*Pleistophora* sp. *Glugea* sp. *Enterospora* sp.	Mu Sk, Mu (Int) Int	Abela et al. (1996), Athanassopoulou (1998) Mathieu-Daude et al. (1992) Palenzuela et al. (2011)
	Apicomplexa	*Cryptosporidium molnari* *Eimeria sparis* *Goussia sparis*	Sto, Int Int Int	Álvarez-Pellitero and Sitjà-Bobadilla (2002) Sitjà-Bobadilla et al. (1996) Sitjà-Bobadilla et al. (1996)

Abbreviations: gills (Gi), skin (Sk), mouth cavity (MC), kidney (Kd), muscle (Mu), intestine (Int), stomach (Sto), systemic (syst)

Culture procedures influence greatly the type and severity of the disease. The structural characteristics of the facilities whether inland, inshore or offshore floating cages or submersible cages are commonly decisive for the onset of the infections and for the appearance of opportunistic pathogens. Related factors involved are fish density, water renewal rate, proximity to the seabed, water temperature and water salinity (Fioravanti et al. 2006, Colorni & Padrós 2011).

There is no effective treatment available for most of the threatening parasites mentioned and therefore strict cleaning, disinfection and sanitary measures to avoid them as well as preventive routine surveys for the early detection are essential in aquaculture facilities. An understanding of parasite's life cycle provides a scientific framework for disease prevention and management. The ultimate goal of a prevention or control program is to break the life cycle of the parasite and to stop future infections.

1.5 MYXOZOA

The phylogenetic position of the phylum Myxozoa has always entailed controversy, first until its placement within Metazoa was confirmed (Smothers et al. 1994) and more recently for its assignment either to Bilateria or to Cnidaria. Though phylogenetic, morphologic and developmental data support the cnidarian hypothesis (Siddall et al. 1995, Jimenez-Guri et al. 2007, Raikova 2008, Morris 2010, Holland et al. 2011, Ringuette et al. 2011, Gruhl & Okamura 2012) ribosomal DNA data also demonstrate Bilateria affinity within Myxozoa (Smothers et al. 1994, Monteiro et al. 2002, Evans et al. 2010).

The phylum Myxozoa gathers over 2,700 species. Most of them are parasites of fish, but they also infect invertebrates, amphibians, reptiles, birds and mammals. Their simple and reduced body form is considered an evolutionary adaptation to parasitism. Characteristic of these parasites are multicellular spores, composed by one or more shell valve cells, ameboid infective cells or sporoplasms and one or more nematocyst-like polar capsules. Such capsules contain a coiled polar filament, extrudible upon contact with the host where it anchors permitting the penetration of the sporoplasm. Myxozoan vegetative (trophic) stages have the form of a closed sac (class Malacosporea) or of a multicelullar, spore-producing, often ameboid plasmodium (class Myxosporea). The latter may be coelozoic or histozoic and intercellular or intracellular. The life cycle pattern involves host alternation as a general rule (Kent et al. 2001, Lom & Dyková 2006). The most pathogenic species for commercially important fish hosts belong to the genera *Ceratomyxa* Thélohan, 1892, *Myxobolus* Bütschli, 1882, *Sphaerospora* Thélohan, 1892, *Enteromyxum* Palenzuela, Redondo et Álvarez-Pellitero, 2002, *Kudoa* Meglitsch, 1947 and *Tetracapsuloides* Canning, Tops, Curry, Wood et Okamura, 2002 (Table 1.4) though not all of them have been recorded from gilthead sea bream.

Classification of Myxozoa was traditionally based on myxospore morphology, namely the number and configuration of valves and polar capsules, but did not take into account life cycle, morphology of actinospores or host and tissue preferences in most cases. Though this taxonomy persists, phylogenetic relationships based on small subunit rDNA have shown the artificial nature of such a classification. Thus, current taxonomic labels in Myxozoa are susceptible to future rearrangement. However, the two main myxozoan clades, Malacosporea and Myxosporea, are well supported by morphological, developmental and genetic data (Lom & Dyková 2006).

Table 1.3 Myxozoa classification of the most important genera pathogenic for aquacultured fish based on classical spore morphology and life cycles (Canning & Okamura 2004).

PHYLUM MYXOZOA Grassé, 1970
- **Class Malacosporea** Canning, Curry, Feist, Longshaw et Okamura, 2000
  - Order Malacovalvulida Canning, Curry, Feist, Longshaw et Okamura, 2000
    - Family Saccosporidae Canning, Okamura et Curry, 1996:
      - *Tetracapsuloides* Canning, Tops, Curry, Wood et Okamura, 2002
- **Class Myxosporea** Bütschli, 1881
  - Order Multivalvulida Shulman, 1959
    - Family Kudoidae Meglitsch, 1960:
      - *Kudoa* Meglitsch, 1947
  - Order Bivalvulida Shulman, 1959
    - ·Suborder Variisporina Lom et Noble, 1984
      - Family Myxiidae Thélohan, 1892:
        - *Enteromyxum* Palenzuela, Redondo et Álvarez-Pellitero, 2002
      - Family Sinuolineidae Schulman, 1959:
        - *Sinuolinea* Davis, 1917
      - Family Ceratomyxidae Doflein, 1899:
        - *Ceratomyxa* Thélohan, 1892
        - *Leptotheca* Thélohan, 1895
      - Family Sphaerosporidae Davis, 1917:
        - *Sphaerospora* Thélohan, 1892
    - ·Suborder Platysporina Kudo, 1919
      - Family Myxobolidae Thélohan, 1892:
        - *Myxobolus* Bütschli, 1882
        - *Henneguya* Thélohan, 1892

1.5.1 Class Malacosporea

Two genera are known to belong to the Malacosporea, i.e. *Buddenbrockia* Schröder, 1910 and *Tetracapsuloides*, both with alternating hosts between freshwater bryozoans and fish (Fig. 1.4). Their trophic, proliferative stages, either closed multicellular sacs (in *T. bryosalmonae* Canning, Curry, Feist, Longshaw et Okamura, 1999) or vermiform organisms (in *B. plumatellae* Schröder, 1910), dwell within the bryozoan body cavity. Within the proliferative stage, upon completion of meiosis, mature, spherical malacospores develop. In both genera, bryozoan malacospores consist of eight soft shell valves encasing four polar capsules and two sporoplasms with secondary cells and in some cases tertiary cells inside. The water born malacospores infect a fish host through the gills or skin, where the sporoplasms leave the spore valves and penetrate (Grabner & El-Matbouli 2010). Once the parasite reaches the fish kidney, sporogony takes place in the interstitium or tubule lumina, where subspherical fish malacospores of four soft shell valves, two polar capsules and one sporoplasm without secondary cells but with sporoplasmosomes, are formed (Hedrick et al. 2004, Morris & Adams 2008). These cause cryptic single cell covert infections or overt infections closing the life cycle.

The highly pathogenic *T. bryosalmonae* is the causative agent of the proliferative kidney disease (PKD), affecting only salmonid fish. Proliferative stages located in the fish kidney cause a severe inflammatory reaction associated with chronic, granulomatous interstitial nephritis and tubular atrophy (Lom & Dyková 2006).

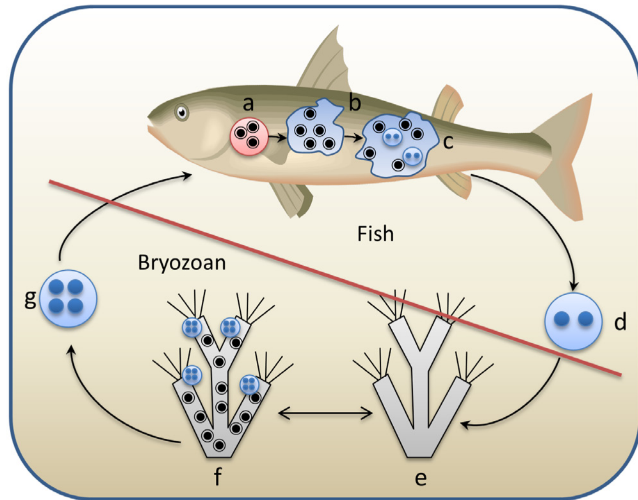

Figure 1.4. Schematic illustration of the malacosporean *Tetracapsuloides bryosalmonae* life cycle alternating between fish and bryozoan hosts. The extra-sporogonic and pre-sprogonic stages (**a-b**) develop in the blood and fish tissues and sporogonic stages in kidney (**c**) produce fish malascospores with two polar capsules which are released to the water (**d**) and infect bryozoans causing covert infections (**e**). In overt infections (**f**) sacs are developed and finally malacospores with four polar capsules are produced (**g**), which are infective for fish. Note that cycling can occur between covert and overt infections (after Sitjà-Bobadilla and Palenzuela 2013).

1.5.2 Class Myxosporea

Most of the myxozoan species known thus far belong to the class Myxosporea, which includes pathogenic species for freshwater as well as for marine fish (Table 1.3). Very characteristic of most myxosporean species is their diphasic life cycle, consisting of a myxospore phase and an actinospore phase, alternating between two hosts. The myxospore phase occurs in the intermediate vertebrate host and results in myxospore production within a sporogonial plasmodium. Typical from these proliferative stages is the cell-in-cell condition, in which endogenously produced secondary cells persist inside primary mother cells. Tertiary cells within secondary cells and quaternary cells within tertiary ones may also occur. The actinospore phase involving a sexual process that results in actinospore production takes place in annelids, primarily oligochaetes in freshwater and polychaetes in marine environments.

1.5.2.1 Life cycle

The biphasic life cycle of myxosporean species was described for the first time for *Myxobolus cerebralis* Hofer, 1903 when the alternation with an actinosporean form in the oligochaete *Tubifex tubifex* Müller, 1774 was discovered (Markiw & Wolf 1983, Wolf & Markiw 1984). Thereafter, many other life cycles have been documented helping to understand the establishment of myxosporean infections and consequently, effective prophylactic measures in aquaculture could be determined and furthermore, experimental infections could be performed. Figure 1.5 shows the myxosporean diheteroxenous life cycle with host alternation. When the waterborne actinospore (Fig. 1.5 b) is released from the annelid and contacts the fish host, the polar capsules discharge the polar filaments and the spore valves open enabling the sporoplasm to penetrate intercellularly the host skin or gill epithelium. Alternatively, the actinospore may contact the fish host through ingestion of the infected annelid. This pre-sporogonic proliferative stage, before or after migration to the target site of sporogony, divides and multiplies itself through plasmotomy forming a plasmodium or a pseudoplasmodium (Fig. 1.5 c-d). The (pseudo)plasmodia have

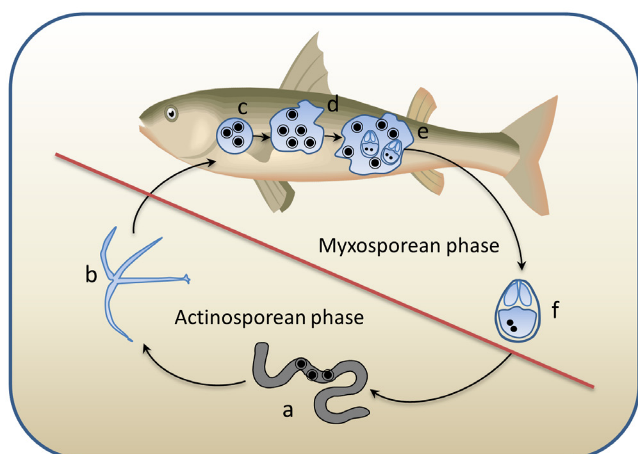

Figure 1.5. Schematic illustration of a myxosporean life cycle alternating between fish and annelid hosts. The actinosporean phase develops in annelids after myxospore ingestion (**a**). Actinospores (**b**) released from the annelids infect the fish host. The myxosporean pre-sporogonic (**c-d**) and sporogonic stages (**e**) develop in the fish producing myxospores (**f**) which are released to the water (after Sitjà-Bobadilla and Palenzuela 2013).

the mentioned cell-in-cell arrangement and may proliferate massively. Endogenously produced generative cells give rise to pericytes and sporogonic cells, the latter divide and differentiate into capsulogenic, valvogenic and sporoplasmogenic cells. These cells develop into sporoblasts and eventually into myxospores inside the pericyte, being all together the so-called pansporoblast (Fig. 1.5 e). Plasmodia are polysporous, while pseudoplasmodia are disporous or monosporous and they usually persist as presporogonic stages along with sporogonic stages for additional parasite proliferation. Pseudoplasmodia are smaller and contain a vegetative nucleus together with secondary cells. No pansporoblast develops from the pseudoplasmodia and sporoblasts originate from secondary cells, the sporogonic cells. Myxospores consist of one to seven myxospore valves which adhere along the suture line and contain polar capsule(s) and sporoplasm(s) (Fig. 1.5 f).

Myxospores released into the environment are ingested by the definitive annelid host (Fig. 1.5 a), in which the binucleated sporoplasm penetrates through the gut epithelium and initiates the merogony phase. After this vegetative proliferation, gametogony produces a pansporocyst with eight zygotes surrounded by eight enveloping cells. These give rise to eight tri-radiate actinospores (Fig. 1.5 b) by successive divisions and differentiation during sporogony, closing the cycle. Actinospores typically have three polar capsules, three valves with caudal projections and a multinucleated plasmodium-like sporoplasm with many infectious cells (Kent et al. 2001, Lom & Dyková 2006). Infectivity of actinospores lasts several days in contrast to the resistant myxospores which may remain infective in some cases more than 20 years (Sitjà-Bobadilla & Palenzuela 2013).

Exceptions to this life cycle pattern do, however, exist and direct horizontal fish-to-fish transmission without actinospore stage has been described for the members of the genus *Enteromyxum* (Diamant 1997, Redondo et al. 2002, Yanagida et al. 2006). Nevertheless, this does not exclude the existence of a putative actinosporean phase for the members of this genus.

1.6 MYXOSPOREAN INFECTIONS IN CULTURED GILTHEAD SEA BREAM

Myxosporea can represent an important threat to the culture of this sparid fish, considering that several species are involved (six different genera), and, moreover that they affect most organs of the fish, including digestive, respiratory, excretory and circulatory systems and lymphohaematopoietic as well as muscle tissues. Almost all tissues in gilthead sea bream can potentially be affected by myxosporean parasites, though infected fish are in some cases asymptomatic and obvious damage is only patent under particular stress conditions and can be influenced by the nutritional background of the host. Furthermore, for the myxosporeans with a broad host range, almost any coastal wild species may become a potential carrier of those parasites endangering aquaculture practices in inshore and offshore facilities.

1.6.1 *Ceratomyxa sparusaurati*

Ceratomyxa sparusaurati Sitjà-Bobadilla, Palenzuela et Álvarez-Pellitero, 1995 is generally not associated with a clinical disease, being its histopathological damage limited to the gall bladder and neighbouring tissues. Trophozoites, disporous sporoblasts and spores are commonly found in the gall bladder and bile of cultured gilthead sea bream from diverse culture systems. Additionally, parasite stages can also be detected in the biliary ducts, blood vessels of the pancreas and the spleen and in the intestinal epithelium (Palenzuela et al. 1997). Though high prevalences of infection can be found, only low dropping mortalities are associated to this species (Álvarez-Pellitero et al. 1995, Sitjà-Bobadilla et al. 1995, Palenzuela et al. 1997, Rigos et al. 1999, Mladineo 2003b). Prevalence of infection fluctuates with season and host age, increasing with higher water temperature and in older animals. In some cases of massive infections in which higher mortalities occur, severely infected fish present enlarged gall bladders and abdominal distension. The histopathological studies reveal intensive desquamation and destruction of the gall bladder epithelium with inflammatory reaction involving leukocyte infiltration. *Ceratomyxa* spp. are typical coelozoic myxosporeans producing sporogonic pseudoplasmodia with two developing spores, which are elongated with two subspherical polar capsules and a binucleate sporoplasm (Sitjà-Bobadilla et al. 1995, Lom & Dyková 2006). Whether an invertebrate host is involved in the life cycle of *C. sparusaurati* as for other members of the genus (Bartholomew et al. 1997), has still not been confirmed.

1.6.2 *Sphaerospora sparis*

This species, ex *Polysporoplasma sparis* Sitjà-Bobadilla et Álvarez-Pellitero, 1995, has recently been ascribed to the monophyletic group *Sphaerospora sensu stricto* based on rDNA sequence data and on the absence of unique morphological and developmental traits, and thus, the genus *Polysporoplasma* has been suppressed (Bartosova et al. 2013). The pathogenicity of *Sphaerospora sparis n. comb.* Bartošová, Fiala, Jirků, Cinková, Caffara, Fioravanti, Atkinson, Bartholomew, Holzer, 2013, found mainly in the trunk kidney of gilthead sea bream, is generally mild, showing no clinical signs and occasionally causing decreased growth and chronic mortality. *S. sparis* forms disporous pseudoplasmodia with a pair of subspherical spores with two subspherical polar capsules. The existence of more than two sporoplasms (up to twelve uninucleate sporoplasms) is a unique feature of a marine lineage *Sphaerospora* subgroup among Myxosporea (Sitjà-Bobadilla & Álvarez-Pellitero 1995, Bartosova et al. 2013).

S. sparis is considered histozoic since disporoblasts and spores are found in renal corpuscles and in the connective tissue of trunk kidney. Different degrees of tissue damage occur linked to spore maturation, eventually leading to the necrosis of infected renal corpuscles. However, the host's inflammatory reaction involving among other cell types melanomacrophages (MMΦs), isolates the parasite in granulomata, which prevent its spreading. Affected fish cope well with the impairment of renal excretion except in stressed or immunodepressed situations (Palenzuela et al. 1999). *S. sparis* has been reported from gilthead sea bream repeatedly without a clear seasonal pattern and with higher prevalence in older fish (Sitjà-Bobadilla et al. 1992, Palenzuela et al. 1999, Rigos et al. 1999, Mladineo 2003b, Athanassopoulou et al. 2004). The putative actinosporean phase of the parasite cycle has not been identified.

1.6.3 *Leptotheca sparidarum*

Leptotheca sparidarum Sitjà-Bobadilla et Álvarez-Pellitero, 2001 is a coelozoic myxosporean found mainly in the trunk kidney and whole urinary system of gilthead sea bream. It is not associated to clinical signs, except for a swollen urinary bladder in few cases. Spores, with two spherical polar capsules and binucleated sporoplasm, develop in disporous pseudoplasmodia. Sporogenesis takes place gradually from the renal tubuli epithelium towards the lumen. Thus, trophozoites are found closer to the epithelium, attached by short filopodia, and spores closer to the inner lumen. Infection by the parasite leads to atrophy of the renal epithelium and thickening of the glomerular capillary walls (Sitjà-Bobadilla & Álvarez-Pellitero 2001). However, few records exist on this myxosporean infection from gilthead sea bream, which seems quite harmless for the fish (Álvarez-Pellitero et al. 1995, Fioravanti et al. 2006). No information on the possible actinospore phase of *L. sparidarum* exists.

1.6.4 *Kudoa iwatai*

Kudoa iwatai Egusa et Shiomitsu, 1983 is the only myxosporean affecting gilthead sea bream that belongs to the order Multivalvulida. It forms macroscopic, whitish, ovoid polysporous plasmodial cysts which produce many quadrate spores of four valves, with four pyriform polar capsules and two uninucleate sporoplasms, one enveloping the other (Lom & Dyková 2006). Though the parasite was originally described from somatic musculature and adipose tissue, plasmodia are also present in other locations like the eye and its periphery, nerve axons, mouth, mesenteries, peritoneum, swim bladder, kidney, ovary, heart and pericardium, in all cases extracellularly located (Paperna 1982, Diamant et al. 2005). The majority of *Kudoa* spp. infect the skeletal muscle of marine fish forming plasmodial cysts and causing myoliquefaction, which gives the name to the disease, i.e. soft flesh condition. Myoliquefaction is due to the proteolytic enzymes released post mortem by the parasite, and makes flesh unacceptable for consumption. However, *K. iwatai* from gilthead sea bream does not induce liquefaction of the muscle tissue and no evidence of inflammation appears associated to plasmodia. In addition, the infection has no apparent effect on the fish, although unpleasant plasmodia appear in a wide range of host tissues. *K. iwatai* infections from gilthead sea bream are in any case only occasional and have been reported in few occasions (Arfara et al. 1995).

1.6.5 *Henneguya sp.*

The histozoic myxozoan *Henneguya sp.* Thélohan, 1892 is found in the gills and the heart of gilthead sea bream with a low prevalence. Spores are ellipsoidal, with caudal projections fused in a tail and with two elongated polar capsules. Free spores and polysporous plasmodia, the latter appearing as cysts, are located in the connective tissue between gill filaments, in gill blood vessels and in the cardiac musculature. *Henneguya* sp. gill infection produces tissue pallor and cardiac infection, accompanied by abundant connective tissue and collagen proliferation with leukocyte infiltration (Bahri et al. 1996, Caffara et al. 2003, Athanassopoulou 2007). These lesions indicate the potential pathogenicity of this myxosporean for gilthead sea bream, though no records on mortalities exist. The complete life cycle including the actinospore phase is known for some *Henneguya* spp., but not for the one affecting gilthead sea bream (Lom & Dyková 2006).

1.7 *Enteromyxum leei*

The genus *Enteromyxum* gathers three species *E. leei* Diamant, Lom et Dyková, 1994, *E. fugu* Tun, Yokoyama, Ogawa et Wakayabashi, 2002 and *E. scophthalmi* Palenzuela, Redondo et Álvarez-Pellitero, 2002, all of them enteric fish parasites constituting a monophyletic group (Palenzuela et al. 2002, Yanagida et al. 2004).

Enteromyxum leei was first described from tank-reared gilthead sea bream and associated to mortality outbreaks (Diamant 1992, Diamant et al. 1994). Thereafter, mortality and morbidity caused by the parasite in aquaculture facilities were reported in many occasions for gilthead sea bream (Álvarez-Pellitero & Sitjà-Bobadilla 1993, Sakiti et al. 1996, Fioravanti et al. 2006, Cuadrado et al. 2007, Fleurance et al. 2008) as well as for other sparids (Le Breton & Marques 1995, Athanassopoulou et al. 1999, Rigos et al. 1999, Golomazou et al. 2006b, Montero et al. 2007, Álvarez-Pellitero et al. 2008, Cuadrado et al. 2008, Rigos & Katharios 2010). Other marine and fresh-water teleosts are also susceptible (Diamant 1998b, Padrós et al. 2001a, Yanagida et al. 2004, Yasuda et al. 2005, Diamant et al. 2006, Sitjà-Bobadilla et al. 2007, Yokoyama & Shirakashi 2007, Yanagida et al. 2008, Katharios et al. 2011). The pathogenicity of this parasite varies among the different susceptible species. *E. leei* is one of the most devastating pathogens in warm water marine culture because of its broad range of susceptible fish (over 46 marine species) and its broad distribution (Mediterranean, Canary Islands, Red Sea, Western Japan). By contrast, the other *Enteromyxum* spp. show high host specificity (Sitjà-Bobadilla & Palenzuela 2012). In addition, *Enteromyxum* spp. are directly transmitted fish-to-fish favouring the propagation of the parasite. Whether an invertebrate host, target for the myxospores, is involved in the life cycle of these parasites, is still to be deciphered. Gilthead sea bream can therefore be infected by *E. leei* by exposure to a contaminated water effluent, by cohabitation with infected fish and by ingestion of pre-sporogonic developmental stages (Diamant 1997). In addition, gilthead sea bream can be experimentally infected by *E. leei* through anal route, as demonstrated within the results of this thesis (see chapter 3).

During the proliferative development, trophozoites develop S and T cells endogenously, which can be released and are responsible for the parasite proliferation within the host by invading new intestinal areas as well as for the transmission to other fish when released through faeces (Cuadrado et al. 2008) (Fig. 1.6 a). Though trophozoites are the first stages found, in advanced infections trophozoites, disporoblasts and free spores coexist. Spores of the *Enteromyxum* genus develop in disporous pseudoplasmodia and are crescent-shaped, with two elongated polar capsules located at the spore's pointed ends and one binucleate sporoplasm. *E. leei* polar capsules (Fig. 1.6 b) are tear-like and located in a 90° angle so that polar filaments are discharged in almost opposite directions. Inside the polar capsules, 7 turns of the polar filament coil can be counted. The binucleate sporoplasm presents the shape of an asymmetrical hourglass and contains a large vacuole in its centre (Diamant et al. 1994). The presence of infective S cells, or accompanying cells, within disporous P cells confers the parasite the possibility of undergoing a proliferative as well as a sporogonic cycle (Cuadrado et al. 2008).

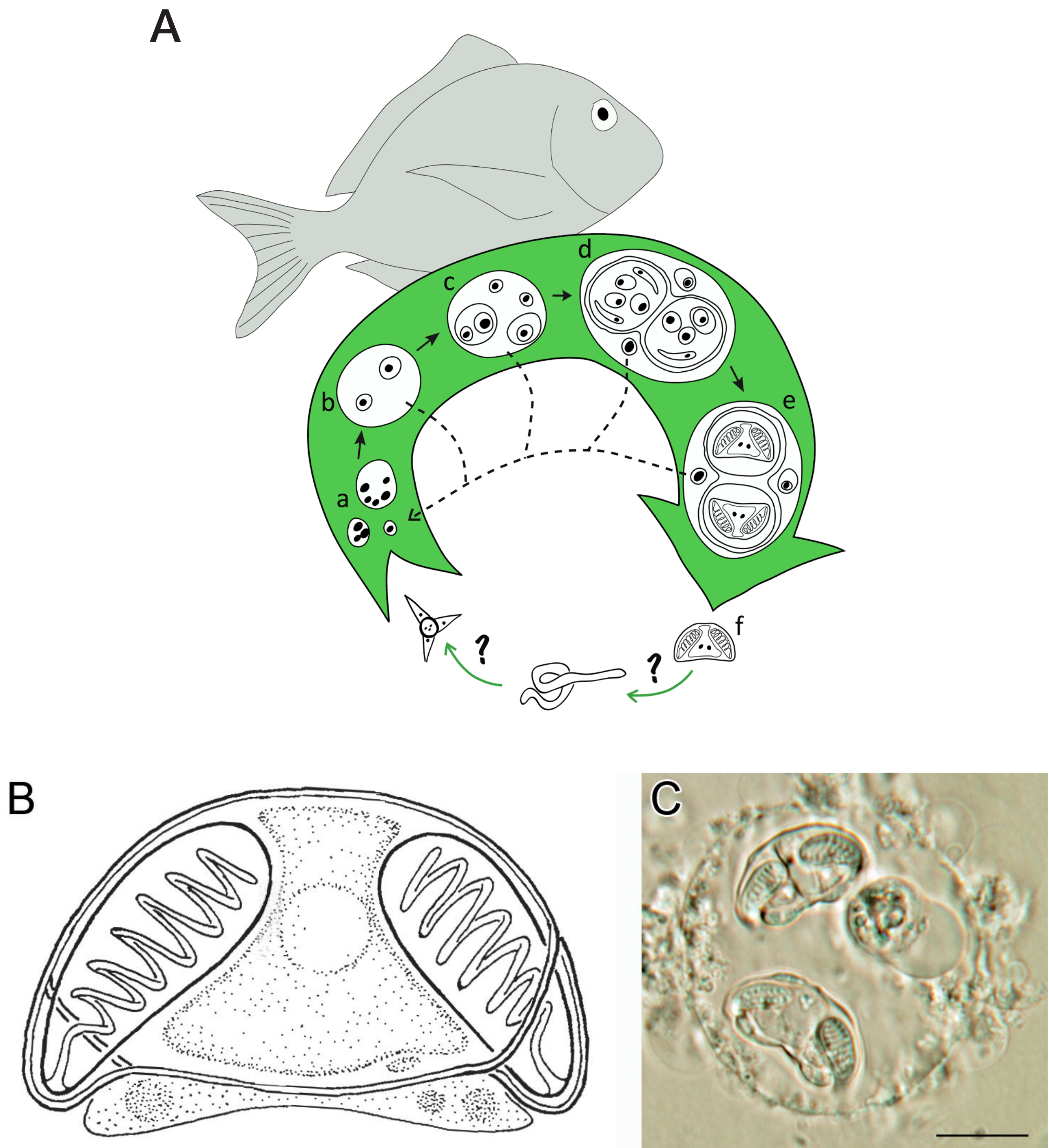

Figure 1.6. (A) Life cycle of *Enteromyxum leei*. In the intestinal epithelium of the fish, proliferative (**a-c**) and sporogonic (**d-f**) development occurs. Stages a-e are responsible for the invasion and dispersion within the fish, as well as for transmission to other fish, when released through faeces. Whether the mature spore starts an alternate cycle infecting an invertebrate host is currently unknown. (**B**) Spore of *Enteromyxum leei* (after Diamant et al. 1994). (**C**) Disporus sporoblast in a fresh smear of posterior intestine. Scale bar= 10 µm.

1.7.1 Enteromyxosis in gilthead sea bream

Enteromyxosis in gilthead sea bream is a chronic disease, evident in land-based facilities but sometimes undetected in cages. The parasite induces severe catarrhal enteritis, which beyond direct mortality (rates up to 20%), causes also weight loss, poor conversion rates, delayed growth and reduced marketability. The most evident clinical sign is the severe emaciation which entails in gilthead sea bream the typical knife-edge body shape, sometimes accompanied by a distended abdomen, accumulation of ascitic fluid and bile (Fig. 1.7).

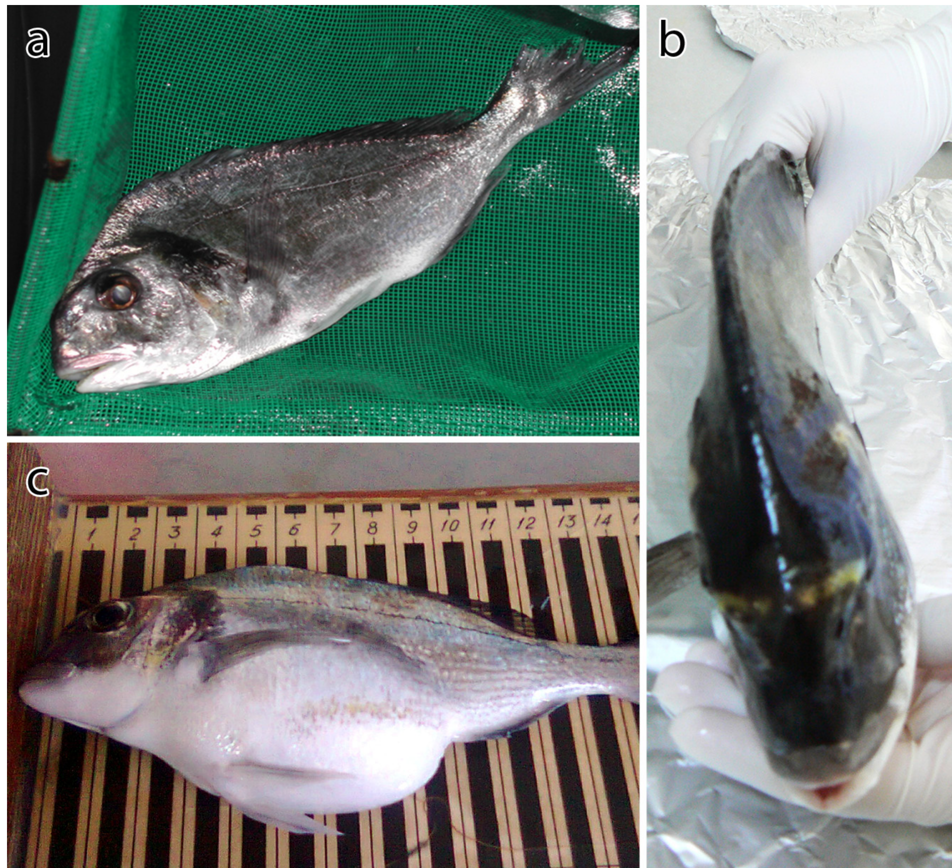

Figure 1.7. Clinical signs of enteromyxosis in gilthead sea bream. (**A**) Extreme weight loss. (**B**) Knife-edge body shape. (**C**) Distended abdomen and ascitis.

Infection in gilthead sea bream is usually restricted to the intestine, following a directional pattern from the rectum towards the anterior intestine and pyloric caeca. At early stages of the infection, the parasite presents a patchy distribution in localized foci. In heavily infected fish, parasite stages have occasionally been observed in the urinary bladder (Diamant et al. 1994), in the gall bladder epithelium, in intra-hepatic biliary ducts and in the gastric lumen (Fleurance et al. 2008) and more frequently in the gall bladder or intestinal lumina together with host cell debris from epithelial sloughing (Cuadrado et al. 2008), in which amoeboid motile stages may be present. Macroscopically, infected intestines show fragile and semi-transparent walls, focal congestion and luminal mucous liquid. Histopathology of enteromyxosis in gilthead sea bream shows intestinal epithelia invaded and disorganized by parasite stages leading to desquamation of the epithelial layer and subsequent disruption of the epithelial barrier and impairment of the absorptive intestinal function. Intraepithelial *E. leei* stages dwell in the paracellular space between enterocytes, extend cytoplasmatic projections and present a convoluted surface to increase their absorptive area, thus enhancing nutrition from host cells (Cuadrado et al. 2008) (Fig. 1.8).

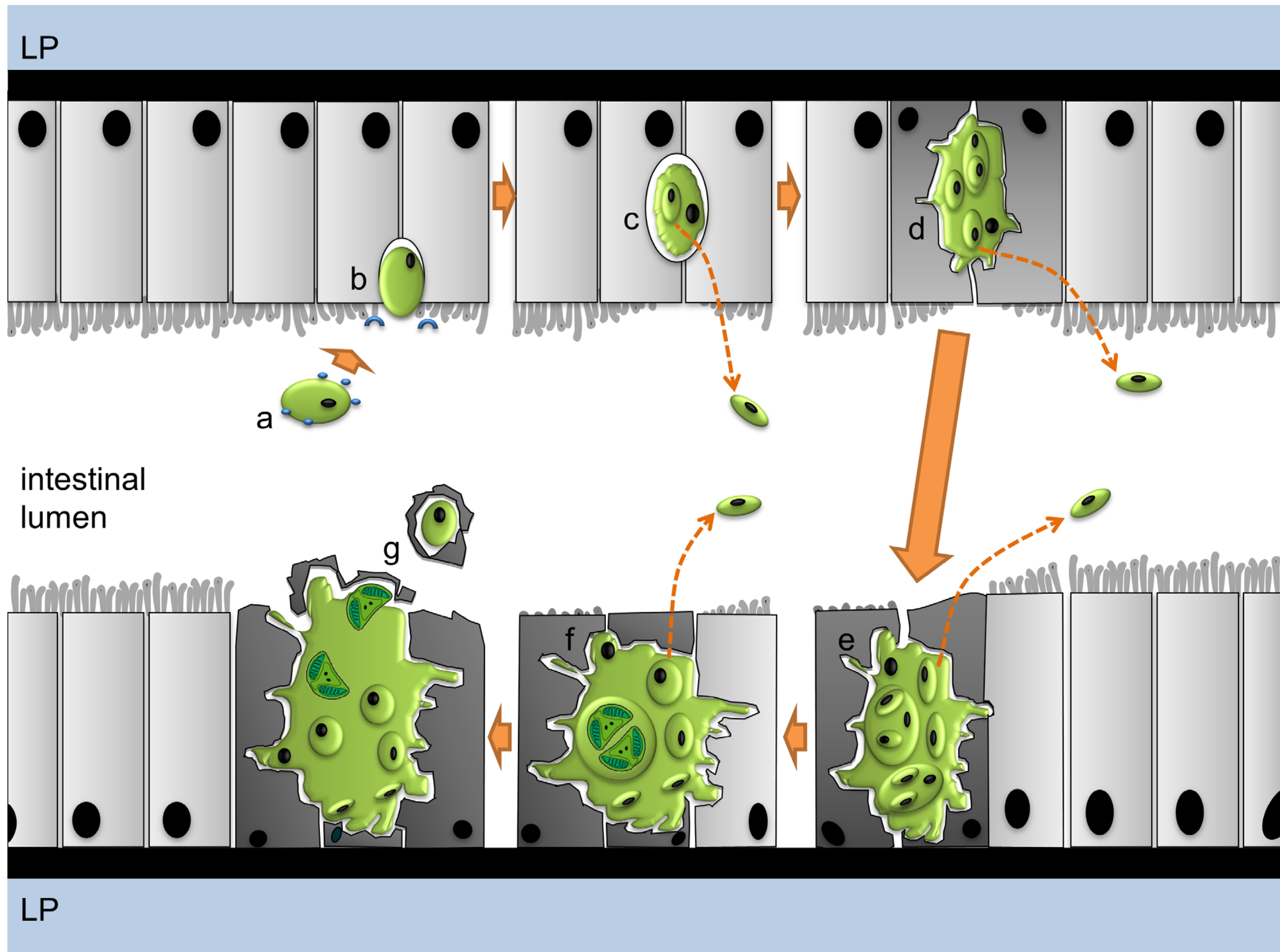

Figure 1.8. Hypothetical invasion mechanism of *Enteromyxum leei*. Parasite cells are represented in green and host enterocytes in grey. Bold arrows indicate the chronologic growth of the parasite, while dashed arrows indicate the generation of free secondary cells ready to colonize new intestinal areas of the same or of other fish. A luminal parasite stage carrying certain antigenic determinants (**a**) recognizes and adheres to specific receptors (like lectins, mucins, glycans, etc.) at the epithelial surface. The parasite penetrates the epithelium through the paracellular space between enterocytes (**b**). The irregular parasite surface (**c**) projects cytoplasmatic protrusions into the neighbouring enterocytes (d) displacing their cytoplasms and nuclei while it grows. Affected enterocytes contacting the parasite increase in number, lose their apical microvilli and start to necrotize (darker colour) (**e-f**). Eventually, enterocytes are disrupted by the parasite which liberates into the lumen desquamated stages still bound to epithelial host material (**g**). LP = lamina propria-submucosa.

The inflammatory response elicited by the host includes hypertrophied submucosae infiltrated by numerous immune cells (Fleurance et al. 2008). Infected gilthead sea bream exhibit a cachectic syndrome as a result of the numerous systemic and local impacts of the parasite on the fish's physiology (Fig. 1.9). Thus, the heavy weight loss is not only a consequence of the damaged intestinal tissue but is also attributed to anorexia, intestinal osmoregulatory failure, anaemia and to the metabolic cost of the immune response itself (Sitjà-Bobadilla & Palenzuela 2012). The variable incidence and severity of the infection in gilthead sea bream seem to indicate a genetically based susceptibility to the disease (Palenzuela 2006, Sitjà-Bobadilla et al. 2007).

Among the factors influencing enteromyxosis, water temperature is determinant for its onset, since the development of the parasite is significantly suppressed below 15 °C (Yanagida et al. 2006). Thus, infection can remain latent during cooler periods and mortalities peak again in the warmer seasons (Le Breton & Marques 1995, Rigos et al. 1999). Within the studies of this thesis, the effect of water temperature on the disease onset in gilthead sea bream is examined (see chapter 3). Another factor involved in the fish susceptibility to enteromyxosis is the diet. It has been suggested, that the proliferation of the parasite is

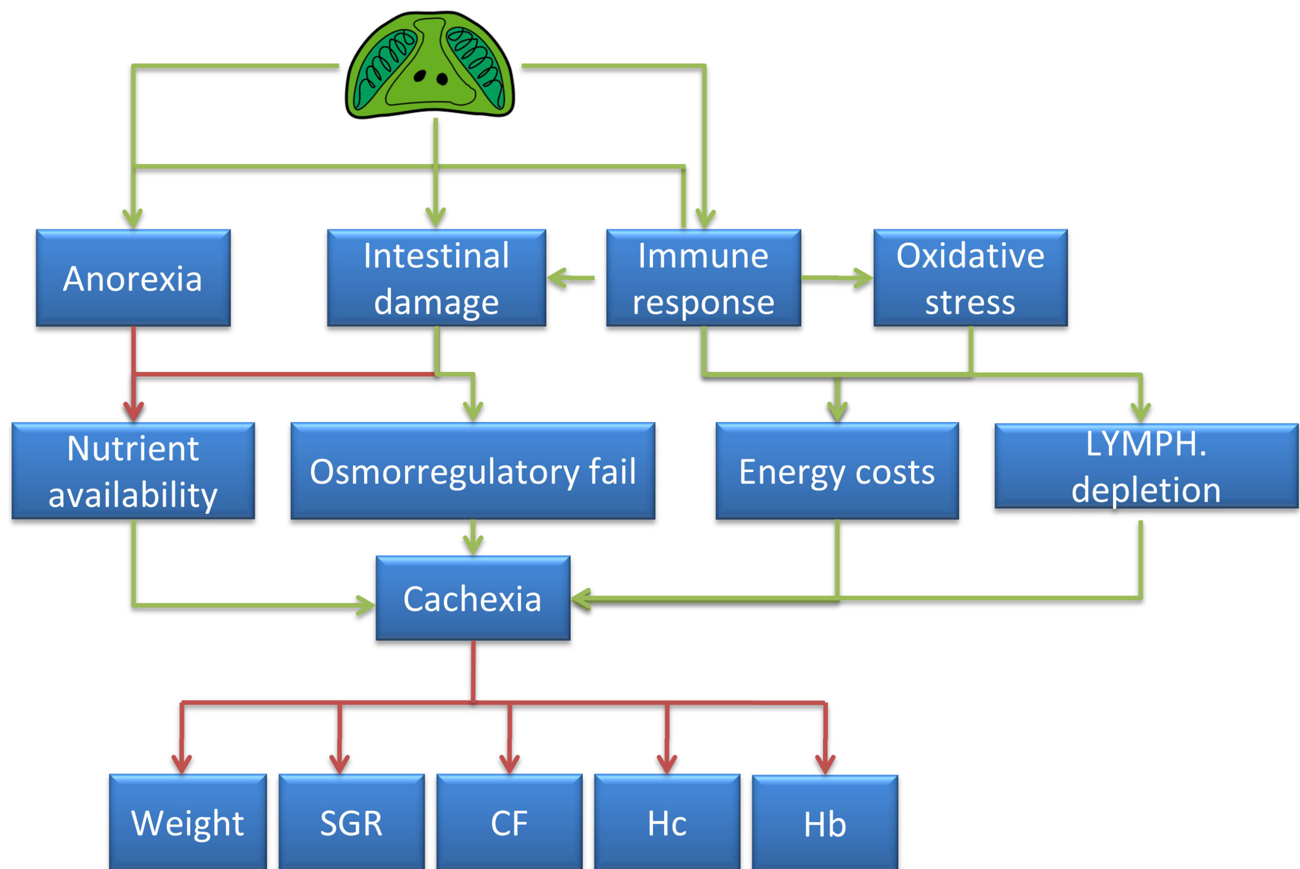

Fig. 1.9. Disease mechanisms of *Enteromyxum leei*. In the diagram, red arrows stand for inducing effects and green ones for inhibitory effects on the pointed box. CF, condition factor; Hb, haemoglobin; Hc, haematocrit; LYMPH, lymphohaematopoietic; SGR, specific growth rate (after Sitjà-Bobadilla and Palenzuela 2012).

favoured in fish fed a diet with excessive fat content (Rigos et al. 1999). The diet plays a paramount role in maintaining the intestinal integrity and functionality, including its microflora, which are essential keys for an effective intestinal epithelial barrier and immune response against offending organisms and substances (Fekete & Kellems 2007, Oliva-Teles 2012). Aspects of the *E. leei* – gilthead sea bream interaction such as the fish immune response, the intestinal integrity and the intestinal glycoconjugate composition have been addressed within this thesis, in the light of the fish nutritional background. Specifically, the effect of fish-based ingredients replacement by vegetable ingredients is studied (see chapters 6, 7 and 9).

Gilthead sea bream enteromyxosis is diagnosed through detection of parasite stages in infected intestinal smears, fresh or stained, histological sections or by means of specific oligonucleotide probes in PCR or *in situ* hybridization techniques (Sitjà-Bobadilla & Palenzuela 2012). An early detection is crucial for isolation or sacrifice of infected fish stocks. The direct fish-to-fish transmission makes enteromyxosis an excellent model to study host-parasite interactions, but is also responsible for the spreading of the disease in cultured stocks, and hampers its prevention and control. There is no approved antiparasitic chemotherapeutic treatment against *Enteromyxum* spp. or any other myxosporean. Against *E. leei*, some coccidiostats that distort parasite morphology (Golomazou et al. 2006a) and hyposalinity treatments that reduce pre-sporogonic stage viability (Yokoyama & Shirakashi 2007), have been tested. The use of lectins and blocking sugars that could avoid parasite adhesion and penetration (Redondo & Alvarez-Pellitero 2010) is a promising issue, which is worth to explore.

1.8 THE IMMUNE SYSTEM OF TELEOSTS

The immune system protects living organisms from threatening pathogens like parasites, bacteria, viruses and fungi, but also from defective components/cells present in their own organism, as occurs in tissue injuries or tumours. The complexity of the defence mechanisms employed depends on the phylogenetic position of each organism, existing an evolutionary trend towards specialisation of the response. However, basic defensive elements and mechanisms were conserved during evolution, like degradative enzymes (lysozyme), cell engulfment (phagocytosis) or secretions (mucus shedding). The protective function of the immune system is based on the ability to differentiate between self and foreign material or healthy and harmed material, thus preserving homeostasis.

Bony and cartilaginous fishes (Osteichthyes and Chondrichthyes) are the earliest vertebrates possessing elements of innate (nonspecific) and adaptive (specific) immunity, shared by all gnathostomata (Bernstein 1998, Flajnik & Du Pasquier 2004, Whyte 2007, Lieschke & Trede 2009). Consequently, the infraclass Teleostei, which comprises almost all living bony fishes, also presents innate and adaptive immune systems, both capable of humoral and cellular responses. The new immune mechanisms firstly acquired by fish and later by all jawed vertebrates established the mainstay of adaptive immunity through the randomly generated and vast antigen-receptor diversity, which provides the potential to evade any invader. Major histocompatibility complex (MHC) processing and presenting genes, thymus and spleen, large numbers of cytokines, chemokines and haematopoietic cell-specific transcription factors, and six different types of rearranging gene (RAG) families that use the same rearrangement mechanism seem all to emerge after the appearance of the jawed vertebrates (Flajnik & Du Pasquier 2004).

As in other vertebrates, the innate immune system of teleosts provides the first line of defence against pathogens and the adaptive immune system contributes to a more specific and efficient response. Both immune mechanisms are integrated parts of a multilevel network, which faces the artificial dichotomy between innate and adaptive systems (Flajnik & Du Pasquier 2004, Álvarez-Pellitero 2011). There is a general agreement about the mechanisms involved in mammalian immune response being also present in teleosts, though available information depends on the fish species and many loose ends in regard to parasite infections are still to be tied up.

1.8.1 Lymphohaematopoietic organs

Head kidney, spleen and thymus are the main lymphohaematopoietic organs of fish, whose cellular populations, resident and migratory, are coordinated during immune response. Among vertebrates, fish are the earliest group that possesses defined lymphohaematopoietic tissues consisting of mixed lymphoid and myeloid elements (Deivasigamani 2007). These organs are considered as scavenging tissues, in which highly endocytic macrophages (MΦ) and endothelial cells account for the clearance of particulate matter and damaged cells in a reticulo-endothelial tissue system (Agbede 2012). Monocytes/MΦs, lymphocytes, granulocytes, mast cells, natural cytotoxic cells, dendritic cells and thrombocytes constitute the lymphoid cell populations of teleosts, which display the humoral and cellular immune responses (Press & Evensen 1999, Reite & Evensen 2006). Mucosal epithelia are the main interface between the host and the microbial world, thus, mucosal-associated lymphoid tissues in form of diffuse lymphoid cells and MΦs in the intestinal, gill and skin mucosae are also found in fish (Iwama 1996).

1.8.1.1 Kidney

The Y shaped teleost kidney is a paired but fused organ situated dorsally in the body cavity along the vertebral column. It consists of the posterior trunk kidney which bifurcates into two lobes at its anterior end and the anterior bilobed head kidney. The aglomerular head kidney, whose excretory function has disappeared, consists of haematopoietic, lymphoid and endocrine tissues, while in the posterior glomerular trunk kidney the haematopoietic capacity is limited and the excretory function predominates (Deivasigamani 2007).

Throughout the head kidney parenchyma, scattered lymphohaematopoietic cells are found between a network of fibroblastic reticular cells and sinusoidal blood vessels. The phylogenetical relationship of head kidney to the bone marrow of higher vertebrates is supported by its capacity for housing and

differentiating blood cell precursors. Thus, head kidney is considered to be a postembryonic source of stem cells (Iwama 1996). RAG expression in head kidney has been found in all developmental stages of rainbow trout, suggesting a continuous B cell development. A specific gene marker for B cell development expressed in this tissue is ikaros and for lymphocyte development (B and T) terminal deoxynucleotidyl transferase (TdT) (Hansen 1997, Hansen & Zapata 1998). B cell receptor (BCR) is a surface immunoglobulin (Ig) characteristic of B cells, which has been phylogenetically conserved among species.

Kidney MΦs, situated in close contact to blood sinuses, and endothelial cells of the blood vessels, are responsible for the blood clearance from both foreign and non-foreign substances. Such endocytosed molecules might be intracellularly degraded, excreted via glomeruli or, in the case of antigenic substances, processed for antigen presentation. Thus, scavenging MΦs participate in the lymphatic function and are also essential for the development of immune responses (Agbede 2012). In the head kidney as well as in the spleen of most teleosts, well-defined aggregations of pigmented MΦs are present within the reticulo-endothelial tissue. Such melanomacrophages (MMΦs) contain citoplasmatic granules of melanin, hemosiderin and lipofuchsin. Melanomacrophage centres (MMCs) have been associated in many species with antigen trapping and processing for subsequent presentation to lymphocytes, which in turn proliferate and trigger the adaptive immune response or maintain the humoral memory. Furthermore, MMΦs are capable of retaining antigen for long periods of time (Grove et al. 2006). It has been suggested that MMCs would be an analogous structure to the germinal centres of higher vertebrates and are frequently considered as their phylogenetic precursor (Vigliano et al. 2006). However, affinity maturation and isotype switch of Igs as it occurs in germinal centres of birds and mammals has yet not been demonstrated in MMC of fish (Lin et al. 2005, Balogh 2010) though head kidney has been shown to be a mayor site of B cell proliferation, antibody secretion and RAG gene expression (Bernstein 1998).

The head kidney of teleosts also accounts as the functional equivalent of the adrenal gland in tetrapod vertebrates, responsible for catecolamine and steroid hormone release. Chromaffin and interrenal cells, innervated by synaptic junctions, form this endocrine tissue, innervated by synaptic junctions, which is essential during stress response and steroidogenesis (Abdel-Aziz et al. 2010). Therefore, head kidney plays an important role in neuroendocrine-immune interactions.

1.8.1.2 Thymus

The thymus is a paired organ in most vertebrates though in teleosts it can appear as more than one pair. In most teleosts it is located near the gill cavity in close association with the pharyngeal epithelium. This organ evolved in fish as an area of the mucosal-associated lymphoid tissue (MALT), which specialized in the development of functional T cells. During ontogeny, the thymus is the first organ to become lymphoid in freshwater teleosts but the last one in marine teleosts and thereafter it undergoes involution, which is an age-, hormone- or season-dependent process (Press & Evensen 1999, Bowden et al. 2005, Zapata et al. 2006). Therefore, the degree of regionalization of the teleost thymus into cortex and medulla structure is not only species specific, but also age-specific or life stage-specific within a single species. However, a clear cortico-medullar differentiation is generally absent in fish. An epithelial capsule encloses a network of supporting epithelial cells. Capsular invaginations project fibroblastic trabeculae that lead capillaries and nerves through the epithelial stroma, which houses mainly MΦs and proliferating T cells. Other cell types also present in the thymus are granulocytes, plasma cells and presumptive dendritic cells (Bowden et al. 2005).

In some teleost species nurse-like epithelial cells have been identified, which interconnect by branched cytoplasmatic processes and house the immature thymocytes in their invaginations. Maturation of thymocytes occurs there in association with the nurse-like epithelial cells, a microenvironment for immunocompetent T cell development through generation of randomly compiled antigen receptors and interaction with MHC molecules (Xie et al. 2006). T cell receptor (TCR) diversity (as well as Ig diversity) is generated by variable (V), diversity (D) and joining (J) gene segment recombination. In this way, genes located in individual V, D or J segments gather to form the mature TCRs. Nurse-like epithelial cells may also participate in thymocyte apoptosis of self-reactive clones which are eventually removed by MΦs. T cell subpopulations have been described in fish in accordance to marker expression of the TCR subunits and

CD co-receptor molecules. Several common gene markers of thymic function and lymphoid development include RAGs, TCR subunits, CD, MHC I and II, T cell specific light-chain kinase and TdT (Bowden et al. 2005, Vesterlund et al. 2006). Summing up, the thymus is a primary lymphoid organ also involved in the control of cell mediated adaptive immune responses.

1.8.1.3 Spleen

The spleen is an unpaired organ, surrounded by a fibro-muscular capsule and located in the body cavity, near the pancreas and towards the anterior intestine. In teleosts, the splenic parenchyma is divided into red and white pulp, arranged in a reticular meshwork, in which MMCs are also present. The red pulp consists of blood sinuses, abundant mature erythrocytes and some MΦs and neutrophils, and the white pulp, is formed by scattered lymphoid accumulations surrounding arterioles. Major arteries entering the spleen ramify into such arterioles called ellipsoids, whose cuboidal endothelium is specialized in trapping blood-borne substances and surrounded by a sheath of leukocytes, MΦs and reticular cells (Espenes et al. 1995, Bernstein 1998, Grove et al. 2006, Leknes 2012). In mammals, these structures are the so-called periarteriolar lymphoid sheath (PALS), but teleost PALS, from where antigen is transported to the MMCs, are poorly developed in most species (Nilsson 1986, Kapoor 2004).

The spleen of teleosts is primarily an iron recycling site, where erythrocyte storage and destruction takes place, as well as erythropoiesis, though less intensively than in the kidney. This organ is a source of hemosiderin, a protein performing iron transport after degradation of the haemoglobin molecule, and needed for the synthesis of new haemoglobin (Soldatov 2005).

The spleen is also a major blood filtering organ crucial for pathogen capture and destruction in its reticulo-endothelial system, a dense meshwork crossed by a slow blood flow. Indeed, splenic somatic index has been suggested as an indicator of disease resistance, speculating that spleen size influences filtering capacity and thus immune function, or that gene expression regulation of organ growth and immune resistance would be closely linked (Hadidi et al. 2008). In any case, the amounts of white pulp lymphoid cells increase after antigenic stimulation and are in some species a major source of antibody production and immunological memory (Iwama 1996, Hadidi et al. 2008, Leknes 2012, Castro et al. 2013). Therefore, the spleen apparently plays an important role in nonspecific and specific defence mechanisms.

1.8.1.4 Gut-Associated Lymphoid Tissue

Teleost mucosal tissues are localized on body surfaces exposed to harsh environments with high microbial load, i.e. skin, gills and gut, and possess an own mucosal immune system. Their mucus-covered surface constitutes a first-line defence against offending organisms and molecules, which acts not only as a physical barrier but contains also multiple bio-active substances, like mucins themselves or Igs, complement, C-reactive protein (CRP), lectins, lysozyme, proteolytic enzymes, alkaline phosphatase and esterase, antimicrobial peptides and hemolysine (Álvarez-Pellitero 2008). In addition to the mucosal-epithelial barrier, a whole set of immune-competent cells is localized in the mucosal epithelia and the submucosa. These MALTs consist of non-encapsulated lymphoid accumulations containing lymphocytes, MΦs, plasma cells, granulocytes and mast cells (Rombout et al. 2011).

The gut-associated lymphoid tissue (GALT) is composed of two different cellular compartments, the intraepithelial compartment and the subjacent lamina propria-submucosa. In teleost fish, the diffusely distributed GALT lacks Peyer's patches or lymphoid follicles present in mammals, but antigen-uptaking enterocytes, the teleost M-cell homologue, have already been identified in Atlantic salmon (Fuglem et al. 2010). In the piscine intestine, recognition of pathogen associated molecular patterns (PAMPs), conserved and diverse pathogen motifs, occurs through Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD) receptors on enterocytes or dendritic cells, and thereafter antigen is actively transported towards the local as well as the systemic immune system. Differential macromolecule uptake from the lumen occurs along the distinct intestine segments (Salinas et al. 2011) and while antigen uptake in the anterior intestine was reported for some species (Odonnell et al. 1994, Dalmo et al. 1995, Dalmo & Bogwald 1996), other authors consider that the anterior intestine meets mainly nutrient absorption and antigen uptake was not detected there (Georgopoulou et al. 1988, Rombout et al. 1993, Fuglem et al. 2010, Rombout et al. 2011). Antigens are in turn transported to MΦs of the epithelium or lamina propria-submucosa,

which posses Ig binding capacity and are frequently observed in association with lymphocytes. High MHC II expression levels have been detected in the medium intestine suggesting an antigen-presenting function of such MΦs with antigenic determinants on their surface (Rombout et al. 1993, Fuglem et al. 2010). In the intraepithelial compartment, mainly Ig-negative T cell infiltrates and a few Ig-positive B cells and plasma cells are found, while abundant plasma cells proliferate in the lamina propria-submucosa (Rombout et al. 1993, Bermúdez et al. 2006). Granulocytes are quickly recruited via cytokine chemotaxis and are very abundant in the lamina propria-submucosa at inflammation sites where they contribute to the immune response by granule release (eosinophils and basophils) and phagocytosis (neutrophils).

Evidence of a local mucosal immune response comes from the detection of specific mucosal antibody responses after intestinal or immersion immunizations, while hardly no systemic immunization is detectable (Rombout et al. 2011). In teleosts, IgM levels found in mucosal secretions are, in part, a result of local synthesis. In addition, an unique teleost Ig isotype, IgT, is specialized in gut mucosal immune responses (Sunyer et al. 2010). Certain in situ lymphopoietic capacity of the teleost intestine is indicated by both RAG and TdT expression in this tissue (Hansen 1997).

The different elements of the mucosal system constitute of both innate and adaptive immunity and interact to coordinate an effective immune response (Álvarez-Pellitero 2011).

Table 1.4. Innate and adaptive elements of teleost fish immune system (Iwama 1996, Whyte 2007, Álvarez-Pellitero 2008).

	HUMORAL	CELLULAR
INNATE	Inhibitors: • Transferrin • Antiproteases • Antimicrobial peptides • Lectins • Interferon Lysins: • Proteases • Lysozyme • C-reactive protein • Complement • Peroxidases Pro-inflammatory cytokines & chemokines Natural antibodies Eicosanoids Mucus mucins	Macrophages-Granulocytes-Mast cells (non-specific cytotoxic cells) • Phagocytosis • Respiratory burst • Halides-H_2O_2-myeloperoxidase • Lysozyme • Histamine • Antimicrobial peptides • Hydrolytic enzymes • NO Natural cytotoxic cells Rodlet cells Innate-like lymphocytes
ADAPTIVE	Antibodies: • Antiadhesins • Antitoxins • Anti-invasins • Activation of classical complement pathway	Antigen presenting cells: • Phagocytes (mainly neutrophils, macrophages) • Dendritic cells • Epithelial cells/M-like-cells B2 cells αβ T cells: • T helper cells • T cytotoxic cells Cytokine activated macrophages (resp. burst & bactericidal activity)

1.8.2 Immune response to parasite infections

Characterization of the host-parasite interaction, including the fish immune response against the parasite, is basic for the development of vaccines and of preventive strategies against diseases, as well as for the selection of resistant fish strains. Both innate and adaptive immunity are involved in the defence mechanisms of fish against parasite infections. In fish, innate immune mechanisms are essential due to their poikilothermic nature, to their limited repertoire and proliferation of antibodies and to their restricted immunologic memory (Magnadottir 2006). Table 1.2 shows a summary of the immune mechanisms involved in teleost immune responses.

1.8.2.1 Innate immune response to parasite infections

The initiation of an innate immune response depends on the recognition of PAMPs by highly conserved pathogen recognizing receptors (PRRs), which are germline encoded and have a broad specificity. Some potential parasite PAMPs have been identified for several fish parasites (Lischke et al. 2000, Clark et al. 2001), namely surface glycolipids (glycosylphosphatidylinositol (GPI) anchors). TLRs are the best characterized class of innate receptors in fish, and after PAMP recognition they initiate multiple intracellular signalling pathways (Takano et al. 2010), though their specific involvement in piscine parasite recognition is still unclear. PRRs may be soluble or expressed at the surface of phagocytes and other cells types, and once activated, they can induce opsonisation, phagocytosis and activation of other immune effectors, like cytotoxic cells or the complement system (Magnadottir 2006). In addition, other transmembrane or soluble lectins recognize glycoconjugate structures on fish parasites as proven for some parasite infections (Kim et al. 1999, Buchmann 2001, Xu et al. 2001). Carbohydrate terminals present in the host-parasite interface have been characterized in various myxozoan-fish models because of their possible role in recognition and adhesion (Morris & Adams 2004, Knaus & El-Matbouli 2005a), including *Enteromyxum* spp. (Redondo et al. 2008, Redondo & Álvarez-Pellitero 2009, 2010). Additionally, the whole set of bioactive or biocidal substances soluble within the mucus, are involved in the innate as well as in the adaptive immune response contributing to limit parasite load (Jones 2001, Buchmann & Lindenstrøm 2002). The study of the distribution and glycosylation of mucins, the highly glycosylated glycoproteins that constitute the mucus barrier on exposed body epithelia, in regard to parasite infection and dietary modulation, is addressed within the current thesis (see chapters 7 and 8).

Humoral immune factors involved in the fish antiparasitic innate response include mainly complement, peroxidases, lysozyme, protease inhibitors (like α2-macroglobulin), lectins, acute-phase proteins, antimicrobial peptides (like piscidins), cytokines (like tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β)), and chemokines (like IL-8) (Jones 2001, Álvarez-Pellitero 2008). The complement system can be activated by three pathways, the classical pathway initiated by antibody-antigen binding, the alternative pathway by direct C3 binding to microbial surfaces and the lectin pathway by mannan-binding lectin or ficolin interaction with microbial glycoconjugates. All three ways generate factor C3 and converge to the lythic pathway, leading to opsonization, activation of phagocytes, inflammation and activation of the membrane attack complex. Teleost complement, composed of more than 35 soluble plasmatic proteins, remains active at low temperatures compensating for the suppressed adaptive immune response (Whyte 2007). Lysozyme, a lytic enzyme produced by phagocytes, is present in body secretions and lymphohaematopoietic tissues and can also activate the complement and phagocytes. Peroxidases, like myeloperoxidase and eosinophil peroxidase from phagocyte degranulation, participate in the oxidative response by forming toxic chlorides and chloramines from H_2O_2 and halide ions. Protease inhibitors are responsible for the clearance or inactivation of parasite proteases contributing to pathogenicity, but they also interact with innate and adaptive mechanisms, like the earlier mentioned humoral factors. Further elements linking both immune responses are cytokines, which can be released by fish leukocytes as inflammatory humoral factors after injury. TNFα is known as a MΦ-activating factor promoting respiratory burst, phagocytosis and NO production and as inducer of immune-related gene expression. IL-1 is fundamental in the early response and leads to inflammation through regulation of a cascade of other cytokines. It is also critical to the differentiation of B cells into antibody secreting plasma cells. More precisely, IL-1β plays a role in immune regulation through T cell stimulation (Jones 2001, Whyte 2007, Álvarez-Pellitero 2008, Secombes et al. 2011).

Alternative complement activity, lysozyme and peroxidases from gilthead sea bream serum, as well as intestinal antiprotease gene expression varied after *E. leei* challenge suggesting their involvement in the innate humoral response, which may be partly responsible for the existence of non-parasitized exposed fish. Modulation of the gene expression of the pro-inflammatory cytokines IL-1β and TNFα was also detected (Cuesta et al. 2006, Sitjà-Bobadilla et al. 2008).

Some PRRs activate tissue resident MΦs which may trigger a cytokine mediated inflammatory response also involving acute-phase proteins and complement factors. Inflammatory response to parasites in fish can lead to the formation of granulomata that encapsulate parasites and their products (Sitjà-Bobadilla 2008). In parasitoses as in other pathogenic processes, innate cellular response during inflammatory reaction involves activation and/or proliferation of phagocytes (granulocytes and MΦs/monocytes, i.e non-specific cytotoxic cells), natural cytotoxic cells, mast cells, epithelial cells, dendritic cells and the enigmatic rodlet cells. Cell-mediated responses include phagocytosis, phagocyte activity, complement activity and receptor-mediated endocytosis as mentioned for scavenger endothelial cells and MΦs of blood sinusoids and ellipsoids (Whyte 2007). After engulfment, tissue MΦs containing parasite debris or MMΦs retaining parasite antigen are frequently found in fish and are associated with tissue melanization. Phagocytic activity includes also oxidative mechanisms to eliminate the internalized microorganisms such as respiratory burst and nitric oxide, which are frequently modulated during parasite exposure. Granulocytes, containing antimicrobial-peptides or lysozyme, mast cells containing histamine and rodlet cells containing antimicrobial-peptides, are present in many fish species and are often recruited into infected tissues, where they release their granules or rodlet content, respectively (Álvarez-Pellitero 2008). The characterization of the leukocytic immune response of gilthead sea bream to *E. leei* carried out in the current thesis includes the study of acidophilic granulocyte, mast cell, plasma cell, B cell and MMΦ distribution at mucosal and systemic levels (see chapters 9 and 10).

1.8.2.2 Adaptive immune response to parasite infections

In parasite infections as for all other pathogens, the display of pathogen-derived antigenic peptides by antigen-presenting cells to the antigen-recognizing lymphocytes sets off the adaptive immune mechanism that leads to humoral antibody secretion with high specificity and affinity for the pathogen. Different Ig isotypes are present in teleost fish, IgM (the most prevalent one), IgT and IgD, mainly produced by plasmablasts and plasma cells and they appear in membrane-bound form on the cell surface or in soluble form as antibodies either in serum or body secretions. The gene encoding the polymeric Ig receptor (pIgR) has been identified for various teleost species and found to be expressed in all lymphoid tissues (Álvarez-Pellitero 2011). Recently, the clonal expansion of splenic IgT-positive B cells in rainbow trout antiviral response suggested either their systemic contribution to defence or their initial activation and proliferation in extra-mucosal sites. However, the limitation of the antibody repertoire in fish compared to mammals seems no longer a fact (Castro et al. 2013). Ig's effector mechanisms include direct neutralization by blocking antigenic receptors, enzymatic active sites or toxigenic determinants, agglutination/precipitation of soluble antigens due to the Ig multivalent binding capacity and opsonization of pathogens that activates the classical complement pathway (Iwama 1996). Serum titres of specific anti-parasitic antibodies frequently increase after parasite infection, but these are not always correlated with protection (Buchmann et al. 2001, Álvarez-Pellitero 2008, Sitjà-Bobadilla 2008).

Pivotal cells involved in the adaptive immune response of all vertebrates are B cells and T cells. Clonal selection of pathogen-specific receptors on these cells is the basis of immunological memory. Conventional lymphocytes are mostly B2-cells and αβ T cells, whose antigen receptors, BCR and TCR respectively, are randomly assembled. There are two types of conventional αβ T cells, *i.e.* T-helper (Th) cells expressing surface co-receptor CD4, which facilitate B cell/plasma cell maturation and antibody production, and T-cytotoxic (Tc) cells expressing CD8. Antigen presenting cells, like MΦs, dendritic cells or granulocytes, process and display intracellular antigens by means of MHC II to CD4+ Th cells and extracellular antigens by MHC I to CD8+ Tc cells (Álvarez-Pellitero 2011). Cytokines mediate activation of and interaction between lymphocytes. The different Th subsets synthesize different cytokines, so Th1 cytokines, (IL-2, IFN-γ, TNFα, TNFβ) particularly effective in defence against intracellular infections, activate MΦs, enhance antigen presentation and induce T cell differentiation, while Th2 cytokines (IL-4, IL-5, IL-10, IL-13) activate B cells/plasma cells adapted for defence against parasites and antibody production, and T-regulatory cytokines (IL-10, transforming growth factor β) modulate T cell activity and

contribute to parasite control (Álvarez-Pellitero 2011). Studies on the distribution pattern of IgM immunoreactive cells and on the IgM gene expression in gilthead sea bream in response to enteromyxosis and dietary factors are carried out in the current thesis (see chapters 9 and 10).

Teleost immunological memory, due to an increase in the number of B cell clones, allows a faster and more effective response following a secondary exposure to a given pathogen. Such immunization is the basis of vaccination (Buchmann et al. 2001). Thus, the antigenic characterization of pathogens seeks the detection of virulence-related proteic or glycoproteic epitopes which can be potential targets for future chemotherapeutants and vaccines. Common surface glycan and glycoproteic traits among myxozoans have been detected for several species including *Enteromyxum* spp. (Muñoz et al. 1999, Muñoz et al. 2000, Knaus & El-Matbouli 2005b, Morris et al. 2006, Redondo et al. 2008, Redondo & Álvarez-Pellitero 2009). This thesis includes studies on the antigenic characterization of the myxozoan *E. leei* as well as the characterization of two polyclonal antibodies against *Enteromyxum* spp. (see chapter 4 and 5).

02

OBJECTIVES

02. OBJECTIVES

Parasitic enteritis produced by the myxosporean *Enteromyxum leei* in gilthead sea bream is among the most important diseases with pathologic impact on marine Mediterranean pisciculture. *E. leei* threatens the fish health status and causes important economic losses to the aquaculture industry. The control of enteromyxosis is particularly difficult due its horizontal fish-to-fish transmission and the lack of effective preventive as well as therapeutic treatments. Additionally, the broad host range of *E. leei* poses many reservoirs able to transmit the disease, including numerous other fish species and potential invertebrate host(s), in which the sexual phase of the life cycle might take place. Therefore, the need for thorough integrative studies of the parasite itself, of its interaction with the fish host as well as of the immune mechanisms it triggers and their contribution to host protection is stressed out.

In this context, the present work was conceived to contribute to identify the defence pathways involved in the processes of parasite entrance, dispersion and multiplication, and to identify predisposing factors for the disease outcome. Moreover, such information will aid to recognize possible therapeutic targets and the basis of immunoprophylactic and immunomodulatory measures.

The specific objectives of this thesis are to:

- Establish an effective and reproducible experimental infection method able to provide *E. leei*-infected gilthead sea bream in a fast and constant way for subsequent studies.
- Obtain the practical tools for the detection and characterization of *E. leei, i.e.* specific antibodies raised against the parasite.
- Characterize the parasite at antigenic level, including its proteic as well as carbohydrate moieties.
- Evaluate the effect of a plant-protein based diet (containing vegetable oils as major lipid source) on the enteromyxosis course and on different immune factors of gilthead sea bream.
- Study the intestinal mucus barrier of gilthead sea bream by analyzing its mucin composition and the effect of enteromyxosis on it.
- Investigate the modulation exerted by *E. leei* on several cellular immune effectors of gilthead sea bream.

The following chapters (3 to 10) correspond to the eight publications that constitute the core of this PhD thesis:

- **Chapter 3**: Itziar Estensoro, María José Redondo, Pilar Álvarez-Pellitero, Ariadna Sitjà-Bobadilla (2010). Novel horizontal transmission route for *Enteromyxum leei* (Myxozoa) by anal intubation of gilthead sea bream *Sparus aurata.* Diseases of Aquatic Organisms 92: 51-58. doi: 10.3354/dao02267.
- **Chapter 4**: Itziar Estensoro, María José Redondo, Pilar Álvarez-Pellitero, Ariadna Sitjà-Bobadilla. Immunohistochemical characterization of polyclonal antibodies against *Enteromyxum leei* and *Enteromyxum scophthalmi*, intestinal parasites of fish (Myxozoa: Myxosporea). Submitted to Journal of Fish Diseases.
- **Chapter 5**: Itziar Estensoro, Pilar Álvarez-Pellitero, Ariadna Sitjà-Bobadilla. Antigenic characterization of *Enteromyxum leei* (Myxozoa: Myxosporea). Submitted to Diseases of Aquatic Organisms.
- **Chapter 6**: Itziar Estensoro, Laura Benedito-Palos, Oswaldo Palenzuela, Sadasivam Kaushik, Ariadna Sitjà-Bobadilla and Jaume Pérez-Sánchez (2011). The nutritional background of the host alters the disease course in a fish-myxosporean system. Veterinary Parasitology175: 141-150. doi:10.1016/j.vetpar.2010.09.015.

- **Chapter 7**: Itziar Estensoro, María José Redondo, Beatriz Salesa, Savasidam Kaushik, Jaume Pérez-Sánchez, Ariadna Sitjà-Bobadilla (2012). Effect of nutrition and *Enteromyxum leei* infection on gilthead sea bream *Sparus* aurata intestinal carbohydrate distribution. Diseases of Aquatic Organisms 100: 29-42. doi: 10.3354/dao02486.

- **Chapter 8**: Itziar Estensoro, Verena Jung-Schroers, Pilar Álvarez-Pellitero, Dieter Steinhagen, Ariadna Sitjà-Bobadilla (2013). Effects of *Enteromyxum leei* (Myxozoa) infection on gilthead sea bream (*Sparus aurata*) (Teleostei) intestinal mucus: glycoprotein profile and bacterial adhesion. Parasitology Research 112: 567-576. doi: 10.1007/s00436-012-3168-3.

- **Chapter 9**: Itziar Estensoro, Josep A. Calduch-Giner, Sadasivam Kaushik, Jaume Pérez-Sánchez, Ariadna Sitjà-Bobadilla (2012). Modulation of the IgM gene expression and IgM immunoreactive cell distribution by the nutritional background in gilthead sea bream (*Sparus aurata*) challenged with *Enteromyxum leei* (Myxozoa). Fish & Shellfish Immunology 33: 401-410. doi:10.1016/j.fsi.2012.05.029.

- **Chapter 10**: Itziar Estensoro, Iván Mulero, María J. Redondo, Pilar Álvarez-Pellitero, Victoriano Mulero, Ariadna Sitjà-Bobadilla. Modulation of leukocytic populations of gilthead sea bream (*Sparus aurata*) by the intestinal parasite *Enteromyxum leei* (Myxozoa: Myxosporea). Submitted to Parasitology.

During the period in which this thesis was performed, I.E. participated in the preparation of an additional scientific publication and presented other results in the following conference papers:

- Jaume Pérez-Sánchez, Itziar Estensoro, María José Redondo, Josep Alvar Calduch-Giner, Sadasivam Kaushik, Ariadna Sitjà-Bobadilla (2013). Mucins as diagnostic and prognostic biomarkers in a fish-parasite model: transcriptional and functional analysis. PLoS ONE 8:e65457.doi: 10.1371/journal.pone.0065457

- Itziar Estensoro, Roberto Bermúdez, Ana Paula Losada, María Isabel Quiroga, Jaume Pérez-Sánchez, Pilar Álvarez-Pellitero, Ariadna Sitjà-Bobadilla (2009). Effect of *Enteromyxum leei* (Myxozoa) on gastrointestinal neuromodulators and cell apoptosis of gilthead sea bream (*Sparus aurata*). 14th EAFP International Conference on Diseases of Fish and Shellfish, Prague, Chech Republic. Abstract book p. 264-265.

- Itziar Estensoro, María José Redondo, Pilar Álvarez-Pellitero, Ariadna Sitjà-Bobadilla (2010). Detection of specific antibodies against *Enteromyxum leei* (Myxozoa: Myxosporea) in gilthead sea bream (*Sparus aurata*) by ELISA and immunohistochemistry. 1st EOFFI (European Organisation of Fish Immunology) Symposium, Viterbo, Italy. Abstract book p. 44.

03

Novel horizontal transmission route for *Enteromyxum leei* (Myxozoa) by anal intubation of gilthead sea bream *Sparus aurata*

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3.0 ABSTRACT

The aim of the present study was to determine whether *Enteromyxum leei*, one of the most threatening parasitic diseases in Mediterranean fish culture, could be transmitted by peranal intubation in gilthead sea bream *Sparus aurata* L. Fish were inoculated either orally or anally with intestinal scrapings of infected fish in 3 trials. Oral transmission failed, but the parasite was efficiently and quickly transmitted peranally. Prevalence of infection was 100% at 60 d post inoculation (p.i.) in Trial 1 under high summer temperature (22 to 25°C; fish weight = 187.1 g), and 85.7% in just 15 d p.i. in Trial 3 using smaller fish (127.5 g) at autumn temperature (19 to 22°C). In Trial 2, prevalence reached 60% at 60 d p.i. in the group reared at constant temperature (18°C), whereas no fish was infected in the group that was kept at low winter temperature (11 to 12°C), although infection appeared (46.1% at 216 d p.i.) when temperature increased in spring. The arrested development at low temperature has important epidemiological consequences, as fish giving false negative results in winter can act as reservoirs of the parasite. Histopathological examination showed a posterior–anterior intestinal gradient in the progression of the infection, in terms of both intensity and parasite maturation. Thus, peranal intubation provides a very uniform, reliable and faster mode of transmission of *E. leei* than the commonly used transmission methods (cohabitation, exposure to infected effluent and oral inoculation), which require long exposure times or give variable and unpredictable results.

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KEY WORDS:
Myxosporea · Experimental transmission · Teleostei · Aquaculture · Temperature · Histopathology

3.1 INTRODUCTION

Since the first description of *Enteromyxum leei* as *Myxidium leei* in cultured gilthead sea bream *Sparus aurata* from southern Cyprus (Diamant et al. 1994), the parasite has been reported along the entire Mediterranean basin, the Red Sea and Japan, being now recorded in >46 fish species (Diamant 1995, 1998, Le Breton & Marques 1995, Sakiti et al. 1996, Paperna 1998, Zrnčić et al. 1998, Padrós et al. 2001, Marino et al. 2004, Yanagida et al. 2004, 2006, Yasuda et al. 2005, Diamant et al. 2006, Sitjà-Bobadilla et al. 2007). This myxosporean produces severe enteritis and is one of the most threatening parasites in Mediterranean fish culture. It causes anorexia, anaemia, emaciation, caquexia and death, which can even lead to the abandonment of some farms in specific culture locations (Palenzuela 2006, Rigos & Katharios 2010). Despite its economic impact, relatively little is known about its transmission and infection routes, and the primary host species in the wild remains unknown.

Thus far, *Enteromyxum leei* has been experimentally transmitted to different fish species via effluent, cohabitation and oral routes (Diamant 1997, Diamant & Wajsbrot 1997, Diamant 1998, Yasuda et al. 2002, 2005, Muñoz et al. 2007, Sitjà-Bobadilla et al. 2007, Álvarez-Pellitero et al. 2008). All these trials depend on a source of infected fish, since in vitro culture of this parasite, as any Myxosporea, has not been achieved. Experimental transmissions by cohabitation and exposure to infected effluent remarkably mimic the natural conditions for the transmission of the disease in cultured fish. However, for gilthead sea bream, it takes ~3 to 4 mo to achieve high prevalence of infection and to develop disease signs. Furthermore, a precise and uniform infective dose and the initial day of entrance of the parasite cannot be assured for each individual. Although oral infection tries to avoid these drawbacks, the obtained results have been very variable and unpredictable (Diamant & Wajsbrot 1997, Sitjà-Bobadilla et al. 2007). Field and experimental data seem to indicate that the distal part of the intestine is the first and main target site of the parasite in gilthead sea bream. Therefore, the aim of the present work was to determine the efficacy of the anal route in the transmission of the myxosporean in comparison with the oral route. The progress of the infection through the digestive tract was also studied.

3.2 MATERIALS AND METHODS

3.2.1 Fish and experimental infections

Naïve gilthead sea bream from a commercial fish farm with no previous records of enteromyxosis were used as recipient (R) fish. Upon arrival, fish were checked for the absence of the parasite and acclimated to the experimental conditions for at least 2 wk before the beginning of the experiment. Water of 37.5 salinity was 1 µm filtered and UV irradiated. Fish were allocated in 200 l fibre glass tanks and fed daily with a commercial dry pellet diet at ~1% of body weight. Donor (D) gilthead sea bream were obtained from experimentally infected stocks that were kept at the facilities of the Instituto de Acuicultura de Torre de la Sal.

R fish were inoculated with 1 ml of *Enteromyxum leei* infective material. The inoculum consisted of freshly obtained intestinal scrapings of D fish (see Sitjà-Bobadilla et al. 2007). D and R fish were starved for 1 d prior to inoculation. Three experimental transmission trials were performed. Trial 1 started in summer and R fish were inoculated either anally (AT-1) or orally (OT) with the same inoculum for 2 consecutive days. In Trial 2 (AT-2), 2 R groups were inoculated once only through the anal route. One group was kept at the natural winter temperature (AT-2-L) and another was reared at a temperature ^{3}18°C (AT-2-H). Trial 3 started in autumn (AT-3) and R fish with lower weight than in Trials 1 and 2 (to check if this route was feasible with smaller animals) were inoculated once through the anal route and kept at a water temperature ^{3}18°C. In all trials, a control (CTRL) group with fish that were not exposed to the parasite, but were inoculated with phosphate-buffered saline (PBS), was established.

The details of fish and experimental conditions, temperature, samplings and diagnostic procedures are summarised in Table 1. Disease signs and daily mortalities were recorded throughout the experiments. In lethal samplings, fish were euthanized by overexposure to MS-222 (Sigma), whereas in nonlethal (NL) samplings, fish were slightly anaesthetized with clove oil (0.1 ml l–1). In both cases, fish were starved for 1 d prior to sampling. In Trials 1 and 2, all the inoculated fish were nonlethally diagnosed at each sampling point, and were euthanized at the last sampling. Therefore, the parasitological status of all the fish and the progression of their infection were obtained at each sampling point.

Table 1. Experimental conditions and sampling details of the experimental transmission of *Enteromyxum leei* to *Sparus aurata* by oral (OT) and anal (AT) routes. Trials with the same number were performed simultaneously using fish with the same initial weight (mean ±SD) that were inoculated with the same infective inocula. Infection was diagnosed using histology (HIS), nonlethal (NL) PCR or fresh smears examination (F). Prevalence of infection values were obtained from the diagnosis of the posterior intestine. The provided temperature values are the mean ± SD obtained for the period between each sampling point and the previous date. Trial 2 was performed at high (H) and low (L) water temperature. p.i.: post inoculation

TRANSMISSION trial	FISH WEIGHT (g)	TEMPERATURE (°C)	INFECTION DIAGNOSIS			
			Days p.i.	Method	No. of fish examined	Prevalence of infection (%)
OT-1	187.1	25.1 ± 0.7	40	NL-PCR	15	0
		22.1 ± 1.2	60	HIS	15	0
AT-1		25.1 ± 0.7	40	NL-PCR	15	73.3
		22.1 ± 1.2	60	HIS	15	100
AT-2-H	280.3	18 ± 0.5	40	NL-PCR	15	20
		18 ± 0.5	60	NL-PCR	15	60
		24.3 ± 2.0	326	HIS	12	0
AT-2-L		11.9 ± 0.4	40	NL-PCR	15	0
		12.1 ± 0.2	60	NL-PCR	15	0
		17.1 ± 4.5	216	NL-PCR	13	46.1
		24.3 ± 2.0	326	HIS	13	0
AT-3	127.5	22.4 ± 0.7	15	HIS	7	85.7
		19.7 ± 1.5	40	HIS+F	14	92.9

3.2.2 Parasite diagnosis and histopathology

The infection status was determined by histology (HIS) or NL-PCR, except in AT-3 at 40 d post inoculation (p.i.), in which half of the 14 examined fish were diagnosed by light microscopic observation of fresh smears of intestinal scrapings (F). For histological examination, pieces of the anterior, middle and posterior intestine were fixed in 10% buffered formalin, embedded either in Technovit resin (Kulzer, Heraeus) or paraffin, 2 to 3 µm sectioned and stained with toluidine blue (TB), haematoxylin & eosin (H&E) or periodic acid-Schiff solution (PAS). In AT-1, additional samples of stomach, oesophagus and pyloric caeca with accumulation of ascitic fluid were taken. NL samples were obtained by probing the rectum with a cotton swab and PCR diagnosis was carried out as described by Palenzuela & Bartholomew (2002) with primers specific for *Enteromyxum leei* rDNA. This procedure has been validated against a gold standard (histological observation of the whole digestive tract), and resulted in a high sensitivity (0.96) and specificity (1) (O. Palenzuela unpubl. data). NL-PCR was also applied to evaluate the parasitic status of donors, and the absence of the parasite in R fish upon arrival at the experimental facilities. The prevalence of infection at each sampling point was calculated by considering all the positive fish detected by PCR, histological or fresh examination. The intensity of infection was semiquantitatively evaluated only in histological sections according to a scale (1+ to 6+) with the following ranges: 1+ = 1–5; 2+ = 6–10; 3+ = 11–25; 4+ = 26–50; 5+ = 51–100; 6+ > 100. For each intestinal portion, the mean intensity of infection was calculated. Parasite stages were classified as spores, sporoblasts and proliferative stages, the latter corresponding to Stages 1 to 3 described by Álvarez-Pellitero et al. (2008).

3.2.3 Statistics

The possible influence of temperature on the presence of *Enteromyxum leei* was studied. Fisher's exact test of significance was applied to the 2 × 2 contingency tables generated in AT-2 (for the infection values obtained at 40 and 60 d p.i.). Statistical analysis was performed using Sigma Stat software (SPSS) at the significance level of $p < 0.05$.

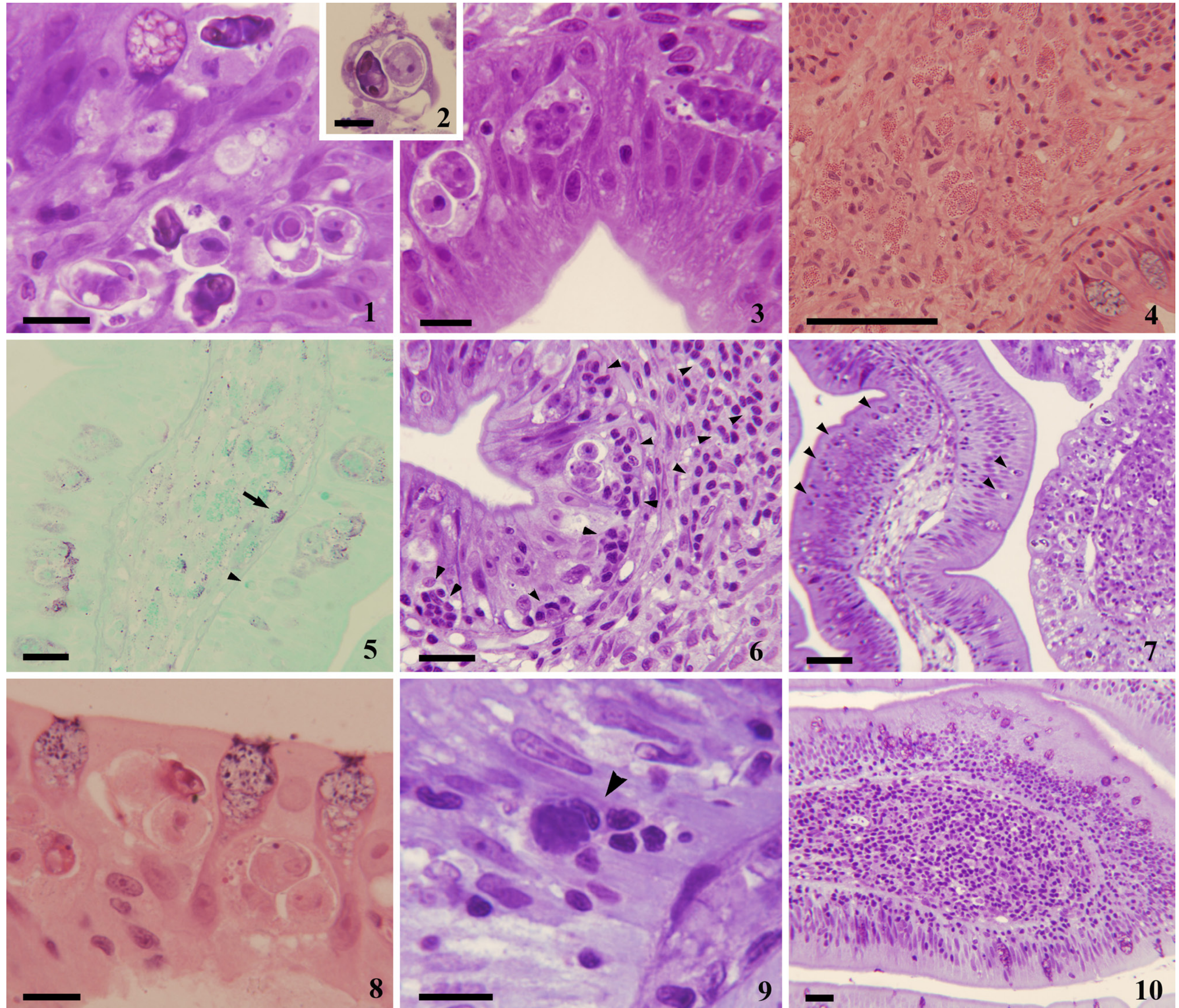
3.3 RESULTS

During the experimental trials, no mortality due to the disease was registered; among the accidental casualties in AT-1 (n = 1), AT-2-H (n = 2) and AT-2-L (n = 1), only the latter was *Enteromyxum leei*-positive (195 d p.i.). None of the R fish in the oral transmission trial or CTRL animals in all the trials was infected by *E. leei*, whereas prevalence of infection in most peranally inoculated groups in the 3 trials was very high, reaching 100% at 60 d p.i. in AT-1 and 92.9% at 40 d p.i. in AT-3. Table 1 summarizes the prevalence of infection in all the trials and samplings. The highest infection values in anal transmission were obtained in AT-1, coinciding with the highest water temperature. In AT-2, in which 2 groups were identically inoculated and kept at different temperatures, prevalence was again higher in the group with higher temperature (AT-2-H). In contrast, AT-2-L fish were not infected until 216 d p.i, when temperature naturally increased in spring. In the last sampling, almost 1 yr after the inoculation, no AT-2 fish was infected. When comparing the prevalence of infection in the 3 trials at 40 d p.i., the highest values were achieved in AT-3 (in which smaller R fish were used). Furthermore, in this trial, the infection was established very early after inoculation (15 d). The Fisher's exact test at 40 d p.i. showed no dependency of prevalence on temperature ($p = 0.224$), whereas a strong dependency was found at 60 d p.i. ($p = 0.00069$). Table 2 shows the prevalence and the mean intensity of infection in each intestinal portion examined histologically in AT-1 and AT-3 at the last sampling. This information was not obtained in AT-2 since fish were not infected in the only sampling point diagnosed by histology. In AT-1, most R animals exhibited accumulation of ascites in the lumen of the intestine, with this accumulation sometimes reaching the pyloric caeca. All R fish had the posterior intestine being massively invaded by the parasite. The anterior intestine and pyloric caeca were also heavily parasitized, and the middle intestine was the least infected portion. All *E. leei* that showed negative results for the anterior intestine were also negative at the middle portion. No parasites were detected in the stomach or in the oesophagus. In AT-3, the parasite did not spread to the anterior intestine until 40 d p.i., although intensity of infection was already very high at the posterior part at 15 d p.i. In both trials, spores and disporous sporoblasts (Fig. 1) were always present in infected posterior and anterior tracts, being frequently detached to the lumen (Fig. 2), whereas they were detected only in 57.1% of the infected middle intestines of AT-1 fish. Instead, in those portions without sporogonic stages, proliferative stages were abundant (Fig. 3). In infected intestines, the parasite was typically patchily distributed mainly in the anterior and middle portions when intensity of infection was low or medium.

Table 2. *Enteromyxum leei*. Prevalence and mean intensity of histologically diagnosed infection in the different portions of the digestive tract in gilthead sea bream experimentally infected through the anal (AT) route. AI, MI and PI refer to the anterior, middle and posterior intestine. *E. leei* stages were classified as spores (SP), disporoblasts (DSB) or proliferative stages (P). p.i.: post inoculation

TRANSMISSION trial	Days p.i.	Tissue portion	Prevalence of infection (%)	Mean intensity	*E. leei* stages
AT-1	60	Stomach	0	–	–
		Oesophagus	0	–	–
		AI	60	5.4+	SP, DSB > P
		MI	46.7	4.9+	P > SP, DSB
		PI	100	5.9+	SP, DSB > P
AT-3	15	AI	0	–	–
		MI	0	–	–
		PI	85.7	6+	P > SP, DSB
	40	AI	1.4	5+	SP, DSB > P
		MI	0	–	–
		PI	85.7	6+	SP, DSB > P

In peranally inoculated fish, the histopathological study revealed the clear damage caused by the parasite in the intestinal tissue of R fish, regardless of the infected portion. Vacuolation and detachment of the epithelium only appeared in AT-1 fish with high intensity of infection. Inflammatory cells occurred in the lamina propria-submucosa, which was hypertrophied, as well as in the basal part of the epithelium, and consisted of eosinophilic granule cells (EGCs) (Figs. 4 & 5) and lymphocytes (Fig. 6). EGCs were classified according to their staining pattern as in Álvarez-Pellitero et al. (2008). EGC Type 1 (EGC1) (fuchsia with H&E, rarely PAS positive and not stained with TB) were abundant in the lamina propria-submucosa (Fig. 4), sometimes also in the epithelium, and were almost in contact with parasite stages. EGC Type 2 (EGC2) (did not stain with H&E or with TB, with some PAS-positive granules, usually with a polarised pattern) were less abundant and mainly localized in the lamina propria-submucosa (Fig. 5). In R fish, rodlet cells were very scarce in areas invaded by the parasite, whereas they were abundant in nonparasitized neighbouring areas, even more than in the intestines of CTRL fish (Fig. 7). The number of goblet cells showed a tendency to diminish in infected intestines, and they were occasionally seen close to parasite stages, almost enclosing them (Fig. 8).



Figs. 1 to 10. *Sparus aurata.* Histological sections of intestines after experimental infections with *Enteromyxum leei* at 60 d post inoculation. Figs. 1 to 8. Anal transmission (AT-1). Fig. 1. Spores and sporogonic stages invading the epithelium of the posterior intestine (PI). Notice a mature spore about to be detached to the lumen. Scale bar: 10 µm. Fig. 2. Detail of a primary cell containing a mature spore and an accompanying cell released to the lumen of the PI, with remnants of epithelial cells. Scale bar: 10 µm. Fig. 3. Proliferative stages in the epithelium of the middle intestine (MI). Scale bar: 10 µm. Fig. 4. Massive proliferation of eosinophilic granule cells (EGC1) in the hypertrophied lamina propria-submucosae of the MI. Scale bar: 20 µm. Fig. 5. EGC2 in the lamina propria-submucosae, showing periodic acid-Schiff (PAS)-positive granules (arrow) that usually have a polarised pattern, and EGC1 with PAS-negative granules in the epithelium (arrowhead) of the MI. Notice the PAS-positivity of *E. leei* stages. Scale bar: 20 µm. Fig. 6. Lymphocytic infiltration (arrowheads) in the epithelium and lamina propria-submucosae of the MI. Scale bar: 20 µm. Fig. 7. Panoramic view of an infected anterior intestine (AI) with a patchy distribution of the parasite (right side). Notice the abundance of rodlet cells (arrowheads) in the non-infected part (left side). Scale bar: 50 µm. Fig. 8. Goblet cells flanking parasitic stages in the epithelium of the AI. Scale bar: 10 µm. Figs. 9 & 10. Oral transmission (OT-1). Fig. 9. Residual parasite stages engulfed by a macrophage (arrowhead). Scale bar: 10 µm. Fig. 10. Massive proliferation of lymphocytes at the base of the epithelium and lamina propria-submucosa of the MI. Scale bar: 20 µm. Sections are stained with toluidine blue (Figs. 1, 2, 6, 7, 9, 10), H&E (Figs. 4 & 8) and PAS (Fig. 5).

In perorally intubated fish, the intestinal epithelium showed empty or vacuolated cells. Other cells containing unidentified debris were compatible with macrophages engulfing cell or parasite debris (Fig. 9). EGCs were abundant in the lamina propria-submucosa, and sometimes infiltrated the epithelium. Lymphocytes were abundant at the base of the epithelium, particularly at the anterior and middle intestines (Fig. 10).

3.4 DISCUSSION

Thus far, anal intubation has been used successfully for vaccine (Crosbie & Nowak 2004, Vervarcke et al. 2005) or immunomodulator (Swan et al. 2008) delivery in fish. However, peranal parasite transmission has only been reported for the microsporidian *Loma salmonae* (Shaw et al. 1998) and this is the first report on this type of transmission for a myxosporean. This mode of infection proved to be very effective, achieving high infection levels at 60 d p.i. (AT-1) or even at 15 d p.i. (AT-3). Since R fish in the AT-2-H and AT-3 trials received the same volume of inoculum, it is tempting to suggest that the higher prevalence of infection achieved after 40 d in AT-3 was probably due to a higher inoculated parasite infective dose per body weight, as fish were smaller and water temperature was similar. These results are higher than the infection levels commonly obtained via cohabitation or effluent routes for gilthead sea bream (Diamant 1997, Diamant & Wajsbrot 1997, Sitjà-Bobadilla et al. 2007) and are comparable to those obtained in sharpsnout sea bream *Diplodus puntazzo*, which is a highly susceptible species (Golomazou et al. 2006, Muñoz et al. 2007). However, in the latter species, 100% infection prevalence was reached in just 10 d after cohabitation with infected donors and the entire intestinal tract was invaded at 20 d post exposure (Álvarez-Pellitero et al. 2008).

The present study shows a clear relationship between prevalence and water temperature in peranal infections. Results confirm those of previous oral infections with *Enteromyxum fugu* and *E. leei* in tiger puffer *Takifugu rubripes*, in which the onset of the disease was suppressed by low water temperatures (<15°C) (Yanagida et al. 2006). In gilthead sea bream farms, the minimum temperature for developing enteromyxosis varies from 18°C (Le Breton & Marques 1995) to 22°C (Rigos et al. 1999), and outbreaks in French farms have only been observed when water temperature is >20°C (Fleurance et al. 2008). The present study also confirms the inhibitory effect of low water temperatures on the development of enteromyxosis in gilthead sea bream, as no fish was infected in AT-2-L during the first 60 d p.i. in which water temperature reached a maximum of only 13.9°C. However, when water temperature naturally increased up to 26°C at 216 d p.i. (mean water temperature between 60 and 216 d was 17.1°C), the infection appeared. As in the tiger puffer, this re-emergence of *E. leei* in gilthead sea bream with increase in water temperature indicates the capability of this parasite to become latent during the cooler period. Water temperature greatly affects the initiation and progression of myxozoan infections. For some myxozoans, favorable water temperatures have been defined (Hedrick et al. 1993), but arrested development or latency in the host at low water temperatures has scarcely been documented for fish parasites (Olson 1981, Beaman et al. 1999). Furthermore, the only available report of arrested development for a Myxosporea during the pre-spore stage was not attributed to temperature (Higgins et al. 1993). This has important epidemiological consequences, since fish can pass as false negatives in epidemiological surveys during wintertime and become reservoirs and/or a source of the parasite to naïve fish when temperature rises in spring and summer. Further studies are needed to determine the parasite stages and the host sites where latency occurs at low temperatures.

Regardless of the temperature, all surviving R AT-2 fish were free of the parasite when sampled 326 d p.i., even though infection was clearly established in previous samplings. This recovery from infection has already been described for enteromyxosis (Yanagida et al. 2006) and other myxosporoses such as PKD (Morris et al. 2005).

Clear differences in the prevalence, intensity of infection and parasite stages were observed among the 3 parts of the digestive tract. The posterior intestine seems to be the first target site for *Enteromyxum leei* in gilthead sea bream, followed by the anterior portion. These differential preferences have also been observed in other infection routes, such as effluent (Estensoro et al. 2010), cohabitation and even oral routes (Cuadrado 2009). Similarly, in natural infections, a higher prevalence of infection was found in the rectum than in the proximal intestine in asymptomatic fish (Fleurance et al. 2008). Therefore, the higher infection levels found in the distal part of the intestine are not due to the peranal infection mode. However, in other *Enteromyxum* spp. oral and cohabitation trials, an anterior–posterior gradient was demonstrated (Redondo et al. 2004, Yanagida et al. 2006, Álvarez-Pellitero et al. 2008). In AT-1, the stomach remained free of parasites, whereas pyloric caeca were infected, as observed by other authors in advanced *E. leei* infections (Fleurance et al. 2008). This observation could indicate that the pyloric caeca might be invaded later on by the parasite, and that we could therefore consider the infection in AT-1 infected fish to be quite advanced in just 60 d.

The histopathological damage caused by the parasite and the inflammatory host reaction were very similar to those described by other authors for different *Enteromyxum leei*-infected hosts (Diamant et al. 2006,

Álvarez-Pellitero et al. 2008, Fleurance et al. 2008). The disruption of the integrity of the mucosa, desquamation and detachment of the epithelium, which correspond to advanced, chronic infections, was observed earlier than in cohabitation and effluent transmission trials. The apparent decrease in goblet and rodlet cells has also been reported in turbot *Psetta maxima* with severe *E. scophthalmi* infections (Bermúdez et al. 2010).

In the current study, peroral transmission failed. The histopathological study showed a cellular activation at the gut level in R fish, and the observed cell debris could correspond to abortive stages that were engulfed by macrophages and were not capable of proliferating in the tissue. In previous studies, oral transmission of *Enteromyxum leei* to gilthead sea bream produced very variable results ranging from 0 to 86.7% prevalence of infection (Diamant 1997, Sitjà-Bobadilla et al. 2007). *E. leei* was also orally transmitted to red sea bream *Pagrus major,* the tiger puffer (Yasuda et al. 2002, Yanagida et al. 2004) and several fresh water species (Diamant et al. 2006). Similarly, a high prevalence of infection by *E. scophthalmi* was obtained orally for turbot (100%, 22 d p.e.) (Redondo et al. 2004). The existence of refractive or nonsusceptible gilthead sea bream strains (Sitjà-Bobadilla et al. 2007) cannot explain the failure of the oral route in the current study, as an identical group of fish was successfully infected using the same inoculum, dose and water temperature by the anal route. Differences in the presence or absence of a true stomach, acidic gastric pH and digestive enzyme activity have been hypothesized to explain the failure in the transmission of *E. leei* by the oral route in some species (Diamant et al. 2006).

In conclusion, this novel peranal transmission greatly improved the infection levels and the timing achieved by other routes and further confirms the direct transmission of *Enteromyxum leei* without the involvement of invertebrate hosts. This ease of transmission makes enteromyxosis an excellent model for the study of host–parasite interactions, but this fact and the described arrested development at low temperatures are also responsible for the spread of the disease under farming conditions, and greatly hamper its prevention and control.

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04

Immunohistochemical characterization of polyclonal antibodies against *Enteromyxum leei* and *Enteromyxum scophthalmi* (Myxozoa: Myxosporea), intestinal parasites of fish

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4.0 ABSTRACT

The enteric myxozoan parasites *Enteromyxum leei* and *E. scophthalmi* are responsible for heavy weight loss in infected fish which leads to subchronic disease and low mortality rates in gilthead sea bream (*Sparus aurata*) (GSB) and to high mortality rates in turbot (*Psetta maxima*). The detection of initial parasite stages in histological sections is particularly difficult, but can be simplified by means of specific antibodies. Rabbit polyclonal antibodies (Pabs) were raised against *E. scophthalmi* and *E. leei* and direct enzyme-linked immunosorbent assay (ELISA) and immunohistochemistry were used to characterize their sensitivity and specificity. Both Pabs were adsorbed (aPab) with non-infected intestines to avoid non-specific labeling of fish tissues and to improve its specificity. The highest titre obtained in ELISA was 1: 32,000 for aPab-Eleei and 1:16,000 for aPab-Escoph. Working dilutions in immunohistochemistry were 1:1,000 for aPab-Eleei and 1:8,000 for aPab-Escoph. Both aPabs labeled proliferative and sporogonic stages with high specificity. aPab-Escoph was very specific, as no cross-reaction with any other tested myxozoan was detected, whereas aPab-Eleei cross-reacted with *Sphaerospora dicentrarchi* and *S. testicularis*, suggesting the presence of shared antigens. These Pabs stand as new tools for antigenic characterization and the diagnosis of both *Enteromyxum* species.

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KEY WORDS:
Gilthead sea bream · Turbot · Emaciation disease · Immunoreactivity · Cross-reactivity · ELISA

4.1 INTRODUCTION

The myxosporeans *Enteromyxum scophthalmi* (Palenzuela, Redondo et Álvarez-Pellitero), affecting turbot, *Psetta maxima* (L.), and *Enteromyxum leei* (Diamant, Lom et Dyková), parasite of several sparids including gilthead sea bream (GSB), *Sparus aurata* (L.), produce significant economic losses in mariculture (Sitjà-Bobadilla and Palenzuela 2012). Severely infect ed fish die after a progressive destruction of the gut by the parasite. These myxosporeans proliferate mainly in the epithelial layer of the digestive tract producing a desquamation of the epithelial cells to the lumen, which is more pronounced in turbot (Redondo, Palenzuela, Riaza, Macias and Álvarez-Pellitero 2002). The production of spores by the highly pathogenic *E. scophthalmi* is scarce because infected fish mostly die before sporogenesis occurs, whereas *E. leei* produces abundant spores. However, primary mother cells (P) containing inner secondary (S) or tertiary (T) daughter cells are very frequent in infected turbot and can easily be obtained from the fluids accumulated in the digestive lumen. On the contrary, parasitic stages of *E. leei* are more firmly attached to the epithelium and are seldom found in lumen fluids.

Many aspects of biology, development and life cycle of myxosporeans remain unknown, and little information on the fish immune response (Sitjà-Bobadilla 2008) and on the use of immunological tools for their diagnosis exists. The diagnosis of these *Enteromyxum* species relies mainly on the detection of parasite stages in gut scrapings or histological sections, which requires considerable experience and is time consuming. Sensitive molecular methods, including PCR (Palenzuela, Agnetti, Albiñana, Álvarez-Pellitero, Athanassopoulou, Crespo, Diamant, Ghittino, Golomazou, Le Breton, Lipshitz, Marques, Padrós, Ram and Raymond 2004; Yanagida, Freeman, Nomura, Takami, Sugihara, Yokoyama and Ogawa 2005; Piazzón, Lamas and Leiro 2012) and in situ hybridization (ISH) (Redondo 2005; Cuadrado, Albinyana, Padrós, Redondo, Sitjà-Bobadilla, Álvarez-Pellitero, Palenzuela, Diamant and Crespo 2007), by means of oligonucleotide probes, have more recently been developed for the diagnosis of enteromyxosis, though their routine use can result expensive and requires specific equipment and trained personnel. Besides, the preservation of the parasite and tissue morphology in sections processed for ISH can be hampered by the application of strong reagents, with subsequent difficulty in defining the exact localization of the parasite in the tissue. Thus, the improvement of the histochemical diagnosis of these parasites and the localization of their main antigens was broached by the authors in the context of a major project to enhance our understanding of marine Myxosporea.

The development of polyclonal antibodies (Pabs) provides a useful tool for the study of the antigenic epitopes of parasites (Hamilton and Canning 1988; Bartholomew, Rohovec and Fryer 1989; Muñoz, Sitjà-Bobadilla and Álvarez-Pellitero 1998; Muñoz, Sitjà-Bobadilla and Álvarez-Pellitero 2000; Yokoyama, Inoue, Sugiyama and Wakabayashi 2000; Belem and Pote 2001), but their use in myxosporean identification is very scarce. Therefore, this paper describes the production of Pabs raised against both *Enteromyxum* spp., and the localization of the antigens recognized by these antibodies by light immunohistochemistry. Furthermore, the antibody's cross-reaction with other parasites is examined.

4.2 MATERIALS AND METHODS

4.2.1 Parasite immunogens

Experimentally infected turbots (see Redondo et al. 2002) were killed by overexposure to the anaesthetic MS-222 (Sigma, St. Louis, MO, USA), and the intestinal fluid was drawn with a sterile syringe. Its content was examined by light microscopy, and fluids from fish (n = 4) containing the minimum amount of host cells were selected for the preparation of the immunogen. After washing the fluids twice in 10 mL sterile HBSS (Gibco-Life technologies, Alcobendas, Madrid) including antibiotics and antimycotics (Gibco-Life technologies) and centrifuging at 1,500 g for 10 min at 4 ºC, the pellets were recovered and parasites were counted with a haemocytometer. Samples ranging between 1.1 x 106 and 6.1 x 106 parasites mL-1 were pooled and stored at -20 ºC with a protease inhibitor cocktail (Roche Diagnostics, Sant Cugat del Vallès, Barcelona, Spain) until used.

Experimentally infected gilthead sea bream (GSB) (n = 4) (see Sitjà-Bobadilla, Diamant, Palenzuela and Álvarez-Pellitero 2007) were killed by overexposure to anaesthetic MS-222 and bled to avoid sample contamination by blood cells. Intestines were opened lengthwise under sterile conditions and the mucosa slightly scraped with a scalpel. The obtained intestinal scrapings were homogenized in sterile HBSS (Gibco-Life technologies) including antibiotics and antimycotics (Gibco-Life technologies) with a syringe and remaining cell aggregations and debris were retained by a cell strainer (40 µm) (SARSTEDT AG & Co., Nümbrecht, Germany). The parasite/cell suspensions were then centrifuged at 1,500 g for 10 min at 4 ºC and the supernatants eliminated. Parasites contained in the pellets were counted with a haemocytometer and ranged between 3.3 x 106 and 36 x 106 parasites mL-1. Samples were pooled and a protease inhibitor cocktail (Roche Diagnostics) was added prior to storage at -20 ºC until used.

4.2.2 Rabbit immunization procedure and antisera production

The Pab against *E. schophthalmi* was generated by immunizing two New Zealand rabbits, *Oryctolagus cuniculus* (L.), subcutaneously with the parasite immunogen using 4.9 x 106 parasites per injection. The primary inoculation was emulsified in Freund's complete adjuvant, followed by three boosts emulsified in Freund's incomplete adjuvant every 20 days (day 20, 40 and 60). Ten days after the second and third boosters (day 50 and day 70), the rabbits were bled and the sera adsorbed and tittered by IHC. Two weeks after the third booster, rabbits were sedated and the final complete bleeding was performed. The sera were aliquoted and stored at -20 ºC until use. Pre-immune rabbit serum was also obtained at day 0.

The anti *E. leei* Pab was produced in the same way, using 8.3 x 106 parasites per injection but an additional fourth booster took place (day 90) prior to a third bleeding (day 100) and to the final bleeding (day 120).

As host cells were not completely removed from both injected antigens, to avoid the detection of the host tissue, rabbit antisera were absorbed by incubating with intestine homogenates of the corresponding non-infected conspecific fish (turbot, GSB), following a procedure similar to that described by Hamilton and Canning (1988), with some modifications. Briefly, non-infected fish intestinal scrapings were homogenized in HBSS with antibiotics and antimycotics and centrifuged at 1,500 g for 30 min at 4 ºC. Supernatants were eliminated and the pellets re-suspended in the antisera (p:V, 1:10). A 2 h incubation at 4 ºC followed by an overnight incubation at 10 ºC was performed. Finally, the antisera were recovered by 5 centrifugations at 10,000 g for 10 min at 4 ºC until particulate material was completely eliminated. Both the raw (Pab) and the adsorbed (aPab) antisera were aliquoted and stored at -20 ºC until used. The specificity and sensitivity of the antisera were determined by ELISA and immunohistochemistry.

All efforts were made to minimize suffering of the animals used for the experiments, in accordance with the national (Royal Decree RD1201/2005, for the protection of animals used in scientific experiments) and the current European Union legislation on handling experimental animals. Fish were kept in IATS facilities and rabbits were kept, immunized and bled in Antibody Bcn S.L. facilities.

4.2.3 ELISA

The sensitivity of the antisera obtained at the final bleeding from each of the two rabbits immunized with each *Enteromyxum immunogen* (and also at the 3rd bleeding for the *E. leei*-injected rabbits) were tested by enzyme linked immunosorbent assay (ELISA). The serum of the rabbit with the best performance for each parasite was chosen for all subsequent analyses. For both parasites, Pab and aPab antisera were screened.

Parasite antigenic preparations: They were obtained from experimentally heavily infected GSB or turbot. Briefly, intestinal mucosal scrapings of GSB or intestinal fluids of turbot were processed as described above for obtaining rabbit immunogens. Several aliquots obtained from different infected fish were pooled, and after addition of antiproteases (1 mM EDTA, 0.1 mM, TPCK,1 mM PMSF; Sigma), whole cell lysates were prepared by nine freeze-thawing cycles at -80 °C. Protein content of the lysate (antigen) was estimated using the BioRad protein assay adapted to microplates (Biorad, Hercules, CA, USA), based on the method of Bradford (Bradford 1976). Bovine serum albumin was used as standard.

Direct ELISA: After the screening of several incubation conditions, antigen concentrations and sera dilutions, the optimized experimental conditions for this assay were as follows. Wells of flat bottomed micro-

plates (96-wells, Nunc Maxisorp, Wiesbaden, Germany) were coated with 50 µL antigen solution containing 3 µg mL-1 protein in coating buffer (carbonate-bicarbonate buffer, pH 9.6), and incubated overnight at 4 °C. The wells were washed three times with TTBS (20 mM Tris-HCl, 0.5 M NaCl, 0.05% Tween 20, pH 7.2) and once with TBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.2), and then blocked with TBS+5% skimmed milk for 2 h at 37 °C. The plate was washed as before and double serial dilutions (1:1,000 to 1:32,000 for the aPab-Eleei or 1:250 to 1:32,000 for the aPab-Escoph) were added as first antibody. After 2 h at 37 °C, the plate was washed again and goat anti-rabbit IgG (H+L) (second antibody) horseradish peroxidase conjugate (Sigma) diluted 1:1,000 was added and incubated for 1 h at 37 °C. After the last washing, TMB peroxidase substrate (3,3',5,5'-tetramethylbenzidine solution plus hydrogen peroxide; BioRad) was added and incubated at room temperature in dark with gentle shaking for 30 min. The reaction was stopped with 1N H2SO4 and the plates were read at 450 nm with an automatic plate reader (Tecan Group Ltd., Männedorf, Switzerland).

4.2.4 Fish tissue samples and immunohistochemistry

Intestinal portions of healthy and *Enteromyxum*-infected of both turbot and GSB were fixed in 10 % buffered formalin and embedded in paraffin. For *E. scophthalmi*, fixed portions of trunk kidney, pancreas and muscle, and frozen blood smears were also included.

Sections (4 µm thick) were collected on Super-Frost-plus microscope slides (Menzel-Gläser, Germany) and allowed to dry overnight. Slides were deparaffinised, hydrated and the endogenous peroxidase activity of the tissues was quenched by incubating in 0.3% (v/v) hydrogen peroxide for 30 min. Incubations were performed in a humid chamber at room temperature and all washing procedures consisted of successive 5 min immersions in TTBS and TBS. After washing, slides were blocked for 30 min with 1.5% normal goat serum (VECTOR Laboratories, Burlingame, CA, USA) and washed again. Sections were then incubated with serial dilutions of the best rabbit antisera either normal (Pab-Escoph and Pab-Eleei) or adsorbed (aPab-Escoph and aPab-Eleei) (for *E. leei* detection 1:1,000, 1:2,000, 1:4,000 and 1:8,000; for *E. scophthalmi* detection 1:8,000, 1:16,000, 1:32,000, 1:64,000). After washing, biotynilated goat antirabbit antibody (VECTOR Lab.) (1:200) was added. After further washing, the sections were incubated with the avidin-biotin-peroxidase complex (ABC) (VECTOR Lab.), and bound peroxidase was finally revealed by adding the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma). The reaction was stopped with deionised water and the sections counterstained using Gill's hematoxylin and mounted in DPX. Negative controls were obtained using serial dilutions of the pre-immune serum or TBS instead of the polyclonal antisera in the first incubation. The staining intensity of the parasites in the immunohistochemical preparations was described by a semi-quantitative scale indicated by + (weak specific label), ++ (moderate specific label), +++ (intense specific label) and – (absence of specific label).

The labelling specificity of aPab-Escoph and aPab-Eleei was tested using tissue sections of several fish species infected by different myxosporeans obtained from paraffin embedded samples as described above. Details on the fish species and tissues can be found in Table 2.

4.3 RESULTS

4.3.1 *Enteromyxum* detection

The reactivity of the antisera obtained from the two rabbits for each *Enteromyxum* species was first tested by ELISA. Figure 1 shows the results for the best antisera from the third and final bleeding of one of the rabbits immunised with *E. leei*, either adsorbed or not. The linearity of the obtained values was checked by common log transformation of the data. The regression coefficients were high ($r^2 \geq 0.99$) for both *E. leei* tested antisera. Figure 2 shows the results for the Pab-Escoph and aPab-Escoph from the best rabbit tested in ELISA. When linearized, the regression coefficients were also high ($r^2 = 0.98$ for aPab-Escoph; $r^2 = 0.95$ for Pab-Escoph). For all antisera, the titre was lower when used adsorbed to intestine. The endpoint of the best aPab-Eleei was >32,000 and that of the best aPab-Escoph was 16,000.

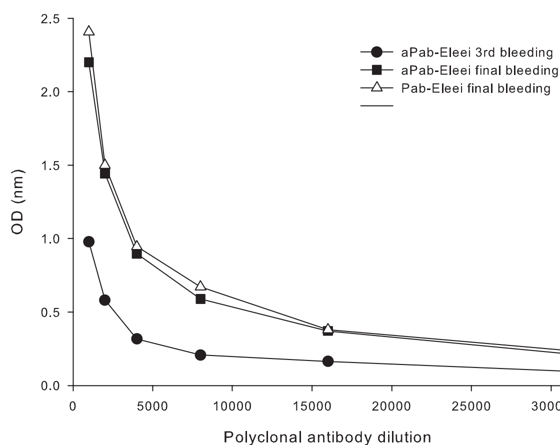

Figure 1. ELISA titration of the polyclonal antibody (Pab) raised against *Enteromyxum leei* (Pab-Eleei). Non-adsorbed sera (Pab-Eleei) and sera adsorbed with normal gilthead sea bream intestine (aPab-Eleei) were tested.

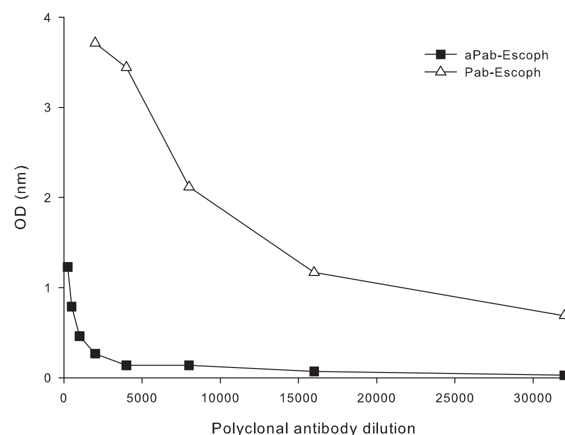

Figure 2. ELISA titration of the polyclonal antibody (Pab) raised against *Enteromyxum scophthalmi* (Pab-Escoph). Non-adsorbed sera (Pab-Escoph) and sera adsorbed with normal turbot intestine (aPab-Escoph) were tested.

The specificity of the best antiserum for each *Enteromyxum* was tested by immunohistochemisty. Serial dilutions of Pab-Escoph (starting 1:2,000 until 1:8,000) were tested on non-infected and infected turbot tissues. At all these dilutions, the serum stained strongly *E. scophthalmi*, as well as the host tissue, especially the intestinal epithelium (enterocytes, brush border and goblet cells) of infected and non-infected fish (Fig. 3a). On the contrary, when the same dilutions of aPab-Escoph were tested, parasite stages were intensively stained and the tissue background staining disappeared completely at 1:8,000. In proliferative stages, aPab-Escoph labelled the surface and the cytoplasms of primary (P) and secondary (S) cells (Fig. 3). P cells were also labelled in sporogonic stages. Additionally, an inconsistent staining of *E. scophthalmi* on the spore valve surface was detected. Labelled *E. scophthalmi* stages were mainly found in intestinal sections (Fig. 3a-l), specifically in the epithelium. These covered a broad range of developmental stages from very initial stages (uninucleated P cell) (Fig. 3j-k) to intermediate stages such as multinucleated P cells with (Fig. 3c-d) or without (Fig. 3e-f) S cells and disporous pseudoplasmodia (Fig. 3g-i). Some stages were observed on the epithelial surface apparently attached to the enterocytes (Fig. 3e-f). Stained parasite stages were also found in the intestinal lumen, together with whole epithelial ribbons of host enterocytes (Fig. 3l). Occasionally, some stages were also observed in blood vessels of the muscle (Fig. 3m) and liver (Fig. 3n-o), in the liver associated serosa (Fig. 3p) and in the trunk kidney parenchyma. In frozen blood smears of infected turbot, parasite stages were not labelled. Further two-fold dilutions of the antiserum showed that the labelling of the parasite was still present at 1:16,000 and disappeared at 1:32,000 (Table 1). When aPab-Escoph was applied to non-infected turbot tissues, no staining was detected.

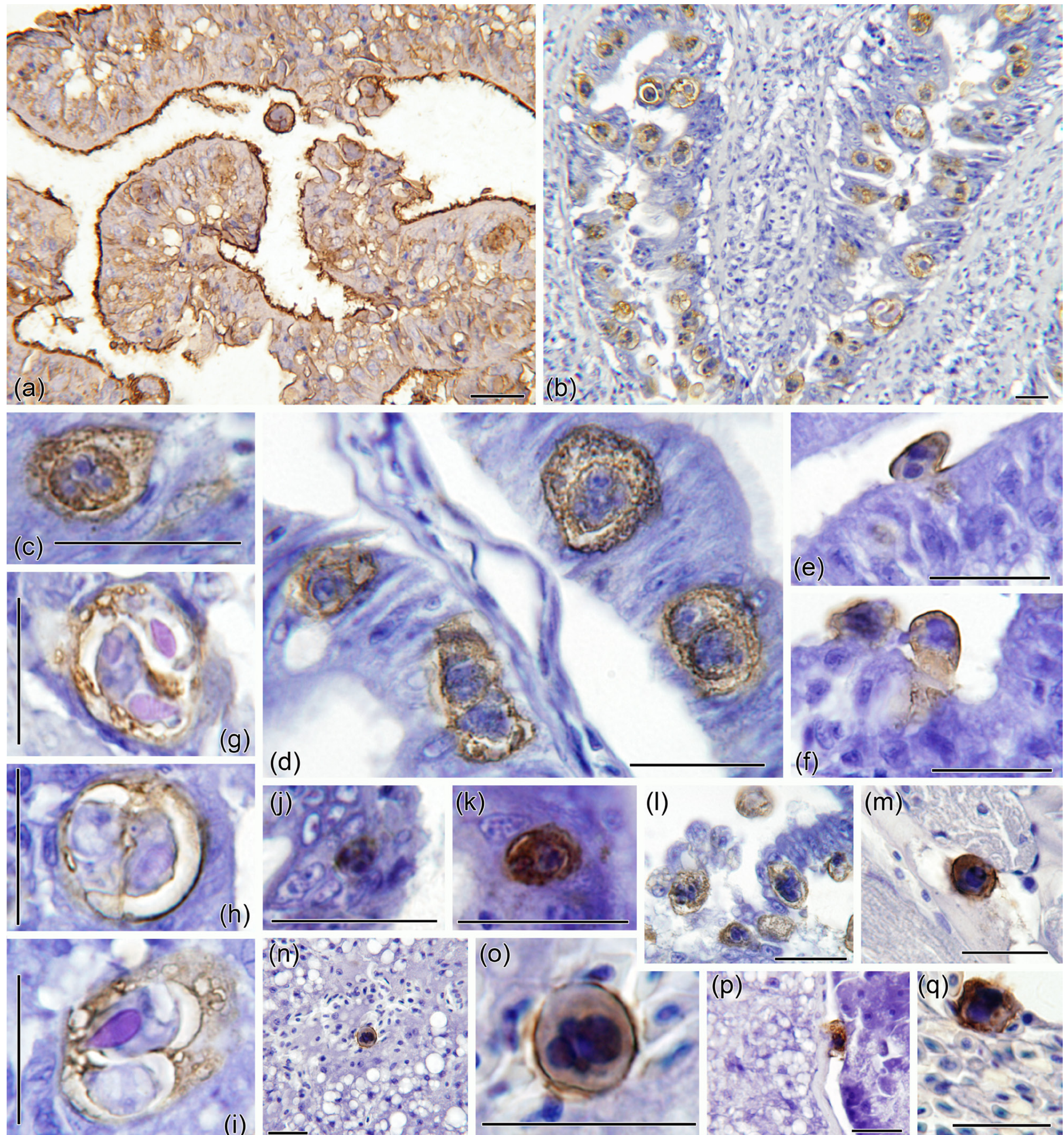

Figure 3. Immunohistochemical staining of *Enteromyxum scophthalmi* in haematoxylin stained paraffin sections of turbot. (**a-l**) Intestinal sections. (**a**) Tissue section incubated with the non-adsorbed polyclonal serum against *E. scophthalmi.* Note background staining in host tissue. (**b-q**) Tissue sections incubated with the adsorbed polyclonal serum against *E. scophthalmi* without host tissue background. (**c**) Intraepithelial stage with immunoreactive cytoplasm of the primary (P) and the secondary (S) multinucleated cell. (**d**) Intraepithelial stages with immunoreactive cytoplasm of the P cells containing one or several S cells. (**e-f**) Stages on the epithelial surface with cytoplasmatic and surface immunoreactivity. (**g-i**) Sporogonic stages with immuroreactive P cells. Note the inconsistent spore valve immunoreactivity. (**j-k**) Small, initial intraepithelial stages. (**l**) Luminal stages desquamated from the intestinal epithelium surrounded by host cell debris. (**m**) Labeled P cell in a blood vessel between muscle fibers. (**n-o**) Stage in a blood vessel of the liver with intense surface staining. (**p**) Labeled P cell in the liver serosa close to the pancreas. (**q**) Immunoreactive P cell in blood surrounded by erythrocytes. Scale bars = 20 µm.

Table 1. Immunohistochemical binding of adsorbed polyclonal antibodies (aPabs) raised against *Enteromyxum leei* and *E. scophthalmi*. aPabs were applied on 5µm paraffin intestinal sections of *E. leei*-infected gilthead sea bream and *E. scophtalmi*-infected turbot. The staining intensity is indicated according to a semi-quantitave scale being + (weak), ++ (moderate), +++ (intense) and – absence of specific label.

PAB	DILUTION	PARASITE	STAINING INTENSITY
aPab-E leei	1:1,000	*E. leei*	+++
	1:2,000		++
	1:4,000		+
	1:8,000		+/–
	1:16,000		–
aPab-Escoph	1:8,000	*E. scophthalmi*	+++
	1:16,000		+
	1:32,000		–

Pab-Eleei stained the parasite strongly, but labelling was also found in the host tissue, especially in the intestinal epithelium (enterocytes, brush border and goblet cells) and in the lamina propria-submucosa (lymphocyte-like cells, red blood cells and connective tissue fibres) (Fig. 4a). However, the non-specific detection of the fish tissues disappeared with aPab-Eleei, which presented a clear and outstanding specific detection of parasitic stages (Fig. 4b-k). aPab-Eleei was tested at serial dilutions (1:500 until 1:16,000) and presented the strongest parasite label without background staining at 1:1,000. aPab-Eleei specifically stained P, S and T cells of proliferative stages, in both the cytoplasm and the cell surface (Fig. 4c-g). P cells containing sporogonic stages, spore valves and accompanying cells were also labelled (Fig. 4i-j). Parasite stages were mostly found in the intestinal epithelium though one stage was apparently located in the lamina propria-submucosa (Fig. 4h). Pre-immune serum did not bind to any parasite stage or to host cells. Further two-fold aPab-Eleei dilutions showed a progressive decrease of the parasite label which disappeared at 1:16,000 (Table 1). No staining was observed in uninfected GSB tissues.

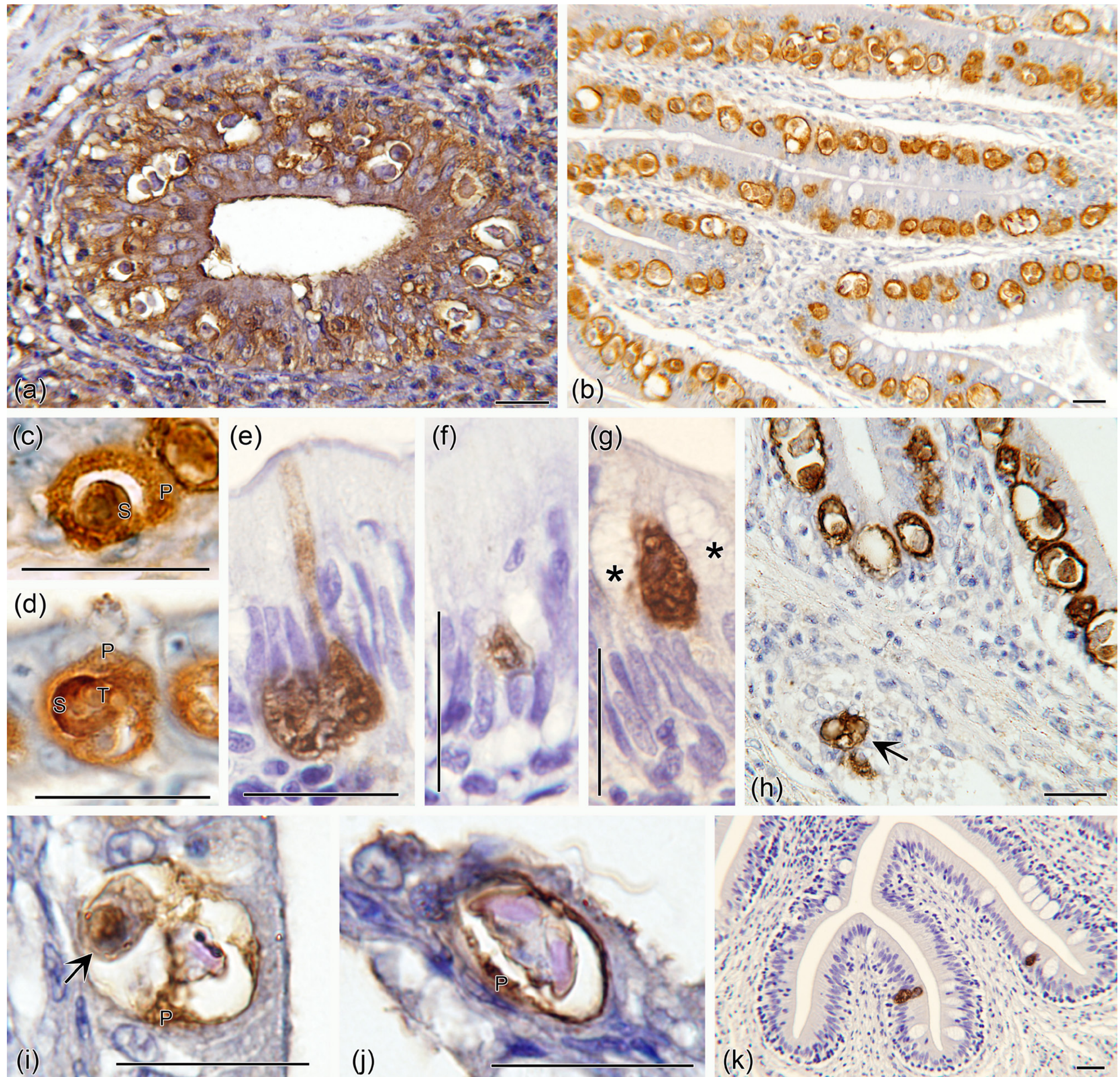

Figure 4. Immunohistochemical staining of *Enteromyxum leei* in haematoxylin stained paraffin sections of gilthead sea bream intestines. (**a**) Tissue section incubated with the non-adsorbed polyclonal serum against *E. leei*. Note background staining in host tissue. (**b-k**) Tissue sections incubated with the adsorbed polyclonal serum against *E. leei* without host tissue background. (**c**) Intraepithelial stage with immunoreactive cytoplasm in the primary (P) and secondary (S) cells. (**d**) Intraepithelial stage with immunoreactive cytoplasm in the P, S and tertiary (T) cells. (**e**) Intraepithelial stage with cytoplasmatic immunoreactivity even in a long plasmodial proyection. (**f**) Small, intraepithelial immunoreactive stage. (**g**) Intraepithelial stage with intense cytoplasmatic immunoreactivity located between goblet cells (*). (**h**) Labelled parasite stages in the intestinal epithelium and lamina propria-submucosa (arrow). (**i**) Disporoblast with immunoreactivity in both P cell and accompanying cell (arrow). (**j**) Disporoblast with immunoreactive P cell and spore valves. (**k**) Overview of intestine with early infection. Scale bars = 20 µm.

For both aPabs, in the absence of rabbit serum or with pre-immune serum no labelling was detected in either parasitized or non-parasitized tissue sections. The best working dilution was 1:1,000 for aPab-Eleei and 1:8,000 for aPab-Escoph, which were further applied to the remaining myxosporean samples.

4.3.2 Cross reactivity

aPab-Escoph did not label any of the myxosporean species tested nor host tissues. In contrast, cross-reaction was obtained with aPab-Eleei though only with both tested *Sphaerospora* spp. In *S. dicentrarchi*, spore valves were labelled (Fig. 5a-c) while in *S. testicularis* the label was inconsistent in the P cells of proliferative stages (Fig. 5d-e), and polar capsules were positive (Fig. 5f). In addition, aPab-Eleei slightly stained host tissues of turbot (enterocytes, intestinal brush border and goblet cells) and those of European sea bass (*Dicentrarchus labrax*) (stomach mucosa, sub-epithelial connective tissue and muscular tissue) (Table 2). In the absence of rabbit serum or with pre-immune serum no labelling was detected in any myxosporean species or host tissues.

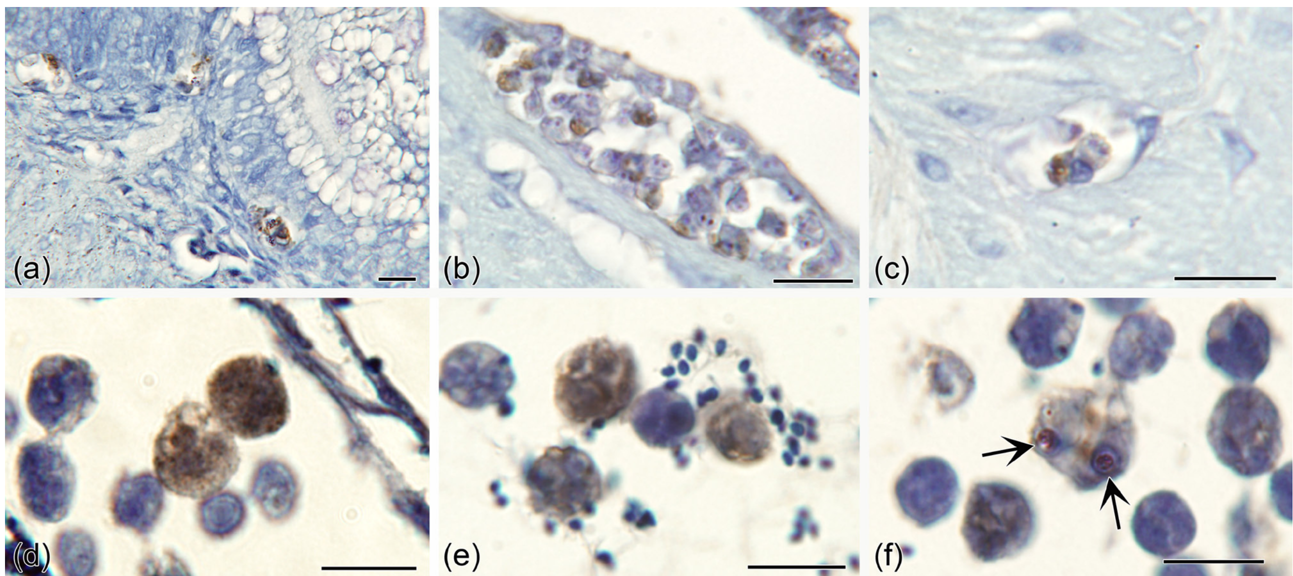

Figure 5. Immunohistochemical staining with the adsorbed polyclonal antibody against *Enteromyxum leei*. Cross-reaction with *Sphaerospora dicentrarchi* (**a-c**) and *S. testicularis* (**d-f**) in haematoxylin stained paraffin sections of European sea bass (*Dicentrarchus labrax*). (**a**) Intestinal connective tissue with immunoreactive *S. dicentrarchi* spores. (**b-c**) Intestinal muscle tissue with *S. dicentrarchi* spores showing immunoreactive valves. (**d-e**) *S. testicularis* proliferative stages showing cytoplasmatic immunoreactivity inconsistently and with different intensities in testis. (**f**) S. testicularis spore with immunoreactive polar capsules (arrows) in testis. Scale bars = 20 µm.

Table 2. Immunohistochemical cross-reaction of adsorbed polyclonal antibodies (aPabs) raised against *Enteromyxum leei* (aPab-Eleei) and *E. scophthalmi* (aPab-Escoph). aPab-Eleei and aPab-Escoph were applied, at 1:1,000 and 1:8,000, respectively, on 5µm paraffin sections of teleost tissues infected by myxosporeans. Shaded lines contain *Enteromyxum* spp.

PARASITE SPECIES	HOST SPECIES	INFECTED TISSUES	ANTISERUM	LABELLING OF THE PARASITE	LABELLING OF THE TISSUE
Ceratomyxa labracis	*Dicentrarchus labrax*	Intestine	aPab-Eleei	-	-
			aPab-Escoph	-	-
Ceratomyxa shasta	*Oncorhynchus mykiss*	Intestine	aPab-Eleei	-	-
			aPab-Escoph	-	-
Enteromyxun leei	*Sparus aurata*	Intestine	aPabEscoph	-	-
Enteromyxun scophthlami	*Psetta maxima*	Intestine	aPab-Eleei	-	BB, GC, RBC
Sinuolinea sp.	*Psetta maxima*	Urinary bladder	aPab-Eleei	-	BB
			aPab-Escoph	-	-
Sphaerospora dicentrarchi	*Dicentrarchus labrax*	Intestine, stomach, liver	aPab-Eleei	valves	E, LP, M
			aPab-Escoph	-	-
Sphaerospora testicularis	*Dicentrarchus labrax*	Testicle	aPab-Eleei	Sporoblasts, polar capsules	-
			aPab-Escoph	-	-
Tetracapsuloides bryosalmonae	*Oncorhynchus mykiss*	Head kidney, muscle	aPab-Eleei	-	-
			aPab-Escoph	-	-
Zschokkella mugilis	*Mugil capito*	Gall bladder	aPab-Eleei	-	-
			aPab-Escoph	-	-

Abbreviations: Brush border (BB), goblet cells (GC), red blood cells (RBC), epithelium (E), lamina propria-submucosa (LP), muscle (M).

4.3 DISCUSSION

The polyclonal antibodies developed in the present work proved to be highly sensitive and specific for the detection of each *Enteromyxum* sp. by immunohistochemistry in formalin fixed tissues. The adsorbed polyclonal antisera immunoreacted with the corresponding proliferative stages (P and S cells), as well as with P cells harboring sporoblasts of each parasite species. Additionally, T cells and spore accompanying cells and valves of *E. leei* were labeled. No staining was observed in spore inner structures (sporoplasm, polar capsules) for none of the *Enteromyxum* species, and valve staining in *E. scophthalmi* was rather weak and inconsistent.

Differences in antibody detection between the diverse life stages have been previously reported for several myxozoans, such as *Ceratomyxa shasta* (Noble) (Bartholomew, Yamamoto, Rohovec and Fryer 1990), *Tetracapsuloides bryosalmonae* (Canning, Tops, Curry, Wood et Okamura) (Adams, Thompson, Morris, Farias and Chen 1995; Marin de Mateo, McGeorge, Morris and Kent 1996) and *Sphaerospora dicentrarchi* (Sitjà-Bobadilla et Álvarez-Pellitero) (Muñoz et al. 1998; Muñoz, Palenzuela, Sitjà-Bobadilla and Álvarez-Pellitero 1999a), and were attributed to stage-specific antigens. In those cases, certain parasite stages predominated in the immunogens and thus the obtained antisera were mainly directed against them. In accordance to this, the current aPab-Eleei detected both, proliferative and sporogonic stages, since the *E. leei* antigenic preparation obtained from GSB contained a mixture of such stages. The enteromyxosis in turbot, by contrast, has a more virulent effect provoking in many cases fish death before spores even develop (Redondo et al. 2002; Sitjà-Bobadilla, Redondo, Macias, Ferreiro, Riaza and Álvarez-Pellitero 2004). Therefore, in the *E. scophthalmi* antigenic preparation, spores were absent though initial sporoblasts might be already present and thus aPab-Escoph detected epitopes of proliferative stages and P cells harboring sporogonic stages, but did not label spores themselves consistently. The strong antigenicity of P cells was in accordance with the intense label of such P cells with sera of *E. scophthalmi*-infectd turbot (Sitjà-Bobadilla et al. 2004). The obtained aPabs did not immunobind to internal components of the spore, which might indicate that the applied freezing-thawing disruption technique was not strong enough to lysate and free neither polar capsules nor sporoplasms when the immunogen was prepared. For the development of future Pabs, more effective lysation techniques should be applied to get a more complete range of antigens. However, the capacity of both current aPabs to label proliferative as well as sporogonic stages will enable the detection of *E. leei* and *E. scophthalmi* at any moment of their life cycle, being a useful tool for diagnosis and antigenic characterization. It should be noted that parasite stages in all tested formalin-fixed tissues, even blood stages contained in tissue vessels, were immunoreactive to the aPab-Escoph. In contrast, *E. scophthalmi* stages in frozen blood smears did not immunoreact. This is probably related to an epitope alteration by the processing techniques, which could either destroy epitopes in frozen unfixed parasites or, contrarily, expose formerly hidden epitopes after formalin fixation.

The adsorption of the raw antisera to non-parasitized fish intestine proved to be a good way of reducing the non-specific reaction with intestinal tissues, eliminating the background completely in immunohistochemistry as previously described (Hamilton and Canning 1988; Belem and Pote 2001). In this way, cross-reactivity of the Pabs with the host tissue was reduced or eliminated and the specificity of the sera further enhanced. This was reflected in the ELISA, by a reduction of the reactivity in adsorbed antisera. The sensitivity of the aPabs in ELISA and in immunohistochemistry, indicated by their endpoints, was found different for both techniques. Strikingly, the intensity of the reaction for the aPab-Escoph was higher than for the aPab-Eleei in immunohistochemistry, while in ELISA it was lower. The lower intensity of detection in ELISA could be the consequence of a lower parasite concentration in the antigenic preparation used for aPab-Escoph titration, compared to the parasite concentration in the *E. leei* antigenic preparation.

No cross-reactivity was observed for aPab-Escoph with other Myxosporea. This serum probably cross-reacted less than the aPab-Eleei due to the less diverse content of parasitic stages in its antigenic preparation. aPab-Eleei did cross-react with both *Sphaerospora* species either with *S. dicentrarchi* valves or with *S. testicularis* (Sitjà-Bobadilla et Álvarez-Pellitero) P cells and polar capsules. The existence of cross-reactions among Myxozoans has been attributed to the share of antigenic epitopes; particularly, carbohydrate moieties are mostly responsible for the common antigenic determinants (Muñoz et al. 1998; Muñoz et al. 1999a; Muñoz, Palenzuela, Álvarez-Pellitero and Sitjà-Bobadilla 1999b).

Our observations reveal the existence of conserved antigenic epitopes in proliferative cells as well as in spores of some Myxosporea. In Muñoz et al. (1998), developmental stages and spores of *S. dicentrachi* apparently shared antigenic epitopes between them and with polar capsules and valves of *S. testicularis* spores, suggesting a similar antigenic composition of these *Sphaerospora* species, which agrees with the present results. Lectin immunohistochemistry revealed a common terminal glycosylation in the *Sphaerospora* polar capsules and valves which contained mannose, glucose and *N*-acetyl-D-galactosamine (Muñoz et al. 1999a), that are also present in proliferative and sporogonic stages of *E. leei* and *E. scophthalmi* (Redondo, Cortadellas, Palenzuela and Álvarez-Pellitero 2008; Redondo and Álvarez-Pellitero 2009). In any case, further studies are required to elucidate the nature of the common epitopes between *E. leei* and *Sphaerospora* spp., which are absent in the congener *E. scophthalmi*.

Other authors have described the existence of cross reactions among parasites. Indeed, monoclonal antibodies (Mabs) raised against *T. bryosalmonae* showed immunoreactivity with several *Sphaerospora* spp. indicating the presence of common antigens (Marin de Mateo et al. 1996), which were further studied and detected by means of DNA probes directed against antigen encoding sequences (Morris, Adams, Feist, McGeorge and Richards 2000; Morris, Adams and Richards 2000). This cross-reacting antigenic protein, located on spore valves or secreted within mucus envelopes of spores was more recently detected in other freshwater myxozoans belonging to the genera *Myxobolus* (Bütschli), *Thelohanellus* (Kudo) and *Sphaerospora* (Thélohan) (Morris, Molnar, Longshaw and Adams 2006). The humoral response to the myxosporeans *Kudoa* sp. (Meglitsch) and *M. aeglefini* (Auerbach) was checked in mice, and low-grade cross-reactivity of the rodent serum with the heterologous parasite antigens was found (De Velasco and Cuellar 2003). This was attributed to similar carbohydrate structures in both phylogenetically distant myxosporeans. For other fish parasites like the scuticocialiate *Philasterides dicentrarchi* (Dragesco, Dragesco, Coste, Gasc, Romestand, Raimond et Bouix), cross-reactivity of turbot sera against different isolates was detected, suggesting recognition of common antigens (Piazzón, Lamas, Castro, Budino, Cabaleiro, Sanmartín and Leiro 2008). Cross-protection of rainbow trout (*Salmo trutta*, L.) against *Ichthyophthirius multifiliis* (Fouquet) following *Tetrahymena* spp. (Furgason) intraperitoneal injection was achieved to some extent, suggesting certain similarities in the surface epitopes (Sigh and Buchmann 2002). In addition, cross-reaction of different Microsporidia with heterologous sera of humans immunized with *Glugea atherinae* (Berrebi) or *Enterocytozoon bieneusi* (Desportes, Le Charpentier, Galian, Bernard, Cochand-Priollet, Lavergne, Ravisse et Modigliani) (Ombrouck, Romestand, Dacosta, Desportes-Livage, Datry, Coste, Bouix and Gentilini 1995) and of mice immunized with *Tetramicra brevifilum* (Matthews et Matthews) or *Pleistophora* sp. (Wales et Wolf) (Leiro, Estévez, Ubeira, Santamarina and Sanmartín 1994) were detected. In any case, cross-reactive antigens are potential targets for multifunctional vaccines against various pathogens.

In conclusion, the Pabs raised against *Enteromyxum* spp. lead to a better understanding of these parasites enabling their in situ localization and the detection of functional antigens. Furthermore, the differences in staining pattern observed in the myxosporean species studied by these Pabs could reflect different evolutionary relationships among them. Nevertheless, further studies are needed to solve the real nature of the epitopes involved in this recognition. Additionally, these Pabs are important tools offering simple methods for the early detection of enteromyxosis and thus can assist in the control of disease outbreaks preventing the parasite's propagation.

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Antigenic characterization of *Enteromyxum leei*

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5.0 ABSTRACT

Enteromyxum leei, an intestinal myxozoan parasite affecting a wide range of fish, was partially purified and the immunogenic composition of its glycoproteins as well as the proteolytic activity of the parasite was studied. Parasite extracts, mainly containing spores, were separated by SDS-PAGE and thereafter, immunoblots were carried out with a polyclonal antiserum (Pab) raised against *E. leei*. Periodic acid/Schiff staining of gels, periodate and proteinase K treated Western blots, and Lectin blots were performed to analyze the terminal carbohydrate composition of the parasite's antigens. Additionally, the cross-reaction of the parasite extracts with a Pab raised against the polar filament of the myxozoan *Myxobolus pendula* was studied. Both Pabs detected proteic epitopes on antigenic proteins and glycoproteins of *E. leei*, ranging between 15 and 280 kDa. Particularly, two glycoproteic bands (15 and 165 kDa), immunoreactive to both Pabs and with glucose and mannose moieties, could correspond to common antigens shared among myxozoans. The 165 kDa band presented also galactose, *N*-acetyl-galactosamine and *N*-acetyl-glucosamine pointing to its possible origin on chitin-built spore valves and to its possible involvement in host-parasite interactions. The 15 kDa glycoproteic antigen would match for its molecular weight with minicollagen, a cnidarian-specific protein of nematocysts with a myxozoan homologue. Several proteases with apparent molecular weights ranging between 43 and 245 kDa were found in zymographies of *E. leei* extracts, which may have a potential role in the parasite's pathogenesis. This is a useful approach for further studies to detect targets for antiparasitic therapy.

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KEY WORDS:
Western blot · Polyclonal antibody · Glycoprotein · Lectin · Protease · Parasite

5.1 INTRODUCTION

Intensively cultured fish are susceptible to parasitic infections, a primary concern for the aquaculture sector (Mennerat et al. 2010). Among fish parasites, the members of the myxozoan genus *Enteromyxum* infect intestines, mainly of marine fish, causing severe enteritis that results in weight loss, poor conversion rates, delayed growth and even death (Rigos & Katharios 2010, Sitjà-Bobadilla & Palenzuela 2012). *Enteromyxum leei* affects, among a wide range of hosts (Sitjà-Bobadilla & Palenzuela 2012), gilthead sea bream (GSB) (*Sparus aurata*), the most important marine finfish in Mediterranean aquaculture (APROMAR 2012). *E. leei* provokes in this sparid a chronic intestinal infection with epithelial destruction and intense inflammatory reaction leading to emaciation, anemia and mortalities (Fleurance et al. 2008, Sitjà-Bobadilla et al. 2008, Cuadrado 2009, Davey et al. 2011, Estensoro et al. 2011).

Little is known about the antigenic composition of this parasite, a key aspect to understand parasite survival and infectivity as well as for the detection of potential targets for the development of chemotherapeutants and vaccines since no efficacious treatment is available against enteromyxoses (Bermúdez et al. 2006, Golomazou et al. 2006, Yokoyama & Shirakashi 2007) and in general for myxosporoses. Previous lectin histochemistry studies have shown that the different *E. leei* stages have different surface-associated carbohydrate moieties (Redondo & Álvarez-Pellitero 2009). Such terminal sugar residues are believed to play a paramount role in host-parasite interactions and may form a protective sheath for the parasite that contributes to evade host recognition (Hicks et al. 2000, Theodoropoulos et al. 2001). Therefore, some parasites, such as *Trypanosoma cruzi* (Buscaglia et al. 2006), carry out an antigen variation strategy through modification of *N*- and *O*-glycosides of glycoepitopes in their surface glycoproteins (Gagneux & Varki 1999, Knaus & El-Matbouli 2005). In fish, as in all vertebrates, the lectin pathway of the complement system is an ancient first line mechanism of defense of the innate immune system, which relies on recognition of pathogen-associated glycan epitopes (Sunyer & Lambris 1998, Nakao et al. 2006, Kania et al. 2010). In previous studies, the glycoproteins of some piscine parasites have been studied by lectin blotting (Feng & Woo 1998b, Kim et al. 1999, Muñoz et al. 2000a, Knaus & El-Matbouli 2005).

Another approach to study the antigenicity of myxozoans is the use of polyclonal antibodies (Pabs) (Bartholomew et al. 1989, Saulnier & deKinkelin 1996, Muñoz et al. 1999b, Muñoz et al. 2000a, Lu et al. 2002, Knaus & El-Matbouli 2005, Zhang et al. 2010). In the current study, the antigenic glycoproteins of *E. leei* were analyzed using two Pabs; one raised against *E. leei* and another raised against the polar filament of *Myxobolus pendula* (Ringuette et al. 2011). The latter was chosen to check its possible cross-reaction with *E. leei* antigens and for its proven capacity to detect common myxosporean and cnidarian antigens.

Parasitic invasion mechanisms imply penetration and colonization of host tissues. Proteolytic enzymes generated by parasites are often involved in such processes, as studied for the myxozoans *Kudoa* sp. (Martone et al. 1999, Funk et al. 2008), *M. cerebralis* (Kelley et al. 2004, Doerfler & El-Matbouli 2007) and *Sphaerospora dicentrarchi* (Muñoz et al. 2000a), as well as for other piscine parasites (Zuo & Woo 1998, Parama et al. 2004, Piazzón et al. 2012). There is no current knowledge about the mechanisms that *E. leei* employs to disrupt cell junctions between enterocytes, to penetrate and invade the intestinal epithelium and to migrate along the digestive tract. Parasite-derived proteinases may play an important role in pathogenesis and lesion formation as well as in evasion of the host immune response in this enteromyxosis, and thus, be the target for antiparasitic chemotherapy (McKerrow et al. 1993).

The antigenic characterization of *E. leei* is an issue of great importance with scarce or none available information. To broaden our knowledge on the antigenicity of this myxosporean, the reactivity of *E. leei* antigens was probed with a set of lectins to determine its terminal carbohydrate composition, and with two different antibodies. Additionally, the proteolytic activity of *E. leei* antigen preparations was determined. Such information may help to develop further studies aiming to select parasite antigens with immunopreventive potential against enteromyxosis.

5.2 MATERIALS AND METHODS

5.2.1 Fish and parasite extracts

Parasite free and clinically healthy gilthead sea bream (GSB) (*Sparus aurata*) from a commercial fish farm were kept in 5 µm-filtered and UV irradiated sea water at temperature always above 18 °C. Some fish were used as control (C) and other as recipient fish (R) for *E. leei* experimental infections (see Sitjà-Bobadilla et al. (2007), Estensoro et al. (2010)). All efforts were made to minimize suffering of the fish used for the experiments in accordance with national (Royal Decree RD1201/2005, for the protection of animals used in scientific experiments) and the current European Union legislation on handling experimental animals.

C and R fish were starved for two days, euthanized by overexposure to MS-222 (Sigma, St. Louis, MO, USA) and bled to avoid sample contamination by blood cells. Infective status of R intestines was checked by light microscopy observation of fresh smears. Intestines were opened lengthwise under sterile conditions and the mucosa slightly scraped with a scalpel. The intestinal scrapings of 7 C and 7 intensively infected R fish were pooled separately, homogenized in sterile HBSS (Gibco-Life technologies, Alcobendas, Madrid) including antibiotics and antimycotics (Gibco-Life technologies) with a syringe and the remaining cell aggregations and debris were retained by a 40 µm cell strainer (BD, Franklin Lakes, NJ, USA). The parasite/cell suspensions were then centrifuged at 2,200 g for 10 min at 4 °C and the pellets resuspended in lysis buffer (Tris-HCl 0.1 M, MgCl2 0.05 M, 1% Triton X-100, sucrose 0.3 M). Suspensions were then centrifuged at 1,100 g for 5 min at 4 °C and the pellet resuspended in ether:HBSS (1:2) before a subsequent centrifugation at 2,500 g for 5 min at 4 °C. Antigen pellets from R pools contained mainly spores and unlysed disporoblasts together with some small sized cellular debris, and those from C pools contained intestinal epithelium cellular debris. They were collected separately and washed twice in cold HBSS (2,500 g, 5 min, 4 °C).

Parasites contained in antigen preparations were counted with a haemocytometer. Protease inhibitor cocktail (Roche Diagnostics, Sant Cugat del Vallès, Barcelona, Spain) was added to the protein extracts. After determining their total protein content *via* Bradford staining (Bio-Rad, Hercules, CA, USA), such extracts were aliquoted and stored at -20 °C until used. The parasite extracts obtained from R fish contained also host cells since complete parasite purification was not achieved. Therefore, all subsequent analyses aimed to detect parasite bands present in R intestinal extracts, that were absent in C intestinal extracts.

5.2.2 SDS-PAGE

C and R antigen preparations were boiled during 5 min before an equal volume of reducing SDS-PAGE sample buffer (0.62 Tris-HCl, 2 % SDS, 10 % glycerol, 0.005 % bromophenol blue, 0.1 M DTT) was added. They were boiled again for 5 min, centrifuged (30 min, 13.000 g, 4° C) and the supernatants containing the soluble protein fraction were recovered and stored at -20° C until used. For the preparation of native antigens, extracts were not denatured by boiling and non-reducing sample buffer lacking DTT was used. Thereafter, antigen preparations were separated by SDS-PAGE either on 12 % or 5 % polyacrylamide gels at 180 V for approximately 50 min. Gels were used either to visualize proteic antigen bands (Coomassie staining), to visualize glycoproteic antigen bands (Periodic acid/Schiff (PAS) staining) or used for further blotting. In all gels, prestained broad range molecular weight (MW) standards (Bio-Rad) (7-209 kDa) were loaded.

For protein staining, 0.25 % Coomassie brilliant blue R-250 (IBF Pharmindustrie Rèactifs, Ville-la-Garenne, France) in 40 % methanol, 10 % acetic acid was employed and thereafter background staining was washed with 40 % methanol, 10 % acetic acid. For PAS staining, gels were fixed in 25 % isopropyl alcohol, 10 % acetic acid overnight; then in 10 % isopropyl alcohol, 10 % acetic acid for 2 hours; 0.5 % periodic acid for 2 hours; 0.5 % sodium arsenate, 5 % acetic acid for 40 min; 0.1 % sodium arsenate, 5 % acetic acid for 20 min, twice; and acetic acid for 20 min. Gels were then stained with Schiff's reagent overnight and washed with 0.1 % sodium metabisulfite in 0.01 N HCl several times until the rinse solution failed to turn pink.

5.2.3 Polyclonal antibodies

The production and characterization of the PabEleei and its adsorption with normal gilthead sea bream intestinal scrapings to avoid background noise due to host cell detection can be found in a previous work

(Estensoro et al. 2013). The adsorbed PabEleei (aPabEleei) was used in Western blots for the current antigenic study.

The Pab against the polar filament of *M. pendula* (PabMPPF) was checked for cross-reactivity with *E. leei* on paraffin histological sections of GSB infected intestines, prior to blotting. Routine immunohistochemical procedure was applied as described in Estensoro et al. (2012) and sections eventually counterstained with Gill's haematoxylin, dehydrated and mounted in DPX (di-N-butyl-phthalate in xylene). PabMPPF was obtained and kindly provided by Ringuette et al. (2011) and is directed against an evolutionary conserved glycoepitope of polar filaments detected in three *Myxobolus* species as well as in some cnidarian nematocysts.

5.2.4 Western blots

The antigen proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (0.45 µm pore size) (Bio-Rad) at 250 mA during 1 hour. Membranes were blocked with 5 % skimmed milk (Merk, Darmstadt, Germany) in TBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.2) overnight at 4 °C and washed 10 min with TTBS (0.05% Tween 20 in TBS). All washing procedures consisted of successive 10 min immersions in TTBS and all washing and incubation steps were performed at room temperature (RT) with gentle shaking. After washing, membranes were incubated for 2 hours either with Pabs or with biotinylated lectins and washed again four times. Detailed information about the used Pabs and lectins can be found in Table 1. Secondary incubations for Western blots were performed with goat anti-rabbit horseradish peroxidase-conjugated antibody (1:2,000) (Sigma) and for lectin blots with avidin-biotin-peroxidase complex (VECTOR Laboratories, Burlingame, CA, USA) during further 2 hours. Blots were washed four times and finally rinsed in TBS before bound peroxidase was visualized by addition of 3,3'-diaminobenzidine tetrahydrochloride chromogen (DAB) (Sigma) for 2 min. The reaction was stopped with deionised water and blots were allowed to dry. Reactive antigen bands in gels and blots were visualized with a Multi-Image light cabinet

Table 1. Detector molecules used in Western blots.

DETECTOR MOLECULE (ANTIBODY OR LECTIN)	ACRONYM	BINDING SPECIFICITY	WORK DILUTION	SOURCE
Rabbit polyclonal Ab anti-*E. leei*	aPabEleei[a]	unknown	1:500	IATS
Rabbit polyclonal Ab anti-polar filament	PabMPPF[b]	non-collagenous glycoprotein of *Myxobolus pendula*	1:30	University of Toronto
Canavalia ensiformis-Agglutinin	ConA	Manα-1>Glcα-1>GlcAcα-1	2 µg/ml	Sigma
Bandeiraea simplicifolia-Lectin	BSL I	D-Gal>D-GalNac	5 µg/ml	Sigma
Glycine max-Agglutinin	SBA	Terminal α,βGalNac>α,βGal	5 µg/ml	Vector Lab.
Triticum vulgaris-Agglutinin	WGA	GlcNAc (β1,4GlcNAc)1-2>β1,4GlcNAc>NeuNAc	10 µg/ml	Vector Lab.
Sambucus nigra-Agglutinin	SNA	NeuAc-α2,6Gal=NeuAcα2,6GalNAc	20 µg/ml	Vector Lab.
Ulex europaeus-Agglutinin	UEA	L-Fucα1,2Galβ1,4	20 µg/ml	Sigma

Abbreviations: antibody (Ab); N-acetylgalactosamine (GalNac); galactose (Gal); D-glucose (Glc); N-acetylglucosamine (GlcNAc); mannose (Man); N-acetylneuraminic acid (NeuNAc); Fucose (Fuc).

[a] The anti-*Enteromyxum leei* polyclonal antibody was obtained and tittered by the Fish Pathology group, IATS-CSIC, Spain. (Estensoro et al. 2013).

[b] The anti-*Myxobolus pendula* polar filament polyclonal antibody (PabMPPF) was produced by the Department of Cell and Systems Biology, University of Toronto, Canada. (Ringuette et al. 2011).

(Alpha Innotech Corporation, CA, USA) and digitally analyzed with Quantity-One Quantitation Software (Bio-Rad). Band sizes were inferred from the linear range of the migration of MW standards.

5.2.5 Periodate oxidation

Periodate oxidation was carried out as described by Woodward et al. (1985) to demonstrate the carbohydrate nature of the epitopes recognized by the Pabs. Following the blotting, membranes were rinsed with 0.05 M sodium acetate buffer (pH 4.5) and incubated for 1 hour in the dark at RT with graded periodate dilutions (0.1 mM; 1 mM; 10 mM) in sodium acetate buffer. Blots were then rinsed with sodium acetate buffer, incubated with 0.05 M sodium borohydride in phosphate buffer saline for 30 min at RT and washed 5 min with TTBS and 5 min with TBS. Thereafter, membranes were blocked, exposed to the primary and secondary antibodies, exposed to DAB and finally dried and visualized as described in the previous section.

5.2.6 Proteinase K digestion

Blots were digested with proteinase K to confirm the polypeptide nature of the antigen epitopes detected by the Pabs, as described by Feng & Woo (1998a). Briefly, blotted membrane strips were incubated for 1 hour at 37 °C with proteinase K (0.2 µg/ml; 2 µg/ml; 20 µg/ml) in Tris-HCl (50 mM, pH 8) and then washed in TTBS and 12 % trichloracetic acid to inactivate the proteinase. Thereafter, membranes were blocked, incubated with the primary and secondary antibodies, exposed to DAB and finally dried and visualized as described in the previous section.

5.2.7 Zymography

The proteolytic activity of the antigen preparations was tested by separating them through SDS-PAGE in 10 % polyacrilamide gels co-polymerized with 0.2 % gelatine. Cell extracts employed therefore were native and not reduced, thus, not boiled and did not contain DTT in the SDS-PAGE sample buffer. After electrophoresis, gels were washed in 2.5 % Triton X-100 (Sigma) for 1 hour to remove SDS and restore proteolytic activity, rinsed with 0.1 M phosphate buffer (pH 5.5) and then incubated with this same buffer for 12 hours at 37 °C to allow proteolysis. Finally, gels were stained with 0.25 % Coomassie brilliant blue R-250 and visualized with a Multi-Image light cabinet (Alpha Innotech Corporation) as previously described. Proteases appeared as clear bands on the blue background where digestion of co-polymerized gelatine occurred.

5.3 RESULTS

5.3.1 SDS-PAGE: Coomassie & PAS

The parasite content of R extracts ranged between 6.1×10^6 and 4.1×10^7 parasite stages/ml and their total protein content between 297 and 904 mg/ml. In Coomassie brilliant blue R-250 stained gels (Fig. 1 A-D), denatured and reduced parasite extracts showed R proteic bands of MWs ranging between 10 kDa and 49 kDa. The detected R antigenic bands were three intense bands of low MW, 10 kDa, 15 kDa and 17 kDa, and three weakly stained bands of 30 kDa, 31 kDa and 42 kDa, respectively. Except the 42 kDa, all these R bands were absent in C extracts. Native R extracts presented a similar band pattern with a slightly lower relative mobility (native proteic bands: 11 kDa, 16 kDa, 18.5 kDa, 31 kDa, 33 kDa, 49 kDa).

In PAS stained gels (Fig. 1 E-F), C extracts did not show PAS positive bands, whereas in R parasite extracts a single high weight glycoprotein band of 193 kDa was weakly stained. The MW of this glycoproteic band was confirmed in 5 % polyacrilamide gels.

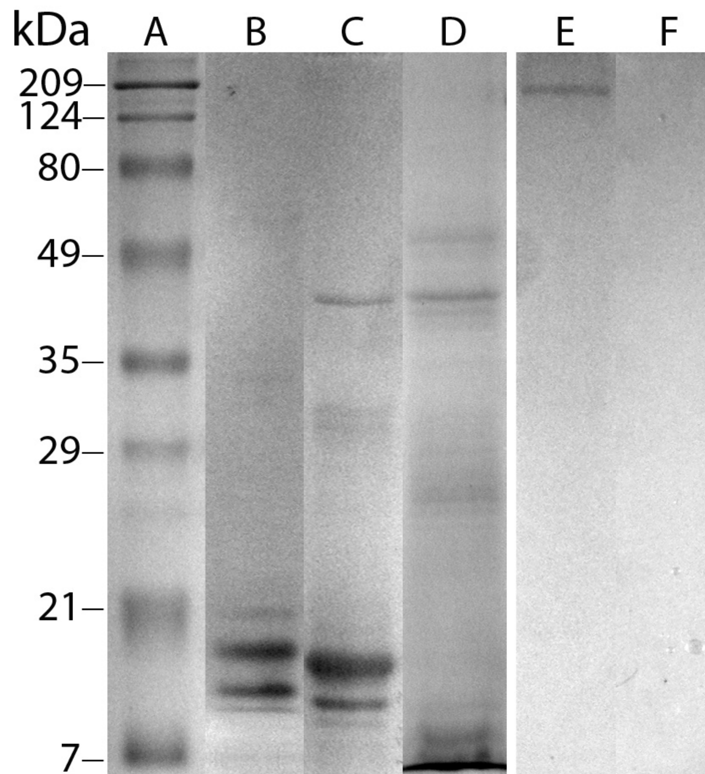

Figure 1. SDS-PAGE soluble protein profile of parasite extracts of *Enteromyxum leei* (lanes **B**, **C**, **E**) and host tissue extracts (lanes **D**, **F**) stained with R-250 Coomassie brilliant blue (lanes **A**-**D**) and PAS (lanes **E**-**F**): Lane **A**, molecular weight standards; lane **B**, native *E. leei* extract; lanes **C** and **E**, denatured and reduced *E. leei* extract; lanes **D** and **F**, denatured and reduced host tissue extracts.

5.3.2 Western blots

The immunoblots with aPabEleei showed several immunoreactive bands in R antigen preparations (Fig. 2 B). In reduced and denatured R extracts, aPabEleei detected reactive bands with MWs of 15 kDa, 23 kDa, 42 kDa, 67 kDa and 165 kDa together with an intense smear ranging from 24 to 37 kDa. In native R extracts, aPabEleei detected only 30 kDa and 43 kDa reactive bands as well as a weakly stained 33 kDa reactive band. In C antigens, this antibody detected only a 29 kDa and a 42 kDa reactive bands.

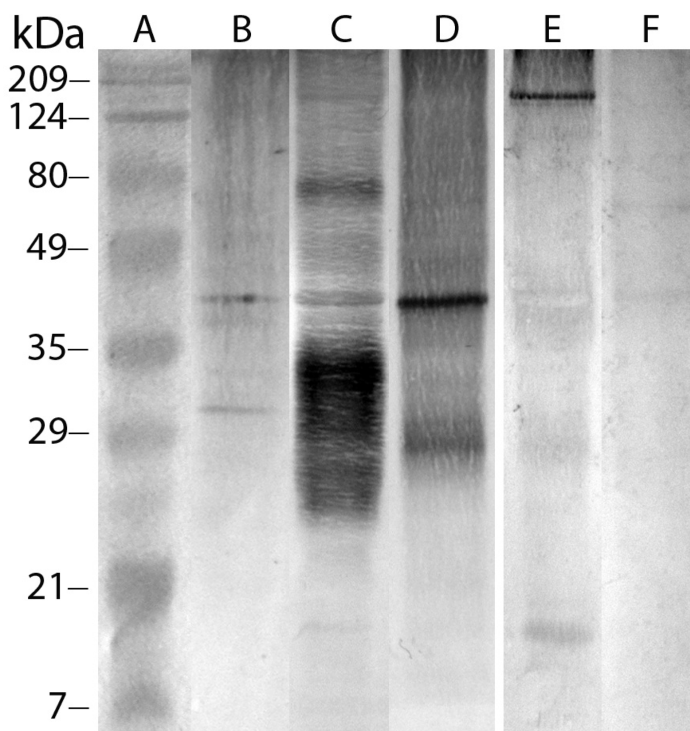

Figure 2. Western blot detection of *Enteromyxum leei* (lanes B, C, E) and host tissue (lanes D, F) extracts with the polyclonal antibody raised against *E. leei* (lanes A-D) and by the polyclonal antibody against polar filament epitopes of *Myxobolus pendula* (lanes E, F). Lane A is the molecular weight standard.

The immunoblots with PabMPPF showed reactive antigens between 15 and >209 kDa in R reduced and denatured extracts (Fig. 2 E-F). Reactive bands had MWs of 15 kDa, 29 kDa, 42 kDa, 154 kDa and 166 kDa and a reactive smear of >209 kDa (mean MW 280 kDa) was also detected. Only a 42 kDa band was detected in C extracts. Using immunohistochemistry, this PabMPPF stained clearly primary and secondary cells of proliferative parasite stages and polar filaments, polar capsules, spore valves and accompanying cells of sporogonic stages of *E. leei* (Fig. 3).

No label was detected in negative control slides omitting primary antibody, secondary antibody and avidin-biotin-peroxidase complex, respectively, or in non-parasitized tissues. For comparative purposes, an image of the staining obtained with aPabEleei is also provided (Fig. 3).

Terminal sugar moieties of glycoproteic bands were determined with six biotinilated plant lectins, which detected a broad array of reactive bands in lectin blots, some of them exclusive of R extracts. The lectin *Canavalia ensiformis*-Agglutinin (ConA) detected mannose/glucose (Man/Glc) residues in glycoproteic bands

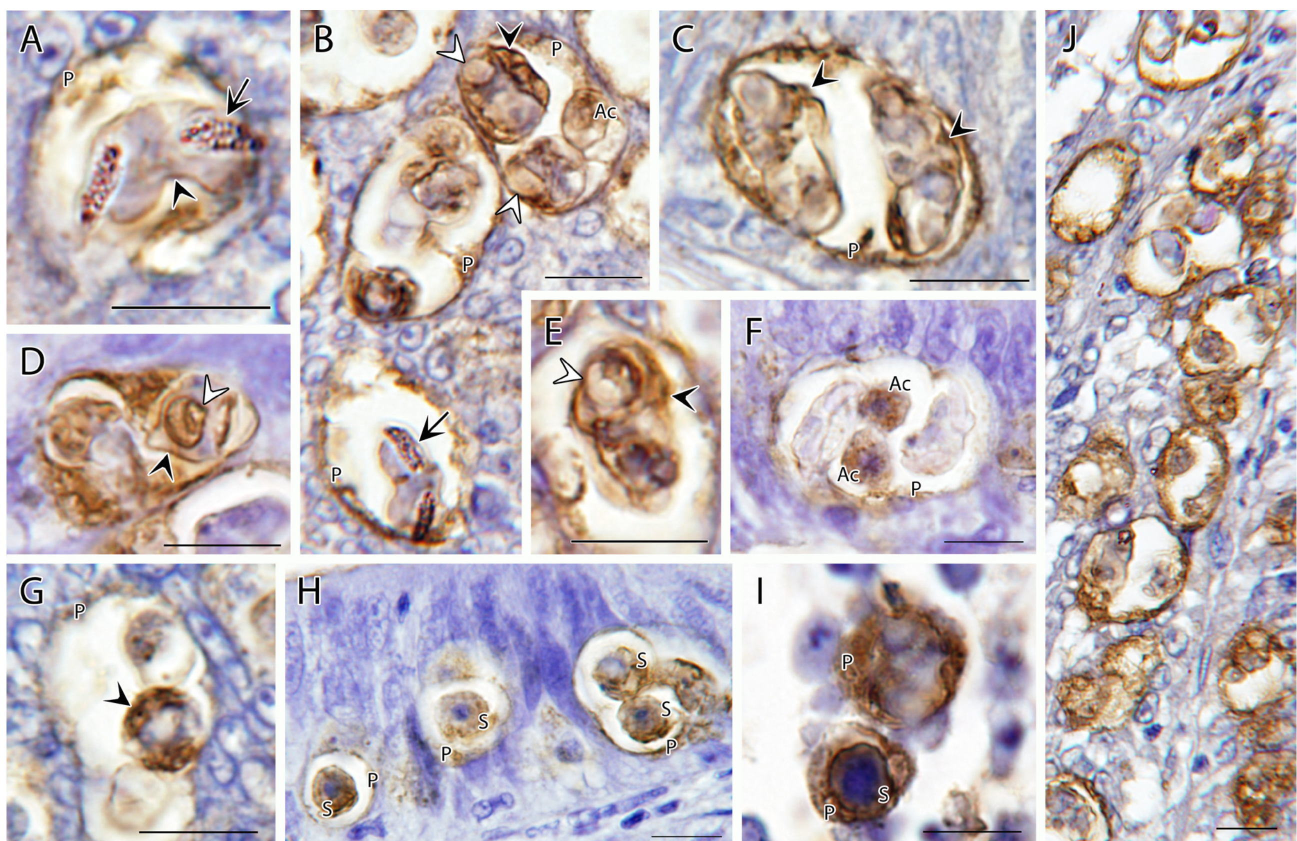

Figure 3. Immunohistochemical labelling of *Enteromyxum leei* with the polyclonal antibody (Pab) against polar filament epitopes of *Myxobolus pendula* (**A-I**) and with the Pab against *E. leei* (**J**) in infected intestinal gilthead sea bream paraffin sections counterstained with heamatoxylin. Immunoreactive parasite components are indicated by arrows (polar filament), black arrowheads (valves and valvogenic cells), white arrowheads (polar capsules), P (primary cells), S (secondary cells) and Ac (accompanying cells). (**A**) Front view of a spore in a disporoblast (DEB). (**B**) Differently orientated DEB with visible valves, polar filaments, polar capsules, P and Ac. (**C**) DEB with lateral view of the two spores with immunoreactive valves and P. (**D**) Polar capsule and valve of a spore in a DEB. Note that the typical protrusion of the spore valve of this species is visible. (**E**) Cross section of a polar capsule in a spore within a DEB. (**F**) Two Ac in a DEB. (**G**) Intensively stained valvogenic cell/s of an immature spore within a DEB. (**H**) Intraepithelial proliferative stages with labelled P and S. (**I**) P and S of free proliferative stages in the intestinal lumen after epithelial desquamation. (**J**) Parasite labelling with the Pab against *E. leei*. Scale bars = 10 µm.

of 15 kDa, 27 kDa, 31 kDa, 33 kDa, 34 kDa, 36 kda, 39 kDa, 46 kDa, 49 kDa, 165 kDa and 311 kDa, which were all absent in C extracts (Fig. 4 B, C). *Bandeiraea simplicifolia*-Lectin (BSL I) detected galactose/*N*-acetyl-galactosamine (Gal/GalNac) moieties in two exclusive bands of R extracts with MWs of 148 kDa and 165 kDa (Fig. 4 D, E). *Glycine max*-Agglutinin (SBA) reacted with *N*-acetyl-galactosamine/galactose (GalNac/Gal) residues in two high MW bands only present in R extracts, 164 kDa and 271 kDa (Fig. 4 F, G). With *Triticum vulgaris*-Agglutinin (WGA), three glycoprotein bands of 158 kDa, 165 kDa and 239 kDa containing *N*-acetyl-glucosamine/neuraminic acid (GlcNac/NeuNac) were visualized exclusively in R extracts (Fig. 4 H, I). The 108 kDa reactive band detected with *Sambucus nigra*-Agglutinin (SNA) (specific for NeuAc) was found in both R and C extracts (Fig. 4 J, K), and no clear results could be obtained with the *Ulex europaeus*-Agglutinin (UEA) lectin (specific for fucose) due to the high background noise.

The optic density profiles obtained for reduced and denatured R extracts blotted with aPabEleei, PabMPPF and ConA are represented in the Supplementary Figure 1.

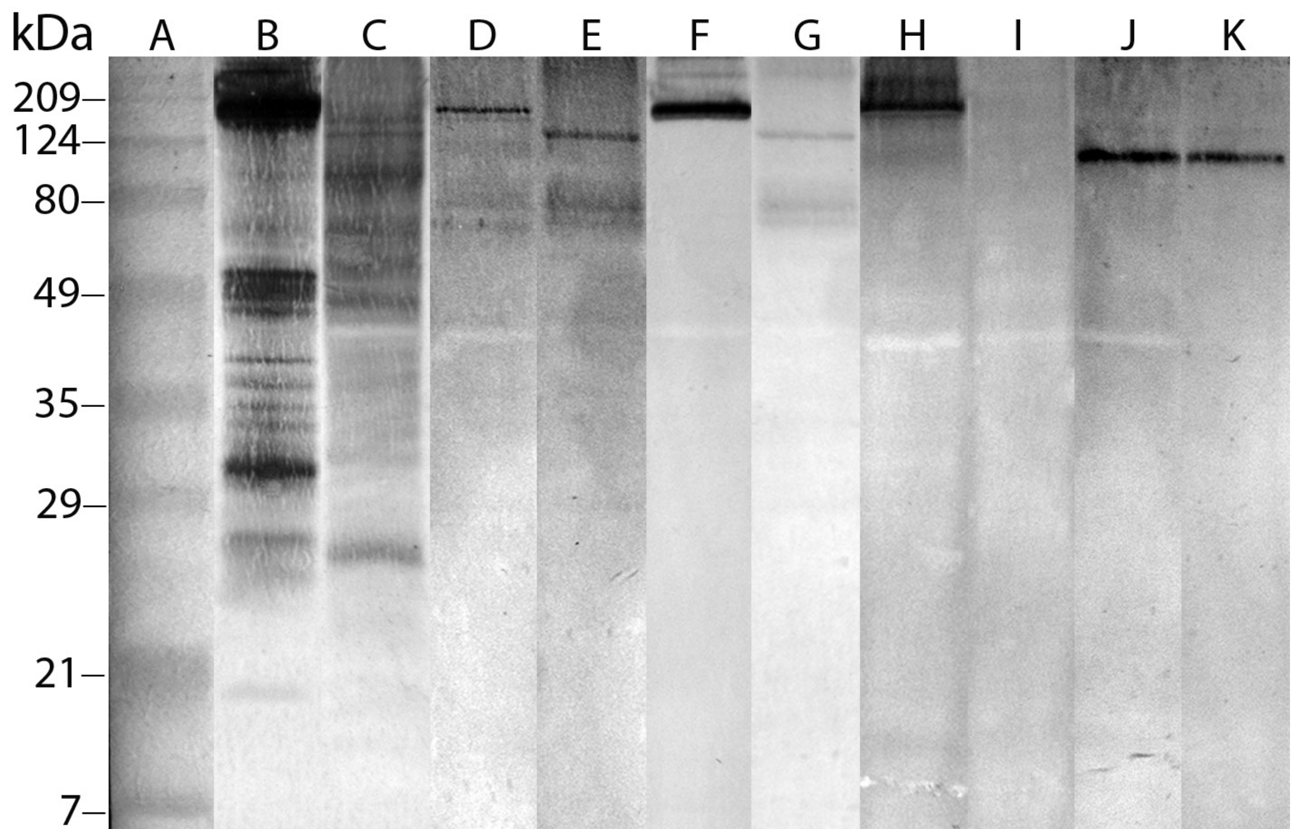

Figure 4. Lectin blot detection of *Enteromyxum leei* (lanes **B, D, F, H, J**) and host tissue (lanes **C, E, G, I, K**) extracts with the biotinylated plant lectins ConA (lanes **B, C**), BSL I (**D**, **E**), SBA (lanes **F, G**), WGA (lanes **H, I**) and SNA (lanes **J, K**). Lane **A** is the molecular weight standard.

5.3.3 Periodate oxidation

Following the treatment with sodium periodate, no reduction of the number of reactive bands recognized by aPabEleei was observed (Fig. 5 B-E). Similarly, the immunoreactive bands detected by the PabMPPF did not decrease after the sodium periodate treatment (Fig. 5 F-I). Band detection with both Pabs was maintained at the different periodate concentrations used.

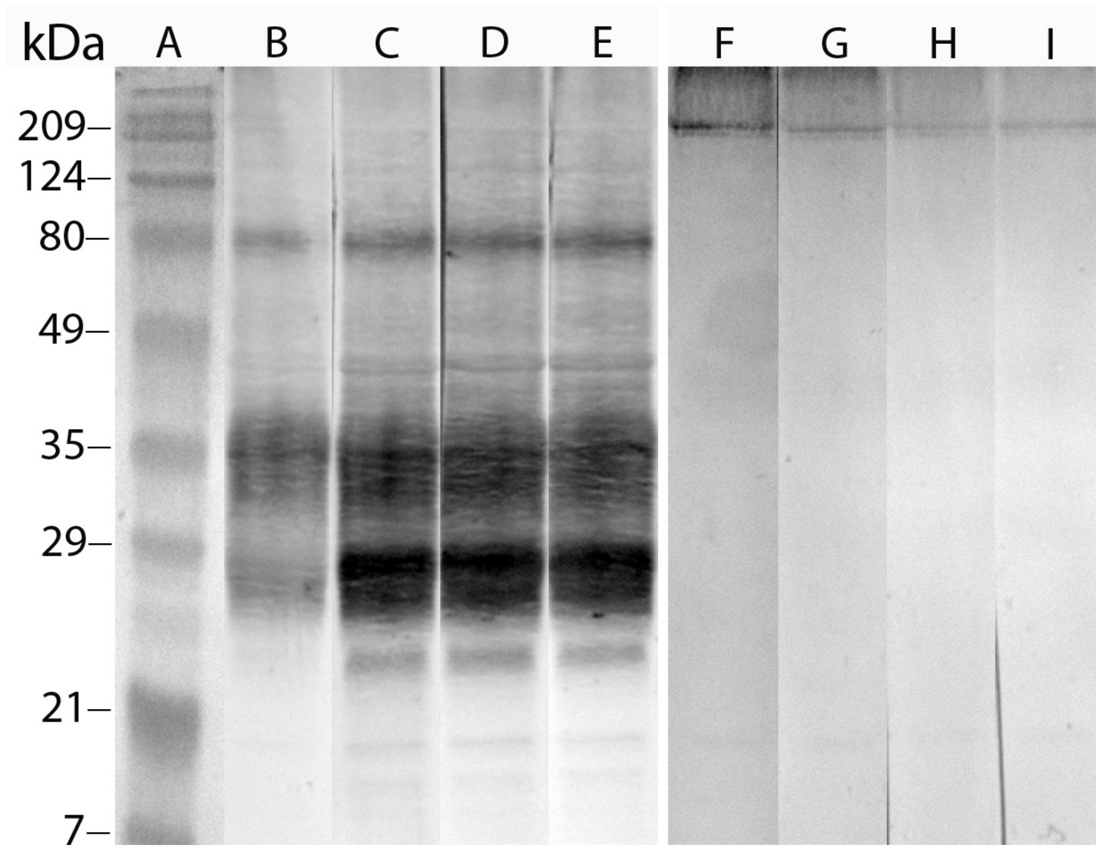

Figure 5. Western blot reactivity of *Enteromyxum leei* parasite extract with the polyclonal antibody against *E. leei* (lanes B-E) and with the polyclonal antibody against *Myxobolus pendula* polar filament (lanes **F**-I) after sodium periodate oxidation (lanes **B** and **F** 0 mM; lanes **C** and **G** 0.1 mM; lanes **D** and **H** 1 mM; lanes **E** and **I** 10 mM). Lane **A** is the molecular weight standard.

5.3.4 Proteinase K digestion

The reactivity of R proteins with aPabEleei in Western blots was reduced after proteinase K digestion (Fig. 6 B-E). A progressive reduction of the antibody's label was observed at increasing proteinase K concentrations and the label almost disappeared after incubation with proteinase K at 20 µg/ml. Likewise, the intensity of the PabMPPF immunoreactive bands was progressively reduced after incubation with increasing concentrations of proteinase K, eventually disappearing at 20 µg/ml.

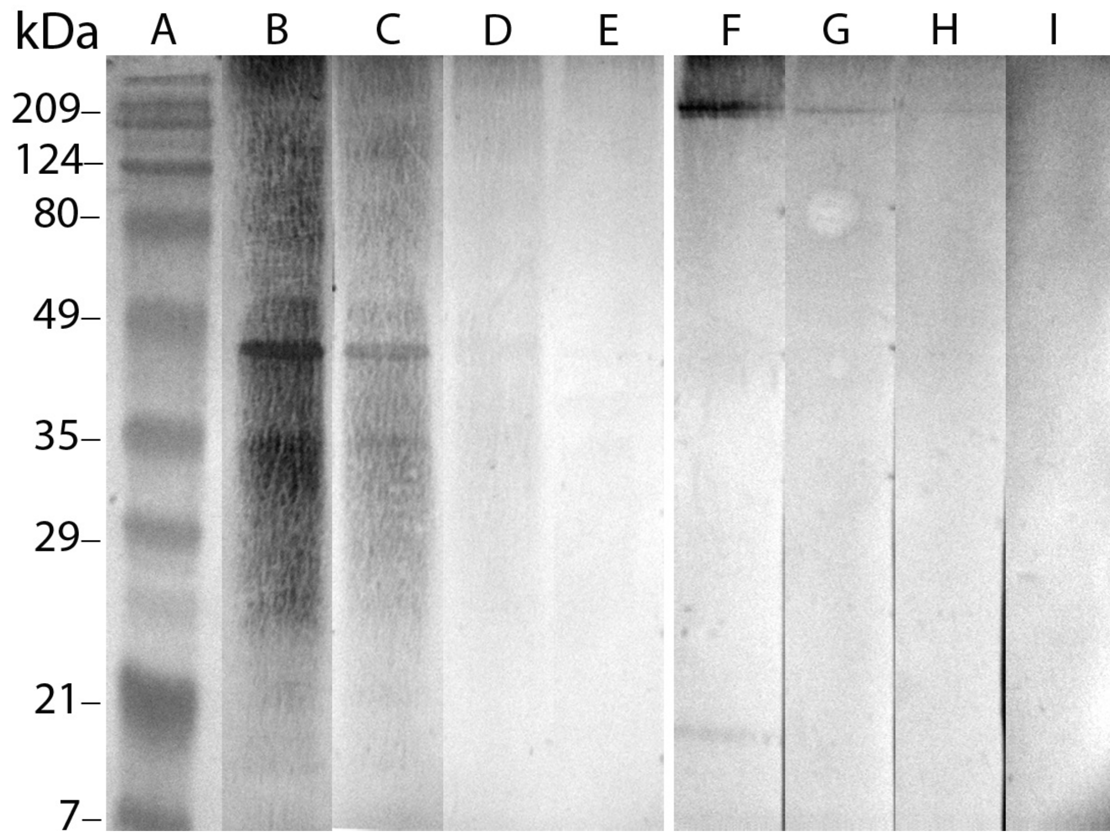

Figure 6. Western blot reactivity of *Enteromyxum leei* parasite extract with the polyclonal antibody against *E. leei* (lanes **B**-**E**) and with the polyclonal antibody against *Myxobolus pendula* polar filament (lanes **F-I**) after proteinase K digestion (lanes **B** and **F** 0 mM; lanes **C** and **G** 0.2 mM; lanes **D** and **H** 2 mM; lanes **E** and **I** 20 mM). Lane **A** is the molecular weight standard.

5.3.5 Zymography

The zymography detected gelatinolytic proteases with a broad MW range, from 27 kDa up to >209 kDa, for C and R extracts (Fig. 7). Accurate assessment of proteinase molecular mass through zymography is, however, not possible since migration is affected by gelatine inclusion in the gel, and moreover, native extracts neither boiled nor reduced were used (Hummel et al. 1996). In any case, proteolytic bands only present in R extracts had apparent MWs of 43 kDa, 49 kDa, 113 kDa and 245 kDa, together with a smear of a mean MW of 174 kDa and two weak bands of 58 kDa and 66 kDa (Fig. 7 B).

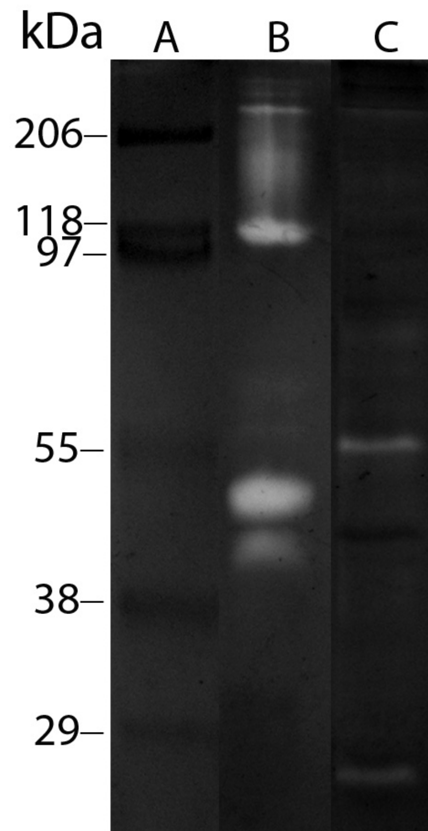

Figure 7. Protease zymography of *Enteromyxum leei* (lane B) and host tissue (lane C) extracts using gelatine non-reducing SDS-PAGE. Lane A is the molecular weight standard.

5.4 DISCUSSION

The antigenic characterization of *E. leei*, a parasite with economic impact on the aquaculture industry, has been a postponed issue due to its complex purification. In the current study, *E. leei* antigens were obtained and analyzed by several immunological and biochemical techniques leading to their identification and partial characterization.

Large amounts of spores together with some disporous sporoblasts were contained in the R parasite extracts yielding concentrations of over $6x10^6$ parasite stages per ml. Spores constitute a large proportion of the parasite stages found in well established *E. leei* infections in gilthead sea bream, either free or as part of sporoblasts. Nevertheless, the role of *E. leei* spores in the life cycle of the parasite and in the infectivity to the fish host is uncertain (Diamant et al. 2006). *E. leei* spores were demonstrated to be immunogenic in rabbit since the aPabEleei specifically detected surface epitopes on spore valves by immunohistochemistry (Estensoro et al. 2013). The immunogenicity of myxospores in organisms different than the corresponding hosts (mice and rabbits) has been already observed in other studies (Clouthier et al. 1997, Chase et al. 2001, Chase et al. 2003). Furthermore, the role of myxospores in resistance development in fish has been suggested. In the case of *M. cerebralis*, common antigens for actinospores and myxospores were discovered and the latter generated an antibody response in fish (Morris et al. 2004). In any case, the presence of sporoblasts in the parasite extracts provided antigens from primary and secondary accompanying cells, both of importance for the parasite's pathogenicity because they are infective for fish (Cuadrado et al. 2008). Obtaining a pure *E. leei* extract was not possible due to its localization inside the intestinal epithelium and its adherence to host enterocytes, but most host derived cells were eliminated and highly enriched parasite extracts were achieved. In all assays, host background in parasite extracts was identified by comparing with parasite free C intestinal extracts. Indeed, a 42 kDa band apparently of piscine origin was detected in all gels and Western blots of R extracts. However, it cannot be discarded that parasite antigens could mimic host antigens to evade immune attack (Bartholomew et al. 1989, Lu et al. 2002, Villavedra et al. 2007).

SDS-PAGE, lectin and Western blotting have proved to be useful techniques for the visualization and characterization of antigen proteins and glycoconjugates of *E. leei.* Protein profile in Coomassie brilliant blue stained gels revealed six low MW antigenic bands (10-49 kDa) of parasite origin which differed slightly in their relative migration when separated under native or under reducing denaturalized conditions. Such variation in the speed of migration suggests a non-linear polypeptide structure of the detected antigen proteins, which hold disulphide bonds (Feng & Woo 1998a). Thus, after the reducing and heating treatment, the unfolded polypeptide presented a higher relative migration.

Further proteic/glycoproteic bands, which were not visualized with Coomassie brilliant blue staining, were detected by Western blotting. Numerous examples exist in literature, in which antibodies or lectins detect additional proteic or glycoproteic bands in blotted membranes, previously undetected in the Coomassie brilliant blue stained gels (Schumacher & Krause 1995, Heimann et al. 1997, Newlands et al. 1999, Muñoz et al. 2000a, Tanaka et al. 2007). The reason is the limited sensitivity of the Coomassie staining to detect trace proteins in antigen preparations, in contrast with the high sensitivity of antibodies and lectins. aPabEleei detected five different *E. leei* antigens in reduced and denatured cell extracts, all of them with proteic antigen determinants since they were not affected by the periodate oxidation, but by proteinase K digestion. The detection of fewer bands in native extracts suggests that aPabEleei does not detect conformational epitopes, but rather unfolded polypeptide epitopes, some of them only exposed after reducing and denaturalizing. Though aPabEleei showed specificity for proteic epitopes some of these bands corresponded to glycosylated proteins also detected by lectins (Table 2) (Supplementary Figure 1). This was the case of the 34 kDa band positive for Man/Glc sugar moieties (ConA), which coincided with the reactive smear to aPabEleei. Intense smears in Western blotting are characteristic of heavily glycosylated molecules (Chase et al. 2001, Villavedra et al. 2007), in accordance with the observed aPabEleei smear coinciding with a ConA reactive band. The 15 kDa protein was detected by both Pabs and was also positive for Man/Glc. At last, the 165 kDa (± 1 kDa) aPabEleei-positive proteic band immunoreacted with ConA, BSL I, SBA and WGA indicating presence of Man/Glc, Gal, GalNac and GlcNac terminals. The affinity of Pabs for proteic epitopes of parasites has been referred while Mabs recognized more frequently carbohydrate epitopes (Bartholomew et al. 1989, Clouthier et al. 1997, Chase et al. 2001, Villavedra et al. 2010), which are the predominant surface molecules. Accordingly, the present aPabEleei recognized exclusively peptidic antigens, which are thought to be generally more

potent immunogens than glycans and are probably the common epitopes shared between parasites, though less often present among surface molecules (Villavedra et al. 2007).

Table 2. *Enteromyxum leei* antigens immunoreactive to the adsorbed polyclonal antibody against *E. leei* (aPabEleei), which were detected by other probes and techniques ± 1 kDa difference.

	Enteromyxum leei antigens (molecular weight)		
	15 kDa	34 kDa	165 kDa
Coomassie	+	-	-
PabMPPF	+	-	+
ConA	+	+	+
BSL I	-	-	+
SBA	-	-	+
WGA	-	-	+
Gelatinolytic activity	-	-	+

Abbreviations: Coomassie brilliant blue stain (Coomassie); Pab against *Myxobolus pendula* polar filament epitopes (PabMPPF); *Canavalia ensiformis*-Agglutinin (ConA); *Bandeiraea simplicifolia*-Lectin (BSL I); *Glycine max*-Agglutinin (SBA); *Triticum vulgaris*-Agglutinin (WGA).

Parasite surface glycoconjugates contribute to protect the parasite from harsh environments and enable host-parasite interactions, like recognition and adhesion leading to penetration of the parasite, but also trapping of the parasite by host mucins leading to its removal. Though not all glycopeptids present in our extracts are surface molecules, some could have a role in such interaction. The detected glycoproteic antigens of 15 kDa, 34 kDa and 165 kDa of *E. leei* contained Man/Glc moieties, which were the most abundant monosacharides among the parasite's antigens, compared to the other monosacharides tested. This result agrees with the ConA staining of *E. leei* proliferative and sporogonic stages observed by light- and electron-microscopy (Redondo & Álvarez-Pellitero 2009) and is a common trait among myxozoans (Muñoz et al. 1999a, Muñoz et al. 2000b, Morris & Adams 2004, Kaltner et al. 2007, Redondo et al. 2008). In fact, Man/Glc moieties were present at the host-parasite interface in enteromyxoses (Redondo et al. 2008, Redondo & Álvarez-Pellitero 2009). The presence of surface Man moieties on offending microorganisms can trigger an innate immune response *via* complement system by the lectin pathway or *via* phagocytosis by Man-binding receptors on macrophages (Kaltner et al. 2007). In fact, it is already known that complement through the alternative pathway and macrophages through the respiratory burst activity are involved in the innate immune response against *Enteromyxum* spp. (Cuesta et al. 2006, Sitjà-Bobadilla et al. 2006, Estensoro et al. 2011) and down-regulation of GSB mannose binding lectin 2 during enteromyxosis was considered as parasite-induced immunodepression (Davey et al. 2011).

The 165 kDa glycoproteic antigen presented, besides the Man/Glc, also reactivity for WGA, binding to GlcNac with high affinity. The presence of chitin, a polymer of D-GLCNac, in polar capsules positive for WGA has been described for some myxozoans (Lukes et al. 1993, Muñoz et al. 1999a, Muñoz et al. 2000b, Kaltner et al. 2007). Myxospore valves also contain substantial amounts of chitin (Lukes et al. 1993, Muñoz et al. 1999a) and thus, bacterial chitinases degrading spore valves of the myxozoan *Thelohanellus kitauei* have been suggested as alternative biological agents to control myxozoan diseases (Liu et al. 2011). Interestingly, polar capsules of *E. leei* were negative for WGA while other spore structures were positive and putatively containing chitin, as happened also with the spores of the congener *E. scophthalmi* (Redondo et al. 2008, Redondo & Álvarez-Pellitero 2009). Since these authors detected no GlcNac in proliferative stages of *E. leei* we can hypothesize that the high MW glycoprotein of 165 kDa is located in spores, the most abundant stage in the present antigen preparation, and could have a structural, protective function as described for chitin in polar capsule walls. In accordance with this, the 165 kDa glycoproteic band of *E. leei* was also labelled by BSL I and SBA, both of them only binding to spores of *E. leei* in light microscopy

preparations (Redondo & Álvarez-Pellitero 2009). These lectins recognize Gal and GalNac carbohydrate residues, frequently involved in host-parasite interactions and which have also been detected in spores of several myxozoans (Muñoz et al. 1999a, Muñoz et al. 2000b, Kaltner et al. 2007, Redondo et al. 2008) and in antigenic bands of *M. cerebralis* (Knaus & El-Matbouli 2005). The 165 kDa carbohydrate-rich antigen was not detected by PAS staining, but primary cells, secondary cells and polar capsules of *E. leei* were PAS positive in histological sections (Álvarez-Pellitero et al. 2008). However, the lack of expected PAS positive bands has been reported before, due to the higher sensitivity of lectins in Western blot over PAS staining in SDS-PAGE gels (Wilkinson & Hames 1983, Schumacher & Krause 1995).

SNA, specifically binding NeuAc, did not recognize any antigenic band exclusive of *E. leei.* However, the intense 108 kDa band observed in R and in C blots, would apparently belong to the host in accordance with the absence of NeuAc residues previously reported for *E. leei* and *E. scophthalmi* by immunohistochemistry (Redondo et al. 2008, Redondo & Álvarez-Pellitero 2009) as well as for *M. cerebralis* by lectin blot (Kaltner et al. 2007). However, surface exposed sialic acids may play a role in self/non-self recognition and therefore some parasites would acquire sialic acid moieties from the host by trans-sialidases as a biological mask to evade immune detection by phagocytes or complement (Feng & Woo 1998b, Kaltner et al. 2007). Therefore, this band might be alternatively interpreted as a syalylated glycoconjugate of the parasite. Nevertheless, even without sialic acids, *E. leei* seems to evade the initial immune response of gilthead sea bream suggesting the involvement of other glycans in the parasite's masking.

PabMPPF crossreacted with *E. leei* epitopes on polar filaments, polar capsules, spore valves, primary and secondary cells. This antibody is directed against a spinalin-like non-collagenous glycoprotein present in *M. pendula* cyst extracts which is resistant to cyanogen bromide digestion. Spinalin is a nematocyst-specific, resistant protein tolerating high mechanical stress and with partial homology to avian keratins. Spinalin presented a MW of 24 kDa in Western blot and was localized in stylets, spines and opercula of cnidarian nematocysts (Koch et al. 1998). Immunoreactive spore structures of *E. leei* to PabMPPF putatively present the spinalin-like peptides which confere a high mechanical strength, but its function in the primary, secondary and accompanying cells of *E. leei* is to be deciphered. The current results show that PabMPPF cross-reacts with several peptidic epitopes of *E.leei*, being two of them glycoproteins also detected by aPabEleei (Table 2). In addition, *M. pendula* blotted with PabMPPF presented a 35 kDa antigenic band (Ringuette et al. 2011) almost coinciding with the 34 kDa glycoproteic band detected in the current study by aPabEleei and ConA. Common antigenic determinants shared between *E. leei* and *Sphaerospora* spp. were already detected with the aPabEleei, but *Myxobolus* was not tested for cross-reactivity (Estensoro et al. 2013). The glycoproteic antigens found in the present study (15 kDa, 34 kDa and 165 kDa) (Supplementary Figure 1) may represent further common antigens shared between *E. leei* and other myxosporeans. Such common myxozoan epitopes have been found previously for other species (Muñoz et al. 1998, Muñoz et al. 1999a, Muñoz et al. 1999b). In support of the phylogenetic affinity between Cnidaria and Myxozoa, Ringuette et al. (2011) also found cross-reactivity of the PabMPPF in polar filament epitopes of *M. pseudokoi*, *M. bartai*, the anthozoan *Nematostella vectensis* and the hydrozoan *Hydra vulgaris* and, in the current study, cross-reactivity with *E. leei* and *E. scophthalmi* (not shown) was demonstrated. Recently, phylogenomic analyses of new genomic sequences of *M. cerebralis* firmly placed Myxozoa as a sister group to Medusozoa within Cnidaria (Nesnidal et al. 2013). Interestingly, minicollagens are phylum-specific genes encoding cnidarian nematocyst proteins and a minicollagen homologue was discovered in the malacosporean *T. bryosalmonae* involving a further link between Myxozoa and Cnidaria (Holland et al. 2011). The inner wall of *H. magnipapillata* nematocyst capsules consists of fibril bundles formed by polymers of small 12-15 kDa minicollagens (Koch et al. 1998), coinciding in MW with the 15 kDa glycoproteic band of *E. leei* recognized by PabMPPF, aPabEleei and ConA. However, at the moment we can only speculate about the existence of a 15 kDa micollagen monomer in *Enteromyxum* and further investigation is required to confirm the nature of this band.

E. leei proteases are probably the key to the pathogenesis of enteromyxosis. Different proteases at different stages of the parasite's life cycle are released to degrade host proteins contributing to tissue damage and parasite proliferation and invasion (McKerrow et al. 1993). Our results showed several functional parasite proteases in *E. leei* parasite extracts (not detected in C intestinal extracts). Their *in vivo* functions may be involved in intra- and extra-cellular digestion of nutrients and/or destruction of immune relevant host molecules. In the myxozoan *Kudoa rosenbushi* infected muscle of *Merlucius hubbsi*, a protease allows spores to enter muscle fibers and to use the breakdown products as a major nutrient source for the pa-

rasite (Martone et al. 1999), and in *Oncorhynchus mykiss* exposed to *M. cerebralis,* the gene expression of *Myx*SP1 protease in gills increases significantly after exposure (Kelley et al. 2004). Moreover, resistance of a *O. mykiss* strain to *M. cerebralis* infection is suggested to derive from a difference in susceptibility to parasite proteases (Doerfler & El-Matbouli 2007). In any case, the further characterization of the detected proteases by its inhibitors and its peptide substrates is still needed to decipher the underlying mechanisms and functions of parasite induced proteolysis during enteromyxosis.

In this first approach to the antigenic characterization of *E. leei,* several glycoproteic antigens were detected, one of them possibly derived from myxospores. Nevertheless, further improvement of the parasite purification technique is needed to avoid all possible interference of host cells with the binding and detection and to deffinitively discard host derived antigens. The function played by each glycoconjugate in the host-parasite interaction as well as the characterization of the individual glycoproteic antigens, deserve further investigation. Eventually, the role played by parasite proteases during intestinal invasion and their importance for virulence and pathogenesis are still to be deciphered in depth.

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Appendix A. Supplementary material
Supplementary Figure 1 is associated with this article (p. 81).

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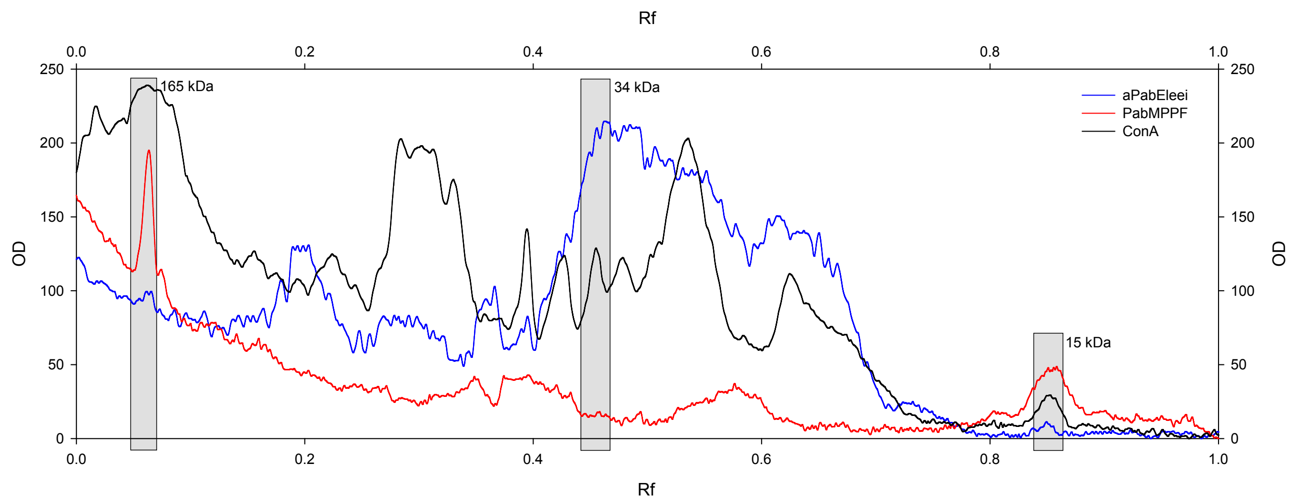
SUPPLEMETARY MATERIAL



Supplementary Figure 1. Optic density (OD) profile along the relative migration distance (Rf) of reduced and denatured *Enteromyxum leei* extracts in Western blots. Peaks corresponding to the bands of same molecular weight detected by the polyclonal antibodies against *E. leei* (aPabEleei) and against *Myxobolus pendula* polar filament (PabMPPF) and detected by *Canavalia ensiformis*-Agglutinin (ConA) are highlighted in shaded boxes.

06

The nutritional background of the host alters the disease course in a fish-myxosporean system

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6.0 ABSTRACT

The aim of the present work was to determine if a practical plant protein-based diet containing vegetable oils (VO) as the major lipid source could alter the disease course when challenged with the myxosporean *Enteromyxum leei*, a wide-spread parasite in the Mediterranean basin causing heavy economic losses. Gilthead sea bream (*Sparus aurata*) fed for 9 months either a fish oil (FO) diet or a blend of VOs at 66% of replacement (66VO diet) were challenged by exposure to parasite-contaminated water effluent. All fish were periodically and non-lethally sampled to obtain biometrical data and to know their infection status. After 102 days of exposure, fish were euthanized and haematological, biometrical, histological, immunological, glutathione and anti-oxidant data were obtained from tissue, blood and serum samples. Anorexia appeared in both exposed groups, but feed intake reduction was higher in 66VO fish. The signs of disease (lower growth, condition factor, specific growth rate, haematocrit) as well as the disease course were worse in fish from 66VO group, with a higher prevalence and intensity of infection, a higher percentage of fish harbouring the parasite in the entire intestinal tract, and a faster establishment of the parasite. Parasite intensity of infection was negatively correlated with growth parameters and haematocrit in both groups, and with complement, lysozyme and hepatic total glutathione in 66VO fish.

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KEY WORDS:
Myxozoa · *Enteromyxum* · Parasites · Immune response · *Sparus aurata* · Anorexia · Vegetable oils

6.1 INTRODUCTION

Parasites are a major constraint on animal production through the world, and recent cases of massive losses in salmon culture due to sea lice are an outstanding example (Costello, 2009). Economic losses are due not only to mortality but also to poor growth performance, low reproduction efficacy, emaciation or other external signs that turn fish into unmarketable products (Sitjà- Bobadilla, 2004, 2009; Guo and Woo, 2009). Gilthead sea bream (*Sparus aurata)* is the main cultured fish species in the Mediterranean area, with a production of more than 150,000 tonnes in 2008 (APROMAR, 2009). Diseases and feed costs are the main limitations for enhancing the productivity. *Enteromyxum leei* is a widely spread myxosporean responsible for one of the most threatening parasitic diseases in Mediterranean fish cultures (Palenzuela, 2006). This parasite invades the intestine of gilthead sea bream producing a slow-progressing disease, which induces anorexia, cachexia and eventually the death of fish. Its impact is further enhanced due to its direct fish-to-fish transmission either by cohabitation with infected fish, by contact with contaminated effluent and per os (reviewed in Sitjà-Bobadilla et al., 2007). Thus far, there are neither preventive nor curative treatments for enteromyxosis. Therefore, there is an urgent need in advancing our knowledge of the parasite itself and the host-parasite interaction.

Substitution of fish meal (FM) and fish oil (FO) by optimised levels of vegetable ingredients stands as one of the current strategies for reducing the cost of fish feeds and the dependency on fisheries to produce aquafeeds (Tacon and Metian, 2008). While using such alternative ingredients, not only the possible effects on growth performance, but also animal health and welfare should be analyzed in an integrative manner. In this context, studies on the effect of FO substitution by vegetable oils (VO) on fish health and the possible nutritional modulation of resistance to infectious diseases are of major importance. In previous works in gilthead sea bream, it has been demonstrated that FO can be replaced in plant-protein based diets up to 66% without detrimental effects on growth, redox balance, immunocompetence or on the intestinal and hepatic architecture (Benedito-Palos et al., 2007, 2008, 2009; Saera-Vila et al., 2009). Thus, a further step was to test whether plant proteins and VO optimised diets could alter the disease outcome when confronted with a pathogen. For this purpose, chronic exposure to *E. leei* by contact with parasite-containing water was chosen to mimic the natural infections. The present work aimed to determine the effects of dietary fat sources on infection levels, growth performance, host immune response and protection against oxidative stress.

6.2 MATERIALS AND METHODS

6.2.1 Experimental design and sampling procedure

Parasite free and clinically healthy gilthead sea bream (GSB) (*Sparus aurata*) from a commercial fish farm were kept in 5 µm-filtered and UV irradiated sea water at temperature always above 18 °C. Some fish were used as control (C) and other as recipient fish (R) for *E. leei* experimental infections (see Sitjà-Bobadilla et al. (2007), Estensoro et al. (2010)). All efforts were made to minimize suffering of the fish used for the experiments in accordance with national (Royal Decree RD1201/2005, for the protection of animals used in scientific experiments) and the current European Union legislation on handling experimental animals.

Naïve gilthead sea bream were obtained from a commercial fish farm and checked for the absence of the parasite (see below). Fish were divided into two experimental groups, which were fed two different diets (Supplementary Table S1). After 9 months of feeding with the corresponding two diets, 60 fish from each diet were individually tagged with passive integrated transponders (PIT-tags) (Trovan, Spain), transferred to the Pathology unit of the Instituto de Acuicultura de Torre de la Sal (IATS) and acclimated for 2 weeks before the parasite challenge. Each diet group was divided again into two groups, control (C, n = 30) and recipient (R, n = 30) in 500-L fibre-glass tanks. The average initial weight before the challenge was 223.7 g. The two R-tanks (one for each dietary treatment) were exposed to *E. leei*-contaminated effluent as previously described (Sitjà-Bobadilla et al., 2007). Briefly, R tanks were set to receive exclusively the effluent water from another tank containing 30 infected fish (donors; average weight = 240.2 g; prevalence of infection = 100%; fed a standard commercial diet). Control fish were kept under the same conditions, but without receiving *E. leei*-contaminated water.

Day length followed natural changes at IATS latitude (40°5'N, 0°10'E) and water temperature was kept always above 18 °C (average = 21.3 °C, range = 18–26 °C, sea water (37.5‰ salinity) was pumped from ashore (open system), 5 µm-filtered and UV irradiated. Each diet group was fed *ad libitum* with the same experimental diet it was receiving before the challenge, and daily food intake in each tank was registered. Disease signs and daily mortality were recorded.

The progression of the infection and the evaluation of growth performance were monitored by sampling both C and R groups at 32, 53 and 88 days post exposure (p.e.). At each sampling, all fish were sized and weighed and non-lethally (NL) sampled for parasite diagnosis with a PCR test (see below). An additional last sampling was performed at 102 days p.e. Then, all R remaining fish and 15 fish from each C group were killed by over-exposure to benzocaine (3-aminobenzoic acid ethyl ester, 100 mg l^{-1} , Sigma, St. Louis, MO, USA) and blood and tissue samples were taken for histological, immunological, and anti-oxidant analyses. Length and body weight, liver and spleen weights were measured and the condition factor (CF = [weight (g) × length $(cm)^{-3}$] × 100), and the hepatosomatic (HSI) and splenosomatic (SSI) indexes were calculated as the ratio between the organ weight and body weight. Specific growth rates (SGR) of all fish were calculated for the period ranging from one week previous to the challenge up to the end of the experiment (109 days) as follows: SGR (%) = 100 × (ln Wt – ln W_0)/t, where W_0 represents weight at the beginning of the period, W_t the weight at the end of the trial and t the number of growth days.

One heparinised blood aliquot was immediately used to measure the respiratory burst activity and another aliquot was drawn into heparinised capillary tubes, centrifuged at 1500 × g for 30 min, and the haematocrit measured. The remaining non-heparinised blood was allowed to clot overnight at 4 °C, centrifuged at 3000×g for 20 min at 4 °C, and serum aliquots were stored at –80 °C until used in immunological, anti-oxidant and glutathione assays.

One replicate tank per group was established to minimize the number of experimental fish (a mandatory requirement of the ethical committee) and to guarantee an infective effluent to the R tanks compatible with a good water quality. According to our previous experience, no tank effect has been found when two tanks are infected from the same D tank under these experimental conditions (Sitjà-Bobadilla et al., 2007, 2008). As all fish were individually monitored along the entire experimental period, each fish was considered the experimental unit (Fisher, 2000).

6.2.2 Parasite diagnosis

Parasite diagnosis was performed by histology (at 102 days p.e.) or by NL-PCR (at 32, 55 and 88 days p.e.). For histological examination, pieces of anterior, medium and posterior intestine were fixed in 10% buffered formalin, embedded in paraffin, 3μm-sectioned and stained with haematoxylin and eosin. NL samples were obtained by probing the rectum with a cotton swab and PCR diagnosis was carried out as described in Palenzuela and Bartholomew (2002) with primers specific for *E. leei* rDNA. This procedure has been validated against a gold standard (histological observation of the whole digestive tract), resulting in a high sensitivity (0.96) and specificity (1) (O. Palenzuela, unpublished data). NL-PCR was also applied to evaluate the parasitic status of donors, and R fish upon arrival to the experimental facilities. The prevalence of infection at each sampling point was calculated considering positive fish detected by either PCR or histology. The intensity of infection was semiquantitatively evaluated in one histological cross section of each of the three intestinal portions (anterior, medium and posterior) and scored 1+ to 6+ according to the number of parasitic stages per microscope field at ×120 with the range: 1+ = 1–5; 2+ = 6–10; 3+ = 11–25; 4+ = 26–50; 5+ = 51–100; 6+ > 100. For each intestinal portion, the mean intensity of infection was calculated. For each parasitized fish, the mean intensity of infection was calculated as an average of the intensity of infection in the parasitized portions.

Fish tagging and NL-diagnosis by PCR allowed individualized monitoring of the infection along the experimental period. Thus, for each R fish euthanized at 102 days p.e., the time at which the parasite was first detected was known. Therefore, each R fish was classified as being first-infected at one of the four sampling times, and four timing-classes were established: late (102 days), medium (88 days), early (53 days), and very early (32 days).

6.2.3 Immunological assays

Induction of the respiratory burst (RB) activity in blood leucocytes was measured directly from heparinised blood, as previously described (Saera-Vila et al., 2009). Briefly, blood was incubated with a luminol suspension containing PMA for 1 h and the resulting integral chemiluminescence in relative light units (RLU) was calculated.

Total serum peroxidases (PO) were chosen as a measure of the serum oxidizing capacity. Serum lysozyme (LY) was measured by a turbimetric assay. The lytic capacity of the serum by the alternative complement pathway (ACP) was determined using sheep red blood cells (SRBC) as targets, and the dilution corresponding to 50% haemolysis/ml was expressed as ACH_{50}. All these assays were performed following the procedure described in Sitjà-Bobadilla et al. (2005), but using 2.85×10^8 SRBC ml^{-1} in ACP.
Total nitric oxide (NO) production in serum samples was determined as the sum of nitrite (NO_2) and nitrate (NO_3), using a fluorimetric assay kit, which detects up to 10nM naphthotriazole (Cayman Chemical, Ann Arbor, MI, USA). Briefly, 10μl duplicated serum samples, previously centrifuged in Microcon-10 filter devices (cut-off value = 10 kDa) (Millipore Corp., Madrid, Spain) at 12,000 × g for 10 min at 4 °C, were incubated with enzyme cofactors and nitrate reductase for 2h. 2,3-Diaminonaphthalene (DAN) and NaOH were sequentially added and the resulting fluorescent product (naphthotriazole) was measured using an excitation wavelength of 360 nm and an emission wavelength of 465 nm in a microplate reader.

6.2.4 Anti-oxidant and glutathione assays

The total antioxidant status (TAS) was measured in serum samples with a commercial kit (Cayman Chemical). Oxidized (GSSG) and total (tGSx) levels of glutathione in frozen liver and intestine samples were determined enzymatically with a commercial kit (Cayman Chemical). The GSH/GSSG ratio was calculated as the quotient of reduced GSH equivalent, as a measure of cellular toxicity. Both assays were performed as previously described (Saera-Vila et al., 2009).

6.2.5 Statistical analysis

The influence of the diet on the presence of *E. leei* was statistically analyzed using a Chi-square test of independence with Yates correction for continuity, considering all the fish sampled at 102 days p.e. For all the data, differences between the four experimental groups at any sampling point were analyzed by one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test. When the tests of normality or equal variance failed, a Kruskal–Wallis one-way ANOVA on Ranks followed by Dunn's method was applied

instead. The same test was applied to determine possible differences in the mean intensity of infection between time-classes at 102 days. p.e. A Student-*t* test was used to analyze the differences between both R groups within each time-class, and within each intestinal portion.

The strength of the possible association of all the studied variables in individual fish was measured with a Spearman rank order correlation test, since the normality test failed for some of them. Thus, all the data of fish from the same diet obtained at 102 days p.e. were pooled and the Spearman correlation coefficients between all the variables (except those conceptually correlated) were calculated. In addition, for some infection parameters and the timing of the infection, a linear regression test was performed to further analyze the relationship between them.

All the statistical analyses were performed using Sigma Stat software (SPSS Inc., Chicago, IL, USA) and in all cases the significance level was set at $P < 0.05$.

6.3 RESULTS

6.3.1 Progression of the infection

Fig. 1A shows the prevalence of infection during the exposure to the parasite. Although the final infection levels were high in both R-groups, the progression of the infection was higher and faster in 66VO-R fish than in FO-R ones. In fact, the relationship between time and prevalence of infection had a pattern of exponential rise to a maximum ($r^2 = 0.9169$, $P = 0.0424$) in the 66VO group, whereas it remained linear in the FO group ($r^2 = 0.953$, $P = 0.0238$). This differential progression was clearly outlined by the statistically significant relationship between the dietary treatment and the cumulative prevalence of infection, as analyzed with a Chi-square test of independence ($P = 0.032$). No C fish was found to be parasitized at any sampling point. Mortality along the experimental period was similar in both R-groups, 4 fish died in 66VO *vs* 5 in FO.

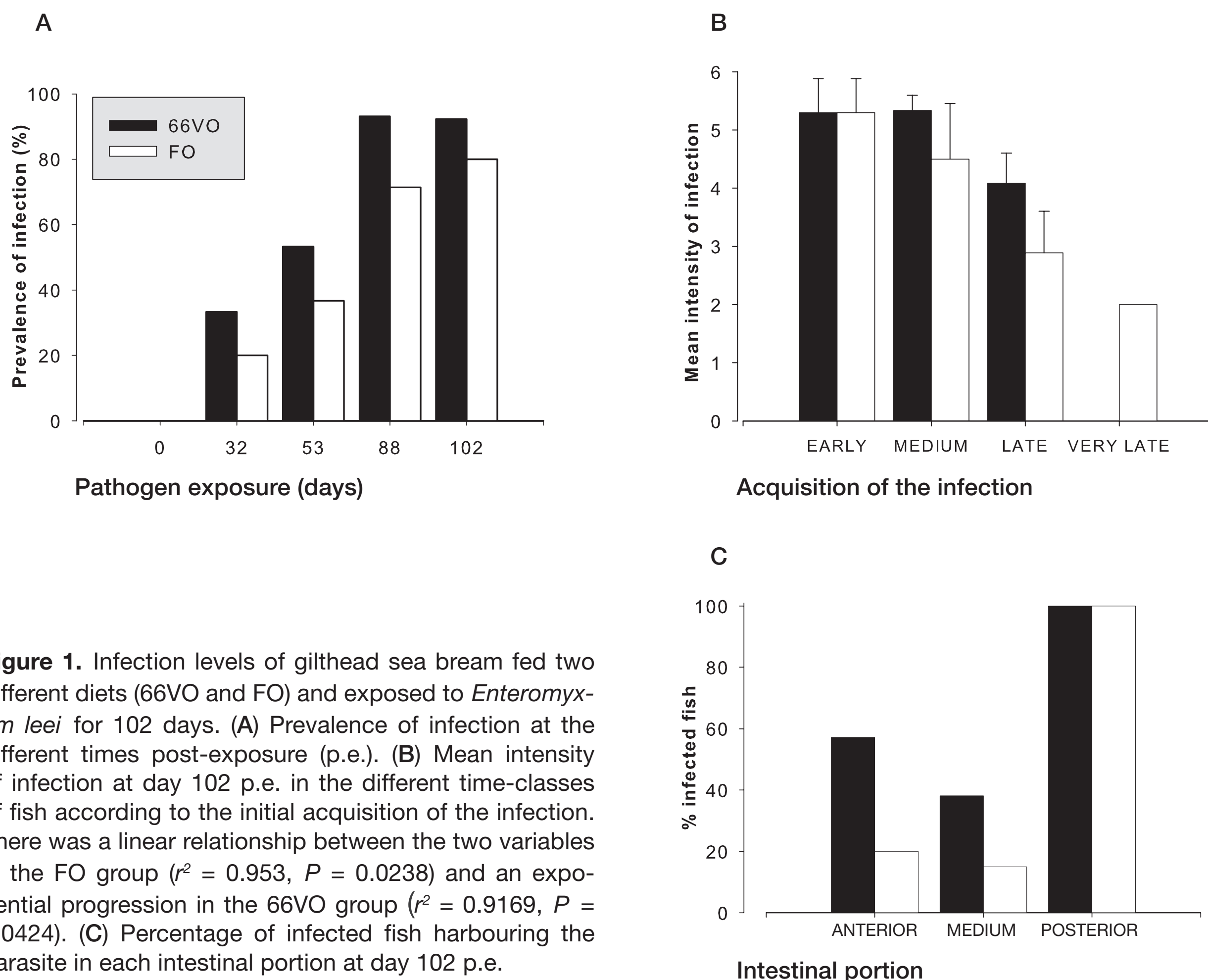

Figure 1. Infection levels of gilthead sea bream fed two different diets (66VO and FO) and exposed to *Enteromyxum leei* for 102 days. (**A**) Prevalence of infection at the different times post-exposure (p.e.). (**B**) Mean intensity of infection at day 102 p.e. in the different time-classes of fish according to the initial acquisition of the infection. There was a linear relationship between the two variables in the FO group ($r^2 = 0.953$, $P = 0.0238$) and an exponential progression in the 66VO group ($r^2 = 0.9169$, $P = 0.0424$). (**C**) Percentage of infected fish harbouring the parasite in each intestinal portion at day 102 p.e.

Fig. 1B shows the mean intensity of infection of infected fish when euthanized (102 days p.e.), considering the four different time-classes related to the first detection of the infection. No fish was detected as newly infected at the last sampling in the 66VO group. In general, the mean intensity of infection was higher in fish of the 66VO-R group than in FO-R fish, but there were no statistically significant differences between them at any time. In both R-groups, it was evident that the earlier the infection was established, the higher the intensity was at the final sampling. Furthermore, a strong positive correlation was found between the intensity of the infection and the number of days of establishment of the infection, in FO-R (r_S = 0.944, P = 0.0000007) and 66VO-R (r_S = 0.783, P = 0.0000007).

At the last sampling point, all the infected fish, regardless of the group, had the parasite established at the posterior intestine. However, the percentage of parasitized fish harbouring the myxosporean also at the anterior and medium sections was clearly higher in 66VO group (Fig. 1C), being always the medium intestine the less frequently affected part.

6.3.2 Biometrical data

No differences in growth performance were detected between control (C) groups fed the two different diets (Fig. 2A). As expected, both groups exposed to the parasite (R) exhibited the physiological effects of the disease, but they were more acute in 66VO-R fish. Growth performance was reduced in both R groups, with a significant lower weight at 88 and 102 days p.e. *vs* their corresponding C groups, and even between 66VO-R and FO-R fish at the last sampling (Fig. 2A). Similarly, body weight gain in the whole experimental period was markedly lower in both R groups, and again the percentage of reduction respect to the C group with the same diet was higher in fish fed 66VO than in those fed FO (54.2% *vs* 29.4%) (Table 1). At 102 days p.e., both R-groups also had significantly lower length and CF than their corresponding C groups (Table 1). When analyzing in detail the decrease in SGR in R groups, all the 66VO-R infected fish, regardless of the time in which they acquired the infection, had significantly lower SGR values than 66VO-C fish. By contrast, only those FO-R fish with early acquisition of the infection had significantly lower values than their respective C group. SGR was lower in all 66VO-R time classes fish than in FO-R, though the difference was statistically significant only in the medium time-class (Fig. 2B). The lower increase of body weight of R fish was due in part to a reduced feed intake in R animals, which was 15% and 22% less than the corresponding C groups in FO and 66VO, respectively (Table 1). Anorexia thus explained about 52% of body weight losses in FO-R animals, but only 41% in 66VO-R group. Anorexia was first detected between 32 and 53 days, increased progressively and was most evident in the last weeks of the experimental period (between 88 and 102 days) when feed intake was 30% and 45% lower in FO-R and 66VO-R than in the corresponding C groups, respectively. Haematocrit values were significantly lower in 66VO-R animals (Table 1). No significant differences were found in the SSI and HSS indexes between any groups (Table 1).

6.3.3 Immune factors (Table 1)

Both R groups, regardless of the diet, exhibited a significantly higher respiratory burst in circulating leucocytes than their corresponding C groups at 102 days p.e. Although the values obtained for 66VO-R fish were higher than those of FO-R animals, differences were not significant. Total serum PO, serum lysozyme (LY) and serum NO were lower in both R groups, but the decrease was only statistically significant for PO in 66VO-R and for LY and NO in FO-R. Both 66VO groups (R and C) had significantly lower values of LY and NO than those of FO-C group. The lytic capacity of the serum by the alternative complement pathway (ACH_{50}) was significantly higher in 66VO-C fish than in the remaining groups, and no differences were observed between both R groups.

6.3.4 Glutathione and antioxidant defence system (Table 1)

Fish from the 66VO-C group exhibited a significantly higher total antioxidant status (TAS) than the remaining groups and no differences were found between both R groups. Concerning the gluthathione values, no significant differences were found in the intestinal values, whereas the hepatic synthesis of glutathione (tGSx) and the hepatic GSH/GSSH ratio were significantly decreased in 66VO-R fish with respect to FO-R and also respect to 66VO-C for the hepatic ratio.

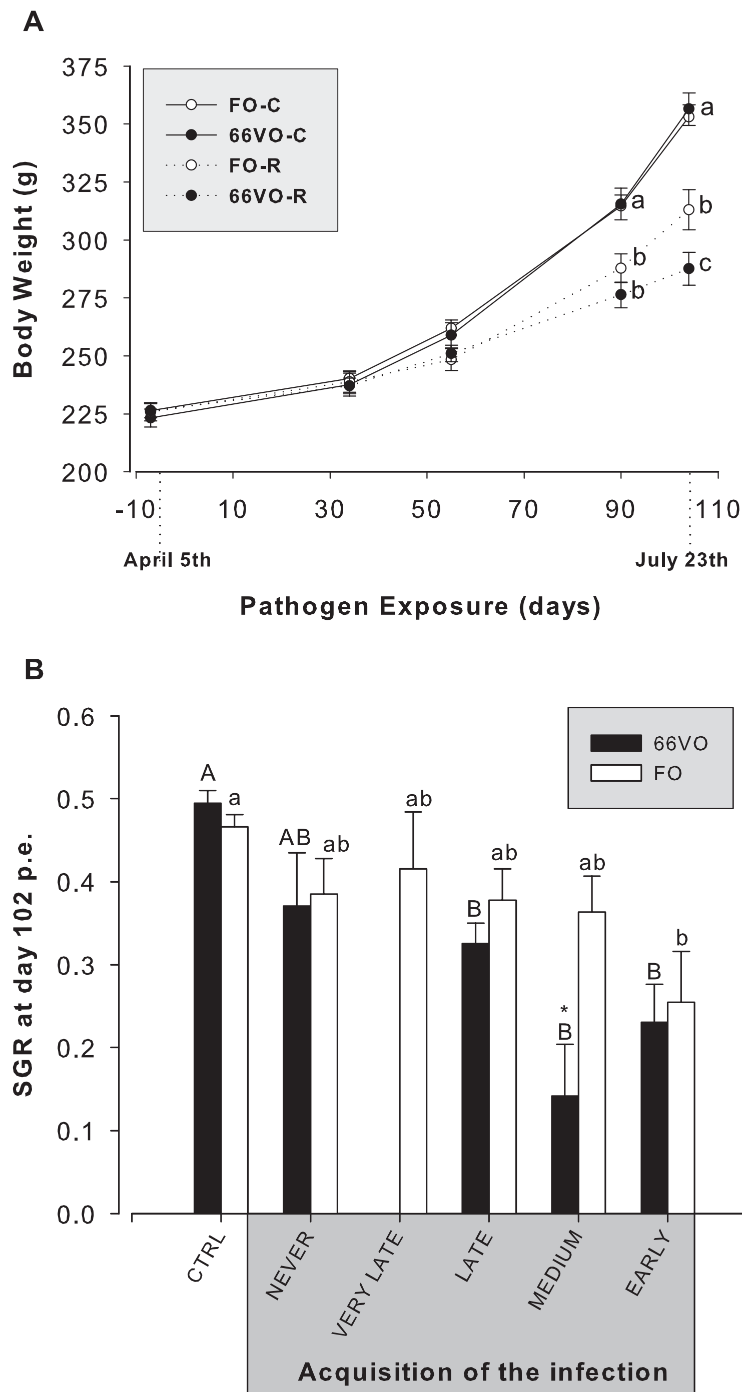

Figure 2. Biometrical data of gilthead sea bream fed two different diets (66VO and FO) and not exposed (C) or exposed (R) to *Enteromyxum leei* for 102 days. (**A**) Progression of body weight along the experimental period. Different letters stand for statistically significant differences among the groups at each sampling time ($P < 0.05$). (**B**) Specific growth rate (SGR) at the end of the experimental period of C and R fish classified in the different time-classes according to the initial acquisition of the infection. Note that some R fish were never infected. Different capital letters stand for statistically significant differences among 66VO fish, and low case letters for FO fish. Asterisk (*) indicates significant differences between 66VO and FO groups within a time-class ($P < 0.05$).

Table 1. Biometric, antioxidant, redox and immunological values (mean ± SEM) of gilthead sea bream fed the two different diets (66VO, FO) and exposed for 102 days to *Enteromyxum leei* (R) or not (C). Different superscript letters stand for statistical differences between groups for each parameter (P < 0.05).

	66VO-C	66VO-R	FO-C	FO-R
Final length (cm)	23.6 ± 0.21 [a]	22.5 ± 0.16 [b]	23.6 ± 0.17 [a]	22.9 ± 0.19 [b]
Weight gain (g/fish)	134.7 ± 4.03 [a]	61.7 ± 6.76 [c]	127.4 ± 3.48 [a]	89.9 ± 6.8 [b]
Feed intake (g/fish)*	180.2	140.3	177.1	149.6
SGR (%)	0.47 ± 0.015 [a]	0.25 ± 0.027 [c]	0.47 ± 0.015 [a]	0.36 ± 0.023 [b]
CF	2.69 ± 0.048 [a]	2.52 ± 0.035 [b]	2.69 ± 0.048 [a]	2.58 ± 0.028 [b]
HSI	0.920 ± 0.053	0.846 ± 0.049	0.961 ± 0.032	0.901 ± 0.029
SSI	0.102 ± 0.129	0.088 ± 0.005	0.119 ± 0.011	0.114 ± 0.012
Hc (%)	46.33 ± 1.64 [a]	33.96 ± 1.79 [c]	41.46 ± 0.97 [ab]	40.58 ± 1.77 [b]
RB (I. RLU)	9905.0 ± 3345.6 [a]	30333.7 ± 6020.2 [b]	10717.9 ± 1796.4 [a]	20617.2 ± 3633.8 [b]
PO (OD_{450} nm)	0.324 ± 0.0669 [ab]	0.208 ± 0.0254 [b]	0.361 ± 0.0337 [a]	0.293 ± 0.0358 [ab]
LY (units/ml)	242.82 ± 66.48 [b]	220.59 ± 10.40 [ab]	306.82 ± 14.75 [c]	200.56 ± 11.33 [a]
ACH_{50}	130.36 ± 19.21 [a]	20.55 ± 1.86 [b]	42.08 ± 17.77 [b]	26.48 ± 4.36 [b]
NO (µM)	4.16 ± 0.87 [a]	3.36 ± 0.38 [a]	8.12 ± 0.31 [b]	3.78 ± 0.89 [a]
TAS (mM Trolox)	0.467 ± 0.025 [a]	0.370 ± 0.009 [b]	0.384 ± 0.017 [b]	0.370 ± 0.009 [b]
LIVER:				
tGSx (nmol/g)	3079.6 ± 178.6 [ab]	2179.2 ± 293.7 [a]	2841.7 ± 178.6 [ab]	3306.4 ± 306.7 [b]
GSSG (nmol/g)	137.9 ± 7.6	143.6 ± 9.7	156.0 ± 10.8	135.0 ± 9.4
GSH/GSSG	20.5 ± 1.7 [a]	13.6 ± 1.6 [b]	17.4 ± 2.4 [ab]	23.1 ± 2.1 [a]
INTESTINE:				
tGSx (nmol/g)	1894.9 ±160.6	1874.2 ±103.1	2076.1 ± 101.3	1914.6 ±86.1
GSSG (nmol/g)	106.7 ± 18.4	93.6 ± 16.3	95.8 ± 22.6	102.4 ±13.1
GSH/GSSG	23.5 ± 5.9	73.1 ± 24.9	39.2 ± 14.2	34.2 ± 10.9

*Total value accumulated during the experimental period. See abbreviations in the text.

6.3.5 Meta-analysis: correlation between all data and the disease course

Spearman's correlation coefficients between all the biometrical, immunological, antioxidant and gluthathione factors for FO and 66VO groups are shown in Supplementary Tables S2 and S3, respectively. The information derived from S2 and S3 is diagrammatically presented in Fig. 3, showing the main correlations found between *E. leei*-intensity of infection and the remaining measured factors. There was a strong negative correlation with biometrical parameters (length, weight, SGR, CF) in both diet groups (Fig. 3, red boxes), also with Hc, HSI and some immunological (ACH_{50}) and glutathione (hepatic GSH/GSSG ratio, liver tGSx) factors only in the 66VO group, and with LY and NO only in the FO group (Fig. 3, red boxes). In addition, a positive correlation was found between the parasite and RB in 66VO fish (Fig. 3, green box). Similarly, a negative correlation was also found between the number of days of establishment of the infection and most biometrical values in both groups, and also with HSI, ACH_{50}, Hc and hepatic glutathione (GSH/GSSG ratio, tGSx) factors in the 66VO group, and with SSI, PO and NO in the FO group (Supplementary Tables S2 and S3).

Hc was positively correlated with ACH_{50} , weight, length, CF and SGR in 66VO fish, and with PO, SGR, HSI and intestinal GSSG in FO fish. By contrast, Hc was negatively correlated with RB in 66VO. In both groups, PO correlated positively with HSI, and RB negatively with SGR. Other correlations were found only in 66VO animals: positive between ACH_{50} and several biometrical values (weight, length and SGR), and negative between RB and HSI, and between RB and ACH_{50} and PO.

From the statistical analysis we can conclude that in 66VO fish, the earlier the infection was established, the higher the intensity of infection was achieved and consequently the lower weight, length, CF, SGR, Hc, LY, ACH_{50} and hepatic glutathione ratio were exhibited.

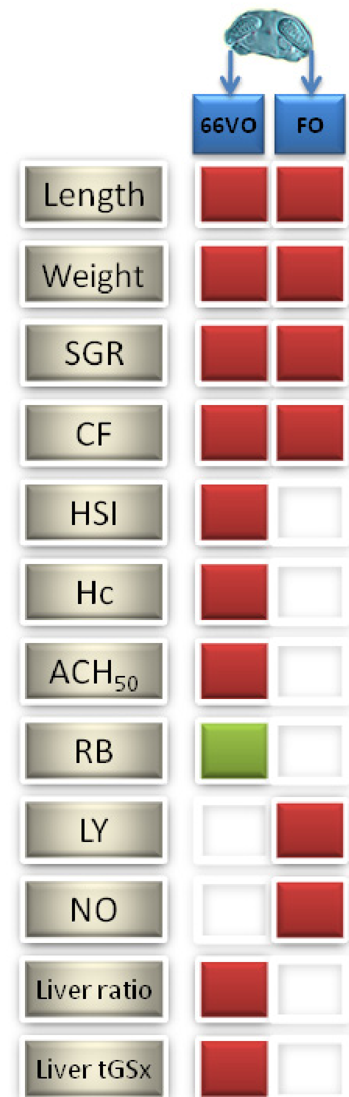

Figure 3. Diagrammatic representation of the main correlations found between the intensity of infection by *Enteromyxum leei* at the posterior intestine and the different biometrical, immunological and antioxidant factors (see the text for abbreviations) in the two gilthead sea bream diet groups (66VO and FO). Red and green boxes stand for negative and positive correlations, respectively, and empty boxes for no correlation (*P*< 0.05). The corresponding Spearman's correlation coefficients can be found in Supplementary Tables S2 and S3.

6.4 DISCUSSION

The present study has shown that feeding fish with a VO diet may increase the disease effects of enteromyxosis in gilthead sea bream. Although the final prevalence and intensity of infection were high in both dietary experimental groups and similar to other effluent-transmission studies (Sitjà-Bobadilla et al., 2007), the progression of the infection and the severity of the disease signs were higher in R fish fed the 66VO diet than in those fed the FO diet. Thus, the effect on weight, length, CF, SGR and Hc, which are the consequences of the emaciative disease provoked by the parasite, were more pronounced in 66VO-R fish. In other fish–*Enteromyxum* systems in which the myxosporeans exhibit a higher pathogenicity, a significant reduction in weight and Hc was detected earlier (Sitjà-Bobadilla et al., 2006; Álvarez-Pellitero et al., 2008). By contrast, in other gilthead sea bream also exposed to *E. leei* by effluent transmission, but fed a standard commercial diet and reared under lower water temperature, no significant differences in weight were detected between C and R, probably due to the lower growth rates and the lower achieved infection levels (Sitjà-Bobadilla et al., 2008)

The 66VO-C fish did not exhibit any detrimental effect of the diet on their biometric and haematological values. Therefore, as already demonstrated in previous growth studies (Benedito-Palos et al., 2007, 2008, 2009), the nutritional background by itself did not produce any detrimental effect on the fish body mass, and it was not responsible alone for the severe disease, but a predisposing factor. Besides, for the first time in a fish–myxosporean model, we find evidence that the body mass loss of R fish is mainly due to a decrease of voluntary feed intake (anorexia). Parasite-induced anorexia is a prominent feature of several host-parasite systems in vertebrates, but the causes and mechanisms of anorexia remain unresolved (Kyriazakis et al., 1998; Colditz, 2008). In fish, anorexia has been scarcely documented with accuracy under cases of parasitic infections (Thomas and Woo, 1992; Dezfuli et al., 2003). Reduced nutrient availability due to decreased feed intake is the major contributor to reduced growth rates in parasitized animals (see Colditz, 2008). However, the decrease in weight gain in R fish was higher than what could be explained by feed intake decrease. Therefore, other factors such as parasite-derived intestinal damage could contribute to the impairment of intestinal absorption and therefore nutrient availability. Indeed, intestinal damage was higher in 66VO-R fish, as more intestinal portions were affected and with a higher intensity of infection, and the typical enteritis syndrome was observed in fish with high intensity of infection (data not shown). It has previously been shown that the massive invasion of the intestinal epithelium by *E. leei* stages produces enteritis with atrophy and blunting of villi which can lead with the detachment of the epithelium from the underlying lamina propria (Fleurance et al., 2008). In addition, the intensity of the lesions produced by *E. scopththalmi* in the intestine of turbot was correlated with the progression of the infection (both prevalence and intensity) and the development of the parasite (Bermúdez et al., 2006, 2010). Finally, body weight loss could also be due to an osmoregulatory failure, as suggested by the pathophysiological evidences in *E. leei*-infected *Takifugu rubripes* (Ishimatsu et al., 2007). Therefore, we are clearly in front of a case of cachexia, in which weight is lost much more rapidly than would be expected from a decrease in feed intake of the same magnitude (Emery, 1999). The host's immune response has a metabolic cost (Ots et al., 2001) and could also have adverse effects on growth and feed intake. In fact, the immune response is responsible for the production of several cachectic cytokines (TNF-α, IL-1 and IL-6) that induce cachexia (Cerami and Beutler, 1988; Argilés et al., 2006). In *E. leei*-infected gilthead sea bream, transcripts of IL-1β and TNF-α were significantly decreased in the intestine at 113 days p.e. (Sitjà-Bobadilla et al., 2008), whereas IL-1β expression was increased in head kidney shortly after exposure (10 days) (Cuesta et al., 2006). Thus, other anorexigenic factors, such as gastrointestinal peptides (Mendieta-Zerón et al., 2008) or growth factors (Carroll, 2008) could be involved. In fact, the number of enteroendocrine cells positive for neuromodulators related with feed intake and digestion, such as neuropeptide Y and substance-P were lower in the intestine of R fish than in C gilthead sea bream (Estensoro et al., 2009).

The main difference between the two diets tested in the current study is the fatty acid (FA) profile, since 66VO diet has lower levels of *n*-3 long-chain polyunsaturated fatty acids (LC-PUFAs), though it meets the theoretical requirements of essential FAs. The complex relationship between nutrition, immune status and parasitic infection is well recognized (Fekete and Kellems, 2007), and dietary FAs are capable of modulating the immune system in mammals (De Pablo and De Cienfuegos, 2000) and *n*-3 LC-PUFAs present in FO in particular have well recognized anti-inflammatory properties (Calder, 2007). However, in fish, changing the dietary *n*-3 LC-PUFA levels can have both beneficial and, in some instances, detrimental effects on disease resistance and immune status. Thus, the intake of high levels of dietary *n*-3 LC-PUFAs suppressed some

immune functions and reduced survival after pathogen challenge (Erdal et al., 1991; Fracalossi and Lovell, 1994; Kiron et al., 1995; Misra et al., 2006). Conversely, in our study, the disease course was worsened in fish fed the 66VO diet (with lower *n*-3 LC-PUFAs), as it occurred in other studies in which significantly higher mortality rates were found in fish fed diets with different types of VOs (with lower *n*-3/*n*-6 PUFAs ratio, or lower *n*-3 LC PUFAs) compared to those fed FO when subsequently challenged with bacteria (Thompson et al., 1996; Bransden et al., 2003) or the ciliate parasite *Ichthyophthirius multifilis* (Vargas et al., 2008).

The involvement of innate mechanisms in the fish immune response to parasitic infections has been documented in several host-parasite models (see Álvarez-Pellitero, 2008). In the current model, ACH_{50} was the only enhanced innate factor in 66VO-C, which is in accordance with the increased ACH_{50} reported in grouper (*Epinephelus malabaricus*) fed a diet partially replaced with corn oil (Lin and Shiau, 2007). In gilthead sea bream, a similar 66VO diet fed during 6 months also produced a slight increase in ACH_{50} (Saera-Vila et al., 2009), but another diet with totally replaced FO by a single VO invoked a decrease in ACH_{50} (Montero et al., 2003). By contrast, ACH_{50} was not affected by feeding with different single or mixed VOs in other fish species (Kiron et al., 2004).

On the other hand, the worse disease outcome in fish fed the 66VO diet could be due to the significantly lower values of serum nitric oxide (NO) and lysozyme (LY). NO is an important molecule in regulating immune functions and has a direct antimicrobial effect. As in FO-R fish, nitrite production of head kidney leucocytes of sharpsnout sea bream (*Diplodus puntazzo*) infected with a myxosporean *Myxobolus* species was hampered (Karagouni et al., 2005). LY is a main defence molecule of fish innate immune system (Saurabh and Sahoo, 2008) and interestingly, in sharpsnout sea bream, a species highly susceptible to *E. leei* in which the infection progresses rapidly, LY was undetectable in either infected or healthy fish (Golomazou et al., 2006; Alvarez-Pellitero et al., 2008). By contrast, serum LY appears to be unaffected in most studies with fish fed diets rich in VOs (Bell et al., 1996; Montero et al., 2003; Kiron et al., 2004; Mourente et al., 2005). These apparent contradictory effects on immune factors are probably due to different experimental conditions (fish meal inclusion, type of vegetable oil, PUFAs ratios, feeding time, etc.), type of immunocytes involved and species model. In most fish trials, the basal diet is replaced either by fish meal or FO, whereas a significant difference of the current trial is that high levels of substitution of fish meal by plant proteins was also achieved.

There are other hypothetical reasons for the worse disease signs in 66VO-R, related to the direct effect on the intestinal tract and mucosal immunity. Some dietary VOs and saturated FAs produce cellular damages in fish enterocytes due to the accumulation of lipid droplets (Olsen et al., 2000), but this damage was observed only with total FO replacement (Benedito-Palos et al., 2008). Other possible changes in the cellular architecture and composition of the digestive tract might favour parasite entrance and proliferation in the intestinal epithelium, through changes at the host-parasite interface (Yoshino et al., 2001), alterations of the gut barrier or the mucosal immunity (Kasper and Buzoni-Gatel, 2001), or alterations of the biochemical makeup of the cell membrane lipid rafts/caveolae microdomains (Chapkin et al., 2008). In fact, the effect of PLs, FAs and soybean meal on tight-junction permeability and therefore in bacterial translocation (Sawai et al., 2001), on fish intestinal mucus and bacterial adhesion (Bakke-McKellep et al., 2007; Schroers et al., 2008) have been reported. Thus, further studies are needed to determine the effect of vegetable diets in the distribution and composition of enzymes, other molecules of interest and leucocyte populations in the intestinal tract.

Normal metabolism and immune and detoxification systems generate reactive oxygen and nitrogen species that if not counterbalanced, lead to oxidative stress, host tissue damage and ageing. Oxidative stress is intimately linked to parasite resistance and fitness (Kurtz et al., 2006) and the removal of these free radicals is achieved through non-enzymatic and enzymatic reactions. The primary enzymatic antioxidant defence system is the glutathione redox system that reduces hydrogen peroxide and lipid hydroperoxides at the expense of oxidizing GSH to its disulfide form (GSSG). The antioxidant and anti-inflammatory effects of *n*-3 LC-PUFAs have been extensively documented (Fang et al., 2002). Nevertheless, in the current study, 66VO-C fish not only had no oxidative/antioxidative imbalance, but also exhibited higher circulating antioxidant defences (TAS) than FO-C fish. This could be due, at least in part, to the presence of some substances, such as flavonoids, in VOs (Beecher, 2003). Flavonoids are potent scavengers of free radicals such as hydroxyl and superoxide radicals, and also act as chelators of transient elements, having therefore biological effects against inflammatory and allergic disorders (Kim et al., 2004). Their presence in VOs could also

explain the lower serum NO values than in FO fish, as they are also known to inhibit the production of NO (Pergola et al., 2006).

Parasite challenge increased reactive oxygen species derived for the immune response in both R fish, due to increased RB, as previously reported in different *Enteromyxum*-infected fish (Sitjà-Bobadilla et al., 2006, 2008; Álvarez-Pellitero et al., 2008). However, TAS was significantly decreased only in 66VO-R animals with respect to 66VO-C group, as occurred in other host-parasite systems (Lightbody et al., 2001). This lower TAS of 66VO-R fish could endanger their health, since their hepatic GSH/GSSG ratio was also decreased, showing a higher risk of lipid peroxidation and oxidative damage. Similarly, in other intestinal inflammatory diseases induced by parasites, there is an imbalance in the oxidant/antioxidant defence mechanisms, which can increase the susceptibility of the intestine to inflammation (Sundaram et al., 2003).

In conclusion, this study has shown that 66VO replacement in gilthead sea bream diet is a predisposing cause that worsens the disease course when fish are experimentally exposed to *E. leei*, the precipitating cause. These results should be confirmed with other fish–pathogen models, as the effects of the diet may be beneficial or not, depending on the virulence factors of the pathogen and the site of infection. Further studies are underway to study the invasion strategy of this parasite and to determine which immune factors are key for controlling this disease.

All the experiments were carried out according to national (Royal Decrete RD1201/2005, for the protection of animals used in scientific experiments) and institutional regulations (CSIC, IATS Review Board) and the current European Union legislation on handling experimental animals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2010.09.015.

Supplementary Table 1 (p. 99).

Supplementary Table 2 (p. 100): Spearman correlation coefficients resulting from the meta-analysis of all measured variables in FO fish. Those coefficients of variables conceptually related have been omitted. The pair of variables with positive correlation coefficients (in green) tends to increase together, whereas negative correlation coefficients (in red) indicate that one variable tends to decrease while the other increases. To facilitate visualization significant *P* values (<0.05) have been highlighted in yellow. *E. leei* = intensity of infection at the posterior intestine; days = number of days of establishment of the infection; H = hepatic; Int = intestinal. For the remaining abbreviations see the text.

Supplementary Table 3 (p.101): Spearman correlation coefficients resulting from the meta-analysis of all measured variables in 66VO fish. Those coefficients of variables conceptually related have been omitted. The pair of variables with positive correlation coefficients (in green) tends to increase together, whereas negative correlation coefficients (in red) indicate that one variable tends to decrease while the other increases. To facilitate visualization significant *P* values (<0.05) have been highlighted in yellow. *E. leei* = intensity of infection at the posterior intestine; days = number of days of establishment of the infection; H = hepatic; Int = intestinal. For the remaining abbreviations see the text.

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SUPPLEMETARY MATERIAL

Supplementary Table 1. Ingredients and chemical composition of experimental diets.

INGREDIENT (%)	FO	66VO
Fish meal (CP 70%) [1]	15	15
CPSP 90 [2]	5	5
Corn gluten	40	40
Soybean meal	14.3	14.3
Extruded wheat	4	4
Fish oil [3]	15.15	5.15
Rapeseed oil	0	1.7
Linseed oil	0	5.8
Palm oil	0	2.5
Soya lecithin	1	1
Binder	1	1
Mineral premix [4]	1	1
Vitamin premix [5]	1	1
$CaHPO_4.2H_2O$ (18%P)	2	2
L-Lys	0.55	0.55
PROXIMATE COMPOSITION		
Dry matter (DM, %)	93.13	92.77
Protein (% DM)	53.2	52.62
Fat (% DM)	21.09	20.99
Ash (% DM)	6.52	6.57

[1] Fish meal (Scandinavian LT).
[2] Fish soluble protein concentrate (Sopropêche, France).
[3] Fish oil (Sopropêche, France).
[4] Supplied the following (mg / kg diet, except as noted): calcium carbonate (40% Ca) 2.15 g, magnesium hydroxide (60% Mg) 1.24 g, potassium chloride 0.9 g, ferric citrate 0.2 g, potassium iodine 4 mg, sodium chloride 0.4 g, calcium hydrogen phosphate 50 g, copper sulphate 0.3, zinc sulphate 40, cobalt sulphate 2, manganese sulphate 30, sodium selenite 0.3.
[5] Supplied the following (mg / kg diet): retinyl acetate 2.58, DL-cholecalciferol 0.037, DL-a tocopheryl acetate 30, menadione sodium bisulphite 2.5, thiamin 7.5, riboflavin 15, pyridoxine 7.5, nicotinic acid 87.5, folic acid 2.5, calcium pantothenate 2.5, vitamin B12 0.025, ascorbic acid 250, inositol 500, biotin 1.25 and choline chloride 500.

Supplementary Table 2. Spearman correlation coefficients in FO fish (see p. 95)

	Length	SGR	CF	HSI	SSI	Hc	*E. leei*	Days	LY	ACH_{50}	PO	RB	NO	TAS	H. Ratio	H. tGSX	H. GSSG	Int. Ratio	Int. tGSX	Int. GSSG
Weight	0.796			0.116	0.062	0.005	-0.42	-0.371	0.490	0.178	0.126	-0.319	0.221	0.107	-0.002	-0.234	-0.217	0.062	0.111	-0.041
	2E-07			0.473	0.710	0.977	0.007	0.019	0.003	0.321	0.474	0.045	0.200	0.538	0.991	0.194	0.230	0.734	0.544	0.82
Length		0.592		-0.067	0.042	-0.028	-0.341	-0.353	0.448	0.289	-0.002	-0.099	0.262	0.206	-0.038	-0.157	-0.068	0.067	0.207	-0.037
		6.2E-05		0.68	0.802	0.878	0.032	0.026	0.007	0.102	0.989	0.543	0.127	0.234	0.833	0.388	0.707	0.711	0.252	0.839
SGR				0.490	0.102	0.4	-0.501	-0.504	0.367	0.182	0.438	-0.365	0.289	0.159	0.029	-0.032	0.033	-0.187	0.065	0.194
				0.001	0.541	0.023	0.001	0.001	0.030	0.308	0.009	0.021	0.092	0.361	0.872	0.859	0.856	0.302	0.722	0.284
CF				0.173	0.120	0.0781	-0.506	-0.419	0.065	0.072	0.282	-0.260	0.178	0.074	-0.059	-0.265	-0.131	0.200	-0.080	-0.148
				0.296	0.484	0.679	0.001	0.009	0.715	0.699	0.117	0.114	0.319	0.679	0.751	0.155	0.485	0.288	0.670	0.431
HSI					0.202	0.508	-0.215	-0.192	0.185	-0.056	0.556	-0.268	-0.073	-0.024	0.091	0.088	-0.017	-0.389	-0.114	0.364
					0.222	0.003	0.182	0.233	0.285	0.756	0.0007	0.094	0.675	0.891	0.618	0.629	0.924	0.028	0.533	0.040
SSI						0.059	-0.259	-0.335	-0.001	-0.039	0.308	0.189	0.283	-0.355	0.331	0.488	0.193	0.165	-0.156	-0.211
						0.756	0.115	0.039	0.995	0.833	0.081	0.255	0.104	0.040	0.074	0.006	0.303	0.380	0.406	0.260
Hc							-0.301	-0.293	0.055	0.286	0.555	-0.210	0.173	-0.186	0.092	0.106	0.195	-0.487	0.171	0.584
							0.094	0.103	0.780	0.138	0.003	0.247	0.385	0.349	0.642	0.594	0.325	0.010	0.389	0.001
E. leei								0.944	-0.348	-0.236	-0.314	0.174	-0.509	-0.173	0.31	0.252	-0.213	-0.118	-0.201	0.043
								2E-07	0.041	0.184	0.070	0.280	0.002	0.318	0.084	0.162	0.240	0.517	0.269	0.811
Days									-0.329	-0.321	-0.386	0.096	-0.619	-0.101	0.252	0.142	-0.292	-0.173	-0.255	0.105
									0.054	0.068	0.024	0.553	8E-05	0.562	0.163	0.436	0.104	0.341	0.157	0.565
LY										0.076	-0.042	0.007	0.177	0.234	-0.025	-0.104	-0.047	0.098	0.300	-0.036
										0.674	0.825	0.969	0.346	0.211	0.889	0.568	0.797	0.589	0.094	0.844
ACH_{50}											0.184	-0.077	0.299	-0.026	0.018	-0.055	0.0355	0.127	-0.163	-0.111
											0.354	0.667	0.120	0.893	0.923	0.766	0.848	0.491	0.377	0.548
PO												-0.196	0.147	-0.055	-0.112	0.061	0.267	-0.081	-0.006	0.087
												0.264	0.403	0.755	0.573	0.759	0.175	0.682	0.977	0.662
RB													-0.083	0.045	0.235	0.184	-0.033	0.207	0.188	-0.140
													0.635	0.797	0.194	0.310	0.858	0.253	0.301	0.442
NO														-0.056	0.154	0.282	0.222	0.174	0.208	-0.18
														0.746	0.431	0.144	0.254	0.374	0.285	0.356
TAS															-0.343	-0.469	-0.116	-0.124	0.278	0.200
															0.074	0.012	0.553	0.527	0.151	0.304
H. Ratio																0.751		0.107	-0.223	-0.127
																2E-07		0.557	0.217	0.485
H. tGSX																	0.229	0.170	-0.079	-0.182
																	0.205	0.348	0.664	0.317
H. GSSG																		0.113	0.282	-0.004
																		0.536	0.117	0.828
Int. Ratio																			0.059	
																			0.746	
Int. tGSX																				0.132
																				0.470

Supplementary Table 3. Spearman correlation coefficients in 66 V0 fish (see p. 95)

	Length	SGR	CF	HSI	SSI	Hc	*E. leei*	Days	LY	ACH_{50}	PO	RB	NO	TAS	H. Ratio	H. tGSX	H. GSSG	Int. Ratio	Int. tGSX	Int. GSSG
Weight	0.863			0.192	0.248	0.668	-0.669	-0.723	0.033	0.445	0.064	-0.329	0.040	0.113	0.265	0.250	0.043	-0.116	0.285	0.170
	2E-07			0.227	0.117	4E-06	7.6E-07	2E-07	0.837	0.004	0.695	0.036	0.805	0.508	0.171	0.189	0.814	0.524	0.113	0.348
Length		0.612		0.038	0.294	0.521	-0.508	-0.509	-0.016	0.362	-0.062	-0.205	-0.014	0.132	0.177	0.270	0.149	-0.219	0.285	0.286
		2E-05		0.811	0.062	0.001	0.0008	0.0007	0.923	0.022	0.706	0.196	0.932	0.441	0.363	0.155	0.421	0.226	0.114	0.112
SGR				0.268	0.209	0.633	-0.752	-0.807	0.037	0.608	0.222	-0.404	0.038	0.175	0.379	0.425	0.143	-0.187	0.025	0.187
				0.090	0.189	3E-05	2E-07	2E-07	0.815	3.5E-05	0.173	0.009	0.815	0.306	0.047	0.022	0.439	0.302	0.892	0.303
CF				0.240	0.031	0.461	-0.486	-0.562	0.011	0.224	0.275	-0.297	0.039	0.111	0.282	0.162	-0.027	0.163	0.161	-0.175
				0.134	0.848	0.005	0.002	0.0001	0.946	0.170	0.094	0.063	0.815	0.518	0.144	0.398	0.885	0.371	0.377	0.336
HSI					-0.159	0.265	-0.366	-0.341	-0.258	0.219	0.453	-0.403	-0.018	0.035	0.386	0.301	-0.096	0.119	-0.247	-0.175
					0.319	0.112	0.019	0.030	0.104	0.173	0.004	0.009	0.911	0.840	0.042	0.111	0.606	0.513	0.171	0.335
SSI						0.266	-0.253	-0.206	0.110	0.066	-0.251	0.174	0.077	-0.116	0.096	0.296	0.396	-0.074	0.279	0.147
						0.110	0.110	0.194	0.490	0.684	0.123	0.274	0.637	0.499	0.624	0.118	0.028	0.686	0.122	0.418
Hc							-0.486	-0.597	-0.065	0.454	0.246	-0.383	0.067	0.151	0.387	0.321	0.201	-0.159	0.352	0.153
							0.002	0.0001	0.703	0.005	0.148	0.019	0.690	0.408	0.062	0.116	0.310	0.416	0.066	0.433
E. leei								0.783	-0.110	-0.476	-0.240	0.400	-0.101	-0.122	-0.510	-0.509	0.021	0.026	-0.100	-0.022
								2E-07	0.490	0.002	0.141	0.001	0.533	0.476	0.006	0.005	0.911	0.885	0.582	0.904
Days									-0.171	-0.642	-0.205	0.396	-0.012	-0.052	-0.430	-0.400	0.010	-0.045	-0.095	0.056
									0.282	7.1E-06	0.209	0.011	0.941	0.762	0.023	0.032	0.958	0.805	0.604	0.757
LY										0.183	-0.235	0.127	-0.191	0.093	0.080	0.046	-0.100	0.011	0.103	0.026
										0.256	0.149	0.425	0.237	0.589	0.683	0.809	0.588	0.951	0.571	0.886
ACH_{50}											0.167	-0.515	-0.010	0.196	0.313	0.324	0.259	-0.037	0.192	0.071
											0.315	0.0007	0.950	0.256	0.111	0.092	0.165	0.841	0.297	0.700
PO												-0.319	-0.006	0.113	0.114	-0.030	-0.109	0.140	0.027	-0.222
												0.048	0.969	0.521	0.576	0.878	0.571	0.456	0.888	0.236
RB													0.057	-0.088	-0.460	-0.420	-0.040	-0.098	-0.049	0.088
													0.726	0.608	0.014	0.024	0.828	0.591	0.789	0.631
NO														-0.287	-0.269	-0.159	0.232	-0.298	0.407	0.350
														0.094	0.172	0.415	0.214	0.102	0.023	0.053
TAS															0.182	0.154	0.130	-0.114	0.167	0.151
															0.351	0.421	0.483	0.533	0.359	0.406
H. Ratio																0.826		0.119	-0.073	-0.189
																2E-07		0.544	0.710	0.331
H. tGSX																	0.521	0.062	0.059	-0.075
																	0.005	0.746	0.758	0.697
H. GSSG																		-0.139	0.353	0.184
																		0.454	0.051	0.319
Int. Ratio																			0.132	
																			0.467	
Int. tGSX																				0.029
																				0.872

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Effect of nutrition and *Enteromyxum leei* infection on gilthead sea bream *Sparus aurata* intestinal carbohydrate distribution

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7.0 ABSTRACT

The effect of a practical plant protein-based diet containing vegetable oils (VO) as the major lipid source on the mucosal carbohydrate pattern of the intestine was studied in gilthead sea bream *Sparus aurata* challenged with the myxosporean parasite *Enteromyxum leei*. Fish fed for 9 mo either a fish oil (FO) diet or a blend of VO at 66% of replacement (66VO diet) were exposed to parasite-contaminated water effluent. Samples of the anterior, middle and posterior intestine (AI, MI and PI, respectively) were obtained for parasite diagnosis and histochemistry. Fish were categorised as control (C, not exposed), early (E) or late (L) infected. Mucin and lectin histochemistry was applied to detect the different types of mucins and sialic acid in goblet cells (GC), the brush border and enterocytes. The number of GC stained with periodic acid Schiff (PAS), alcian blue (AB), aldehyde fuchsin-alcian blue (AF-AB), for the detection of neutral, acidic, sulphated and carboxylic mucins, and with the lectin *Sambucus nigra* agglutinin (SNA), were counted in digital images. The 66VO diet produced a significant decrease of GC with neutral and acidic mucins at AI and MI, and also of those with carboxylic mucins and sialic acid at MI. Sulphated mucins and sialic acid were less abundant at the AI than at MI and PI in C-66VO. *E. leei* infection had a strong effect on the number of GC, as E and L infected fish had a significant decrease of GC positive for all the stains versus C fish at PI. Time and diet effects were also observed, since the lowest values were mostly registered in E-66VO fish at PI. In conclusion, though GC depletion was mainly induced by enteromyxosis, an effect of the diet was also observed. Thus, the diet can be a predisposing factor that worsens the disease course.

KEY WORDS:
Goblet cell · Replacement diet · Parasite · Myxozoa · Myxosporea · Mucin · Histochemistry · Lectin

7.1 INTRODUCTION

Intensive farming practices favour the emergence of infectious diseases, posing a major problem in aquaculture industry, and these practices are likely to select for fast-growing, early-transmitted, and hence probably more virulent parasites (Mennerat et al. 2010). Parasites such as sea lice (Costello 2009), myxosporeans (Kent et al. 1994, Moran et al. 1999, Ferguson et al. 2011, Okamura & Feist 2011) or *Cryptobia salmositica* (Woo 2003) account for massive losses in fish culture. Fishborne zoonotic parasites are also acquiring worldwide relevance in aquaculture (Lima dos Santos & Howgate 2011). In the Mediterranean basin, gilthead sea bream *Sparus aurata* is the main cultured fish species, with a total production of more than 130 000 t in 2010 (APROMAR 2011), and *Enteromyxum leei* is one of its threatening parasitic diseases (Palenzuela 2006). This parasite invades the intestine of gilthead sea bream, producing a slow-progressing disease, which induces anorexia, cachexia and eventually death. Its impact is further enhanced due to its direct fish-to-fish transmission (reviewed in Sitjà-Bobadilla et al. 2007). Thus far, there are neither preventive nor curative treatments for this enteromyxosis. Therefore, there is an urgent need to advance the knowledge of the parasite invasion mechanisms and the host–parasite interaction.

The increased consumer demand for healthy, safe and high quality fish products together with the need to reduce the cost of fish feeds and the dependency on fisheries to produce aquafeeds (Tacon & Metian 2008) has led to the substitution of fish meal (FM) and fish oil (FO) by optimised levels of vegetables. The possible effects of such alternative diets have to be approached in an integrative manner, and therefore growth performance and animal health and welfare have to be studied altogether. Previous works on gilthead sea bream have demonstrated that FO can be replaced by a mixture of vegetable oils with up to 66% plant-protein based diets (66VO) without detrimental effects on growth, redox balance, immunocompetence or on the intestinal and hepatic architecture (Benedito-Palos et al. 2007, 2008, 2009, Saera-Vila et al. 2009). However, when 66VO fish were challenged with *Enteromyxum leei*, the disease outcome was greater than in FO-fed animals (Estensoro et al. 2011a).

In an effort to understand the possible underlying mechanisms involved in the worse progression of the infection in 66VO fish, we have started a series of detailed studies of the gut immunology and architecture in both diet groups. The current study is focused on the carbohydrate features of the mucus layer of the intestinal tract because the previous information on *Enteromyxum scophthalmi* and *E. leei* suggests a role of some carbohydrate moieties in the interaction with their hosts (Redondo et al. 2008, Redondo & Álvarez-Pellitero 2009). The mucosal surface of the gastrointestinal tract is a complex organisation of epithelium, immune cells and resident microbiota (McCracken & Lorenz 2001, Rombout et al. 2011). The intestinal epithelium is covered by a mucus layer, with mucins acting as the main structural component. Mucins are secreted by goblet cells (GC) and are mainly found at the periphery of epithelial cells and their extracellular environment or covering epithelial cells. Thus, they form a mesh-like structure that impedes the diffusion of offending macromolecules, constituting an immune defence barrier (Dharmani et al. 2009). Pathogens generally initiate infection by the specific recognition of host epithelia surfaces. Receptors present in the mucin layer can act as binding sites in the subsequent adhesion, which is essential for invasion. In their infection strategy, pathogens often use sugar-binding proteins, such as lectins and adhesins, to recognise and bind to host glycoconjugates where sialilated and fucosylated oligosaccharides are the major targets. In addition, microbial products can alter the production of mucins and many enteric microbes and their toxins are known to have a potent secretagogue effect on GC in mammalian models. This rapid mucin secretion can be an important mechanism of protection by eliminating the pathogens. By contrast, other pathogens induce mucus depletion, producing deleterious side effects (Moncada et al. 2003, Linden et al. 2008).

Changes in the number of GC cells as a consequence of infection have been reported in several fish–parasite models (Fleurance et al. 2008, Bermúdez et al. 2009) and qualitative or semi-quantitative analyses have been done in *Enteromyxum leei*-infected gilthead sea bream (Fleurance et al. 2008, Redondo & Álvarez-Pellitero 2010b), but no quantitative kinetic study with a high number of fish is available. Furthermore, there is no information on the effect of the diet on mucins and terminal carbohydrate residues in the intestinal mucosa of gilthead sea bream. For such purpose, in the present work, histochemistry and lectin histochemistry were applied to study the changes induced by *E. leei* infection at different times of infection and by long-term feeding with a diet with high levels of plant protein and oil sources.

7.2 MATERIALS AND METHODS

7.2.1 Experimental set up and sample collection

Gilthead sea bream were fed for 9 mo either a FO diet or a blend of VO at 66% of replacement (66VO diet) until they reached an average initial mean weight of 224 g (age = 15 mo). They were then allocated to 2 control (C) tanks and 2 recipient (R) tanks (1 for each dietary treatment). R fish were challenged by exposure to *Enteromyxum leei*-contaminated effluent as previously described (Sitjà-Bobadilla et al. 2007). Briefly, R tanks (n = 30 fish tank^{-1}) were set to exclusively receive the effluent water from another tank containing infected fish, whereas C tanks (n = 30 fish tank^{-1}) were kept under the same conditions but without receiving *E. leei*-contaminated water. In both groups (C and R), fish were kept in 5 µm filtered and UV-irradiated sea water (37.5‰ salinity) at a mean temperature of 21.3 ± 0.25 °C (range = 18.5 to 26 °C). All fish were individually tagged with passive integrated transponders and were non-lethally sampled periodically by probing their rectums with a cotton swab. Non-lethal PCR diagnosis was carried out to ascertain their infection status, as described in Palenzuela & Bartholomew (2002) with primers specific for *E. leei* rDNA. This procedure has been validated against a gold standard (histological observation of the whole digestive tract), and resulted in a high sensitivity (0.96) and specificity (1) (O. Palenzuela unpubl. data). After 102 d post exposure (dpe), 10 fish from each C group and 15 fish from each R group were euthanised under anesthesia (3-aminobenzoic acid ethyl ester, 100 mg l^{-1}) (Sigma), and samples of anterior, middle and posterior intestine (AI, MI and PI, respectively) were taken for histochemistry. For more details see Estensoro et al. (2011a).

R fish were classified in 2 categories: parasitised at early (E) or late (L) times of infection after exposure, which were compared with C animals (not exposed to the parasite). As the final prevalence of infection was high in both groups (84 and 96.2% in FO and 66VO fish, respectively), the number of non-parasitised fish was very low and not statistically useful to be included in the analysis. Fish from the E group were infected at 32 or 53 dpe and had high intensity of infection in several intestinal sections, whereas L fish were infected just 1 sampling before the end of the experiment (88 dpe) and had low infection levels in AI and MI in most cases. The mean intensity of infection was high at the PI in both diet groups but was clearly higher at the AI and MI in R-66VO fish than in R-FO ones. See Estensoro et al. (2011a) for more details.

All the experiments were carried out according to national (Royal Decrete RD1201/2005, for the protection of animals used in scientific experiments) and institutional regulations (CSIC, IATS Review Board) and the current European Union legislation on handling experimental animals.

7.2.2 Mucin and lectin histochemistry

Pieces of the AI, MI and PI intestine were fixed in 10% buffered formalin, embedded in paraffin, 4 µm sectioned and stained using the following histochemical techniques: periodic acid Schiff (PAS) to demonstrate neutral mucins (magenta-stained); alcian blue (AB) recognising predominantly acidic mucins (blue-stained); and aldehyde fuchsin-AB (AF-AB) for localisation of the sulphated (purple-stained) and/or carboxylic type (blue-stained) of acidic mucins. For the detection of *N*-acetylneuraminic acid (α2-6)galactose and *N*-acetylneuraminic acid (α2-6)-*N*-acetyl-D-galactosamine (= sialic acid), paraffin sections were collected on Super Frost-plus microscope slides (Menzel-Glaser) without additives and allowed to dry overnight. We chose to detect sialic acid because this terminal carbohydrate residue was previously shown to be modulated by enteromyxosis in gilthead sea bream intestine (Redondo & Álvarez-Pellitero 2010a).

Slides were deparaffinised and hydrated, and the endogenous peroxidase activity was blocked by incubation in hydrogen peroxide (0.3% v/v for 30 min). After rinsing with Tris-buffered saline containing 0.05% Tween20 (TTBS, 20 mM Tris-HCl, 0.5 M NaCl pH 7.2), sections were incubated with the biotinylated lectin *Sambucus nigra agglutinin* (SNA) (Sigma) solution (20 µg ml^{-1}) in TTBS for 1 h at 20°C. After rinsing, the sections were incubated with the avidin-biotin-peroxidase complex (ABC, Vector Laboratories) for 30 min at 20°C and bound peroxidase was finally revealed by adding DAB chromogen (3,3'-diaminobenzidine tetrahydrochloride) (Sigma) for 5 min. The reaction was stopped with deionised water, and the sections counterstained using Gill's haematoxylin and finally mounted in di-N-butyl-phtalate in xylene (DPX). Adequate controls were included as described in Redondo & Álvarez-Pellitero (2010a).

For each fish, intestinal section and staining, 10 microscope fields at 25× were digitally photographed and the number of positive goblet cells (GC) for each staining was counted using Photoshop's (Adobe Systems)

count tool. Thus, for each staining technique, 1500 images were processed. The mean and SEM of each group was calculated. In addition, for SNA lectin, a semiquantitative evaluation of the staining intensity in the brush border (BB) and the epithelial layer was performed. The staining intensities were evaluated on a scale of 0 to 6 (0 = no staining; 1 = very weak; 2 = weak; 3 = moderate; 4 = strong; 5 = very strong, 6 = strongest).

7.2.3 Statistics

For each intestinal section and diet, differences between the 3 infection categories (C, L, E) were analysed by 1-way analysis of variance (ANOVA-I) followed by Student-Newman-Keuls test. When the tests of normality or equal variance failed, a Kruskal-Wallis 1-way ANOVA on ranks followed by Dunn's method was applied instead. The same test was applied to determine possible differences between C fish along the 3 intestinal sections, each diet group separately. A Student's *t*-test was used to analyse the differences between both diet groups in each intestinal section and infection category. A 3-way ANOVA (ANOVA-III) was used to globally analyse the effect of the 3 factors involved in the presence of carbohydrates: the intestinal section, the time of infection and the diet. As the intensity of infection seemed to gather the effect of the 3 factors previously analysed, the strength of its possible association with the number of GC positive for each stain in individual fish was measured with a Spearman rank order correlation test (since the normality test failed for some of them) collating all the data of the 3 intestinal sections from all the diet groups. When significant correlations were found, additional ANOVA-I tests were performed to establish the differences between the different intensity of infections. All the statistical analyses were performed using Sigma Stat software (SPSS) and the significance level was set at $p < 0.05$.

7.3 RESULTS

7.3.1 Mucin histochemistry

Neutral mucins, as revealed by PAS staining, were similarly abundant in GC at the 3 intestinal sections among C animals, as no differences along the sections were observed in either of the diet groups (Figs. 1A & 2). However, C animals fed the 66VO diet had a significantly lower ($p < 0.05$) number of PAS+ GC than C-FO fish in the AI and MI. No diet effect was detected in the PI in C fish. The infection also had a clear effect, as early (E) and late (L) infected R fish had significantly lower numbers of GC than C fish, regardless of the intestinal section in the FO fed group, whilst in the 66VO group this difference was only statistically significant in the PI section. However, a clear decreasing trend was observed with the time of infection, and the lowest value was found in the PI of E infected fish fed the 66VO diet (Figs. 1A & 2).

Acidic mucins, stained by alcian blue (AB), were also abundant in GC in the 3 intestinal sections, and their distribution showed a pattern similar to that observed for neutral mucins, with no differences among C fish along the intestinal tract in any of the diet groups (Figs. 1B & 3). C-66VO animals also had a significantly lower ($p < 0.001$) number of AB+ GC at AI and MI than C-FO fish. R-infected fish (both E and L) had significantly lower values than C fish in the PI, regardless of the diet group, but only for FO group in the MI. Again, the lowest count was registered in the PI of E-R-66VO fish (Figs. 1B & 3).

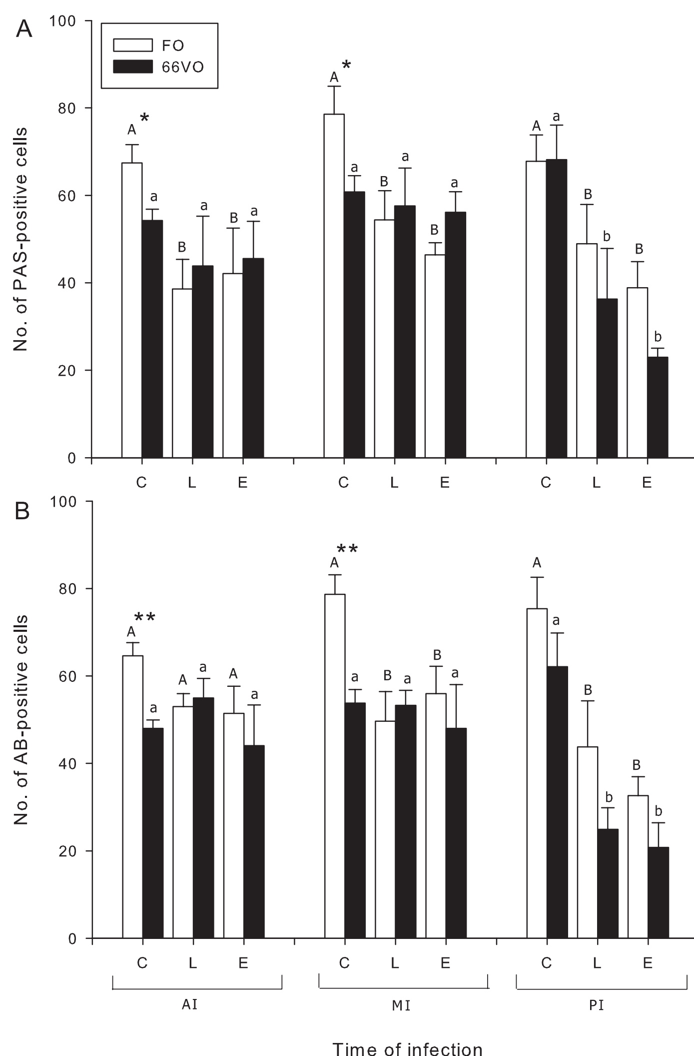


Figure 1. *Enteromyxum leei* infecting *Sparus aurata*. Number (average + SEM) of goblet cells (GC) containing (**A**) periodic acid Schiff positive (PAS+) neutral mucins or (**B**) alcian blue positive (AB+) acidic mucins in the anterior (AI), middle (MI) and posterior (PI) intestine sections for control (C), late infected (L) and early infected (E) fish. Different letters indicate significant differences ($p < 0.05$) between time of infection groups within the fish oil (FO) diet (uppercase letters) and within the vegetable oil (66VO) diet (lowercase letters). Asterisks indicate significant differences between diet groups: *$p < 0.05$, **$p < 0.001$

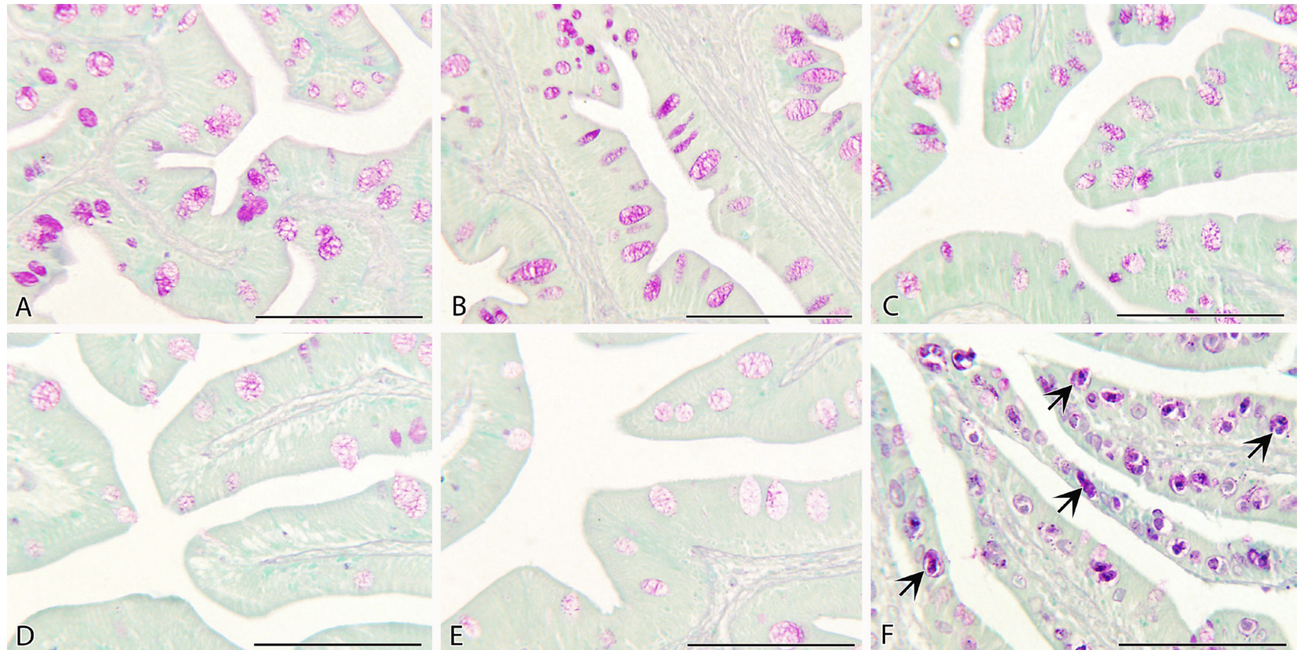


Figure 2. *Enteromyxum leei* infecting *Sparus aurata*. Photomicrographs of gilthead sea bream intestines in paraffin sections stained with periodic acid Schiff (PAS). Neutral mucins contained in epithelial goblet cells and PAS+ structures are stained magenta. (**A–C**) Control, unexposed fish fed the fish oil (FO) diet: (**A**) anterior, (**B**) middle and (**C**) posterior intestines. (**D–F**) Fish fed the vegetable oild (66VO) replacement diet: (**D**) anterior intestine of a control fish, (**E**) middle intestine of a control fish and (**F**) posterior intestine of an early infected recipient fish. Arrows: Dark stained PAS+ structures in *Enteromyxum leei* stages. Scale bars = 100 µm.

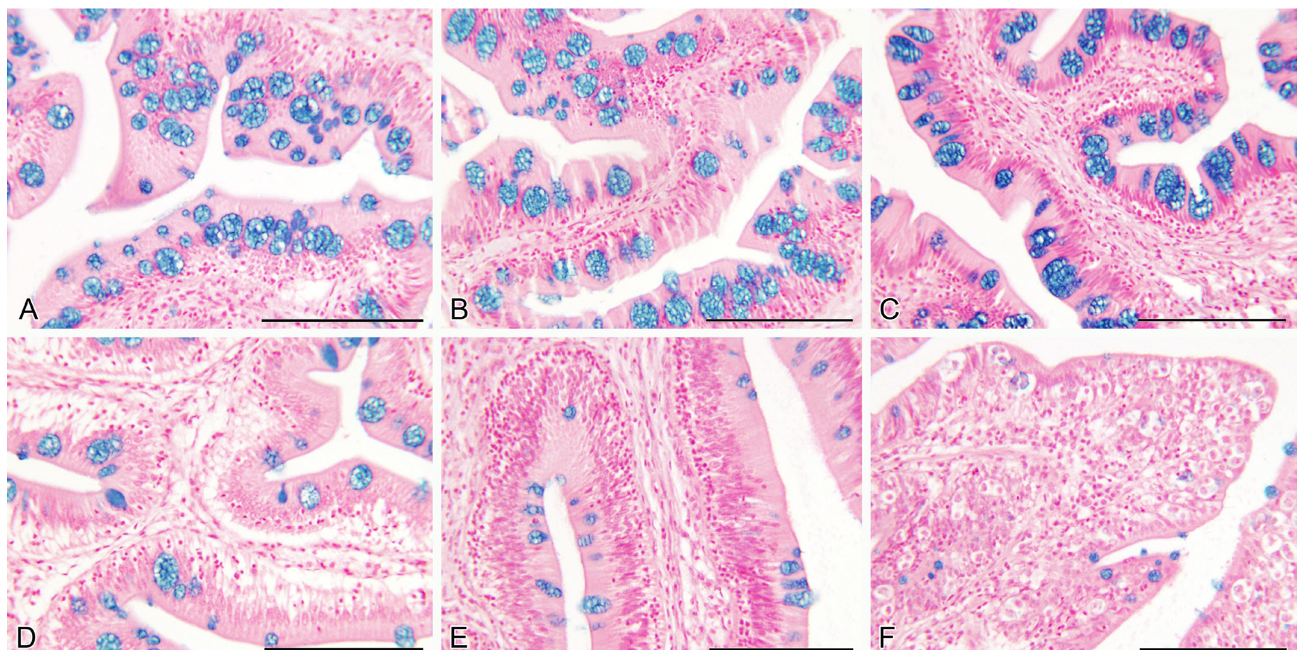


Figure 3. *Enteromyxum leei* infecting *Sparus aurata*. Photomicrographs of gilthead sea bream intestines in paraffin sections stained with alcian blue (AB). Acidic mucins contained in goblet cells are stained blue. (**A–C**) Control, unexposed fish fed the fish oil (FO) diet: (**A**) anterior, (**B**) middle and (**C**) posterior intestines. (**D–F**) Fish fed the vegetable oil (66VO) replacement diet: (**D**) anterior intestine of a control fish, (**E**) middle intestine of a control fish and (**F**) posterior intestine of an early infected recipient fish. Scale bars = 100 µm

The AF-AB staining allowed a detailed analysis of the acidic mucin types present in GC. As shown in Figs. 4 & 5, most of them were carboxylic, followed by mixed and sulphated. Carboxylic mucins presented a similar pattern of distribution in C animals in both diet groups, but a statistically significant decrease of carboxylic GC was detected in C fish fed the 66VO diet compared to C-FO group in the MI. Similarly to acidic mucins, E- and L-R infected fish had significantly lower values than C fish at the PI in the R-66VO group, whereas only E-R-FO fish had a significant decrease in this section. At MI, L-R-FO fish also had significantly lower values than C fish (Figs. 4 & 5).

Sulphated mucins were present in small amounts in the 3 intestinal sections. No statistically significant differences were detected in most comparisons, except that C-66VO fish had significantly lower values in the AI than in the MI and PI (Fig. 4B). The pattern of distribution of mixed carboxylic–sulphated GC differed from those of other stains, as only at AI, C-66VO fish had significantly higher values than C-FO ones, and E-R fish from the FO group had significantly higher values than their corresponding C group (Fig. 4C). No differences were found among the 3 intestinal sections in C animals of both diet groups.

Hardly any mucin staining was observed on the brush border and no staining was detected in enterocytes with the above techniques applied. Among acidic mucins, a size gradient was observed in positive GC; carboxylic-GC+ were larger than mixed ones, and sulphated the smallest of the three. In addition, in most parasitised sections, GC appeared smaller than in C ones (Figs. 3F & 5F).

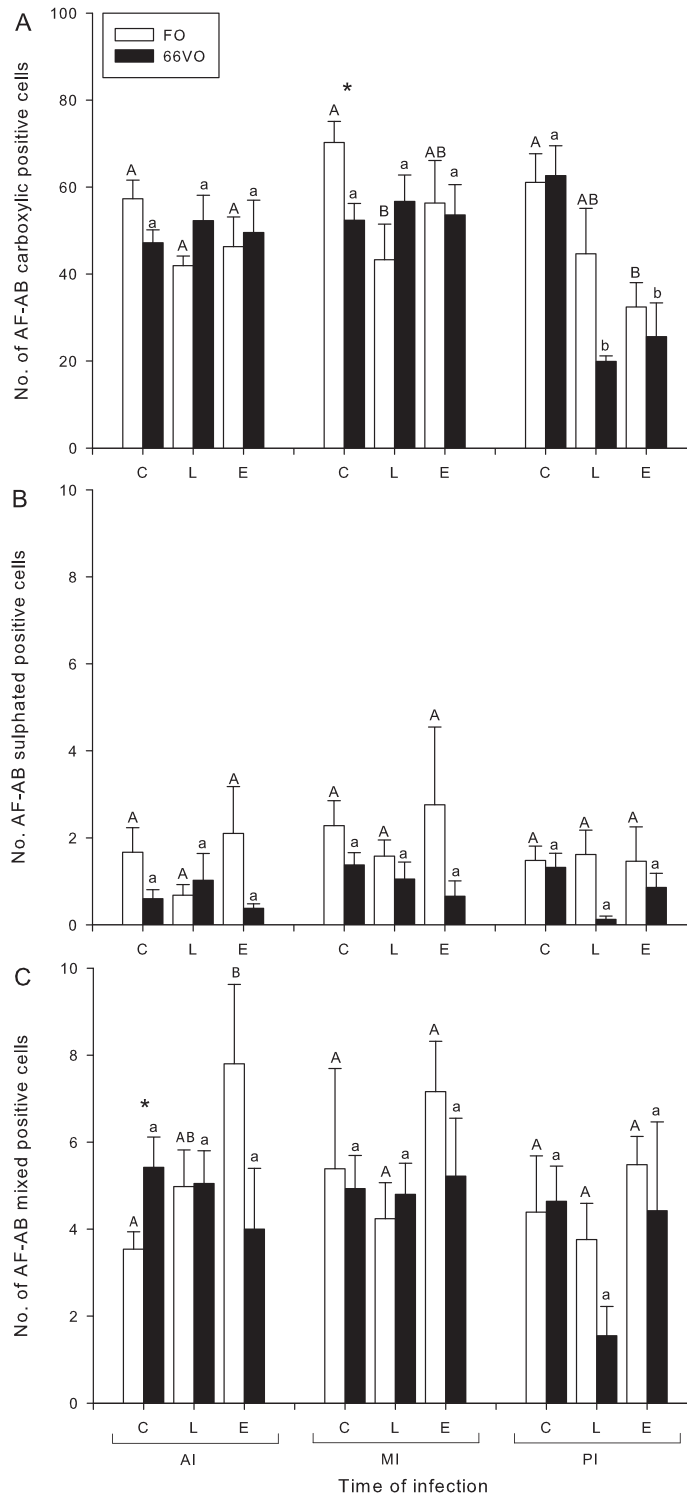


Figure 4. *Enteromyxum leei* infecting *Sparus aurata*. Number (average + SEM) of aldehyde fuchsin-alcian blue positive (AF-AB+) goblet cells (GC) containing (**A**) carboxylic, (**B**) sulphated or (**C**) mixed sulphated–carboxylic mucins in the anterior (AI), middle (MI) and posterior (PI) intestine sections for control (C), late infected (L) and early infected (E) fish. Different letters indicate significant differences ($p < 0.05$) between time of infection groups within the fish oil (FO) diet (uppercase) and within the vegetable oil (66VO) diet (lowercase letters). Asterisks indicate significant differences between diet groups: *$p < 0.05$

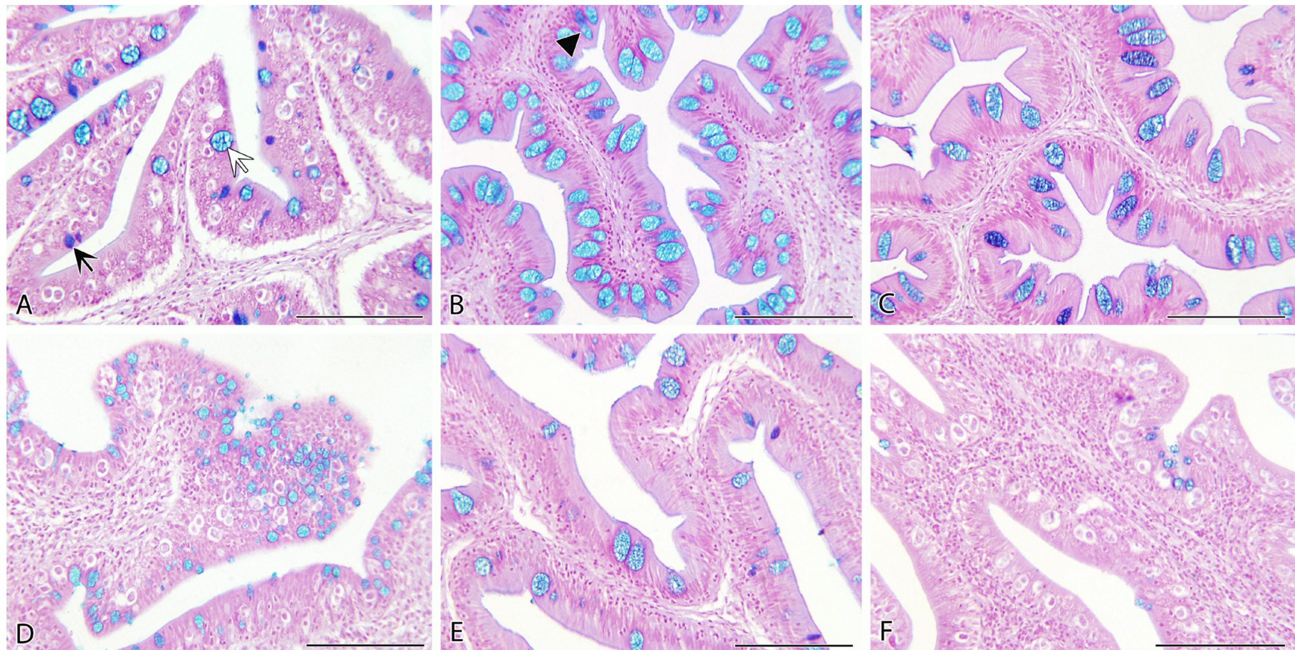


Figure 5. *Enteromyxum leei* infecting *Sparus aurata*. Photomicrographs of gilthead sea bream intestines in paraffin sections stained with aldehyde fuchsin-alcian blue (AF-AB). Carboxylic, sulphated and mixed carboxylic–sulphated acidic mucins contained in goblet cells are stained (white arrow) blue, (black arrow) purple or (black triangle) blue-purple, respectively. (**A–C**) Fish fed the fish oil (FO) diet: (**A**) anterior intestine of an early infected recipient fish, (B) middle intestine of a control fish and (**C**) posterior intestine of a control fish. (**D–F**) Fish fed the vegetable oil (66VO) replacement diet: (**D**) anterior intestine of an early infected fish, (**E**) middle intestine of a control fish and (**F**) posterior intestine of an early infected fish. Scale bars = 100 µm

7.3.2 Lectin histochemistry

Sialic acid was detected with the SNA lectin in GC, the BB and the enterocytes in the 3 intestinal sections, though with clear differences among them. In C-66VO fish, the values obtained in the AI were significantly lower than those of MI and PI, and the number of SNA+ GC at MI was significantly lower than that in C-FO fish (Figs. 6 & 7). The infection produced a significant decrease of SNA+ GC at PI both in E- and L-R fish regardless of the diet group, but only in the FO group at the MI. Again the lowest value was for the PI of E-R-66VO (Figs. 6 & 7).

The intensity of staining for sialic acid in BB and the apical part of enterocytes was significantly lower at AI than at MI and PI, regardless of the diet, and no differences were observed between diet groups within each intestinal section (Figs. 6 & 7). The infection significantly reduced the staining intensity of the BB in E-R-FO at MI and AI (Fig. 6) and that of the apical enterocyte layer in E- and L-R-66VO at PI (Fig. 6).

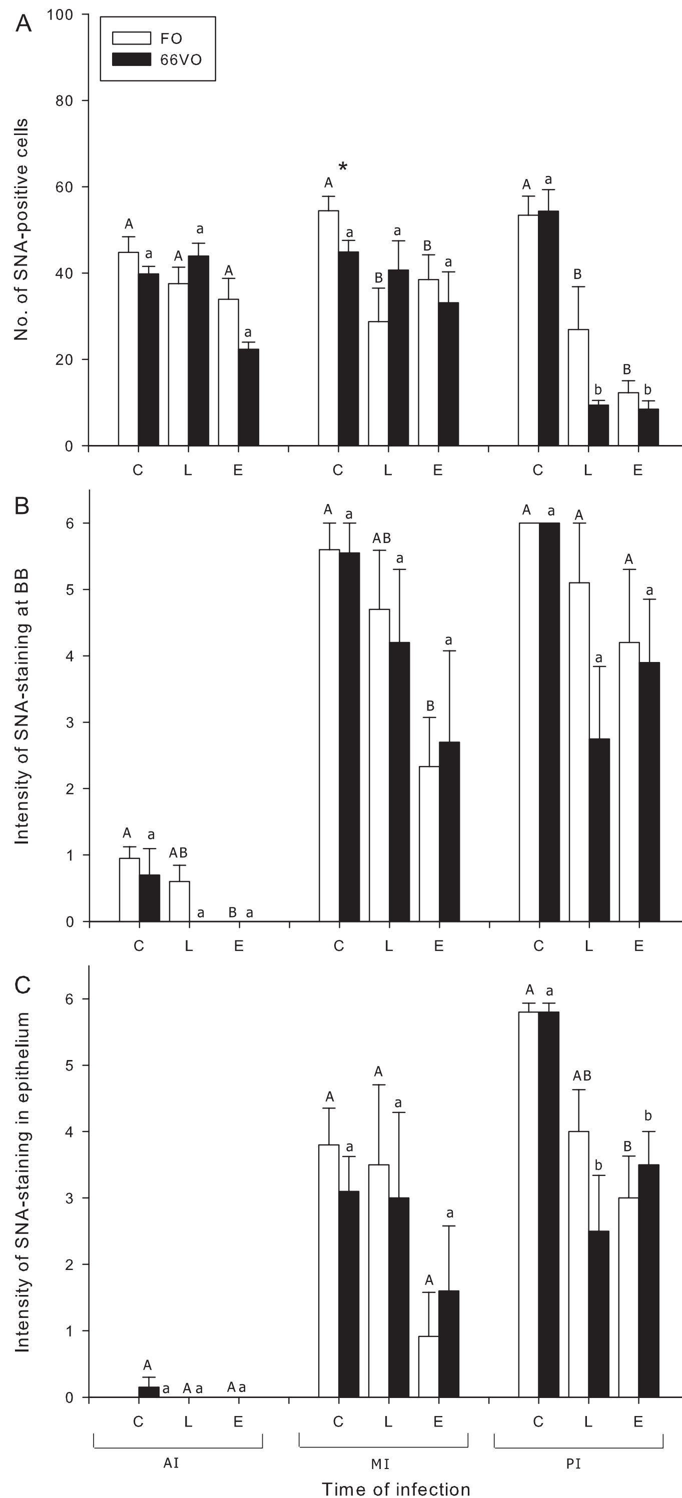


Figure 6. *Enteromyxum leei* infecting *Sparus aurata*. Number (average + SEM) of *Sambucus nigra agglutinin* positive (SNA+) staining for sialic acid in (**A**) goblet cells (GC), (**B**) brush border (BB), or (**C**) the epithelium in the anterior (AI), middle (MI) and posterior (PI) intestine sections for control (C), late infected (L) and early infected (E) fish. Significant differences ($p < 0.05$) between time of infection groups within the fish oil (FO) diet in uppercase and within the vegetable oil (66VO) diet in lowercase. Asterisks indicate significant differences between diet groups: *$p < 0.05$

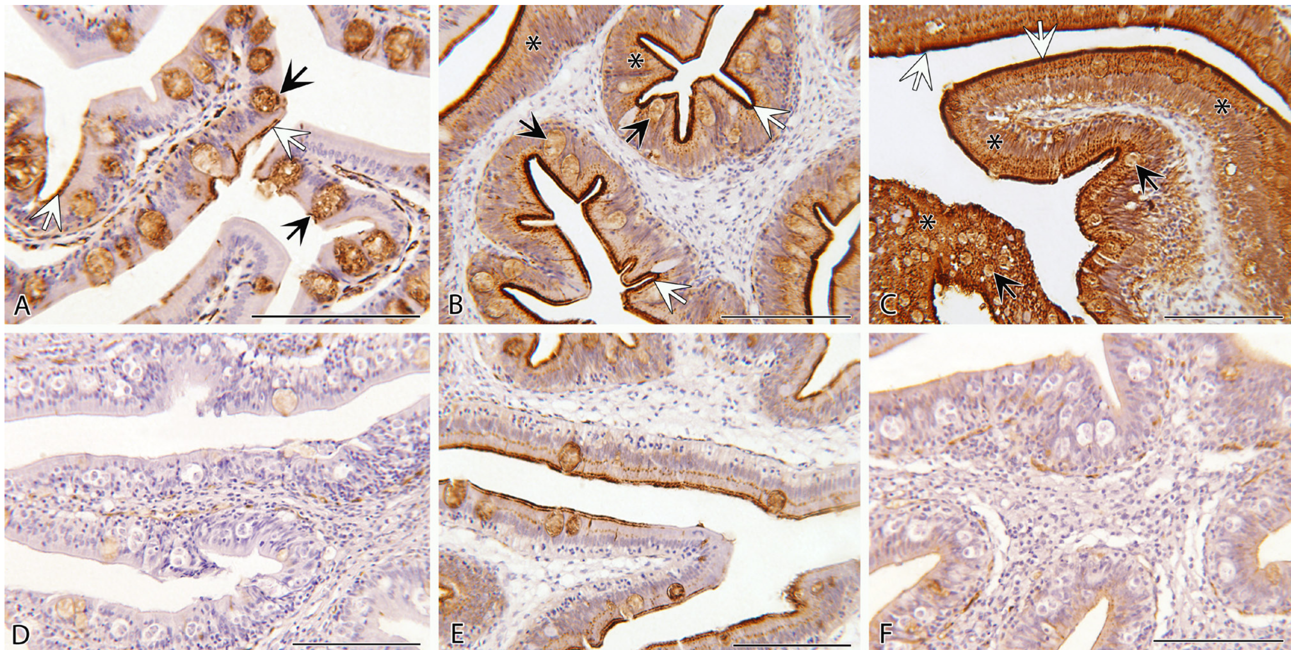


Figure 7. *Enteromyxum leei* infecting *Sparus aurata*. Photomicrographs of gilthead sea bream intestines in paraffin sections stained with the biotinylated lectin *Sambucus nigra agglutinin* (SNA). Lectin-labeled sialic acid contained in (black arrow) goblet cells, (*****) the enterocytes and (white arrow) the brush border appears brown in colour. (**A–C**) Control, unexposed fish: (**A**) anterior intestine of a fish fed the fish oil (FO) diet, (**B**) middle intestine of a FO fish and (**C**) posterior intestine of a vegetable based diet (66VO) fish. (**D**) Anterior intestine of an early infected FO fish. (**E**) Middle intestine of a control 66VO fish. (**F**) Posterior intestine of a late infected 66VO fish. Note the decrease of SNA labeling in parasitised sections (**C**, **F**) and in the middle intestine of control 66VO (**E**). Scale bars = 100 µm

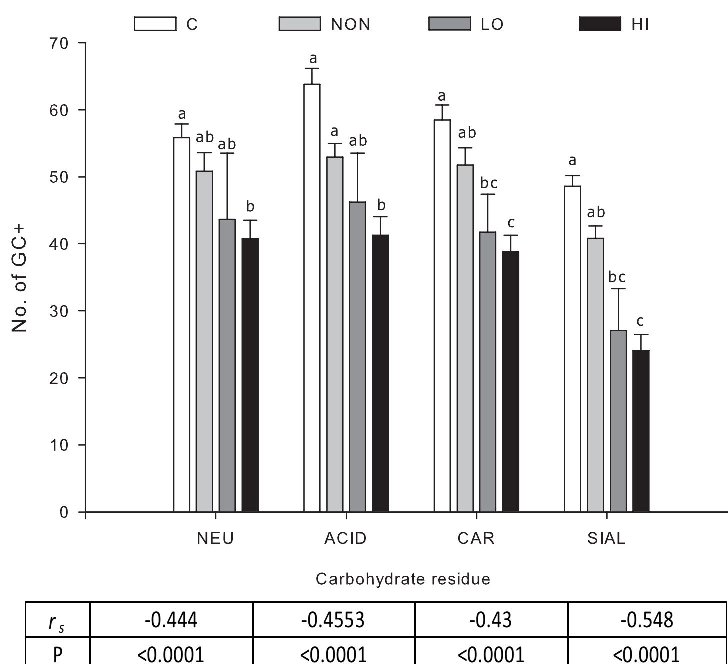
7.3.3 Meta-analysis of the factors affecting carbohydrate distribution

The ANOVA-III allowed a global and complex analysis of the relationship between the 3 studied factors involved in mucin presence: diet, time of infection and intestinal section. Table 1 summarises the significance of these 3 factors for each of the applied stains. Intestinal section resulted in an effect in 5 out of the 7 staining patterns analysed, but it only explained up to 8.46% of the total data variance. When trying to isolate which group differed from the others, in acidic and carboxylic mucins, PI had significantly lower values than AI and MI, whereas for sialic acid in BB and enterocytes, AI values were significantly lower than those of MI and PI. Time of infection was a strong factor with statistical significance in 5 out of 7 staining patterns, and in most cases it was due to the lower values of E- and L-infected fish versus C animals. It was the strongest factor, as it explained up to 26.08% of the total data variance, and p-values were <0.001 in all cases. Diet accounted for the global significant differences only for acidic and sulphated mucins, and it explained up to 8.42% of the total data variance.

Significant differences between diet groups were due to the lower values of 66VO. As can be seen in Table 1, there were also significant interactions between the different combinations of 3 factors for several stains; the effect of different levels of a given factor depended on what level of another factor was present. The most common interaction was between infection time and intestinal section. No significant interaction was found between diet and time of infection, and diet and intestinal section. However, triple interactions were significant for carboxylic mucins and sialic acid found in GC, the former explaining 7% of total data variance. Intensity of infection was correlated negatively with the number of GC+ for neutral, acidic, carboxylic mucins and sialic acid ($p < 0.0001$), being the strongest correlation with sialic acid ($r_S = -0.548$) (Fig. 8). The intestinal sections with the highest intensity of infection had the lowest GC+ counts for neutral, acidic, carboxylic mucins and sialic acid, and differed significantly from sections of C fish and even from non-infected sections of R-fish except for neutral mucins (Fig. 8).

Table 1. *Enteromyxum leei* infecting *Sparus aurata*. P-values (<0.05) and calculated % for each sum of squares for the 3-way analysis of variance (ANOVA-III) applied to data from mucin, including periodic acid Schiff (PAS) for neutral mucins; alcian blue (AB) for acidic mucins; and aldehyde fuchsin-AB (AF-AB) to differentiate sulphated, carboxylic and mixed sulphated–carboxylic mucins, and lectin *Sambucus nigra agglutinin* (SNA) for sialic acid histochemistry in the 3 intestinal sections of gilthead sea bream fed with the 2 diets and exposed or not to *Enteromyxum leei.* Residue was localised either to the goblet cells (GC) or the epithelium. The 3 factors used in the analysis were diet (fish oil: FO; blend of vegetable oil at 66% of replacement, 66VO), intestinal section (anterior, AI; middle, MI; posterior, PI) and time of infection (Time; control, C; early infection, E; late infection, L). The analysis was not applied to sialic acid in the brush border (BB) as normality and the equal variance tests failed. ns: non-significant (p > 0.05).

CARBOHYDRATE RESIDUE & LOCALIZATION	TIME		INTESTINAL SECTION		DIET		TIME x DIET	TIME x SECTION		DIET x SECTION	TIME x SECTION x DIET	
	p	%	p	%	p	%	p	p	%	p	p	%
Neutral mucins in GC	< 0.001	26.08	0.016	4.96	ns	ns	ns	ns	ns	ns	ns	ns
Acidic mucins in GC	< 0.001	23.09	0.002	6.43	< 0.001	5.83	ns	< 0.001	11.66	ns	ns	ns
Carboxylic mucins in GC	< 0.001	15.40	0.001	8.46	ns	ns	ns	0.004	9.87	ns	0.026	7.00
Mixed mucins in GC	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Sulphated mucins in GC	ns	ns	ns	ns	0.002	8.42	ns	ns	ns	ns	ns	ns
Sialic acid in GC	< 0.001	26.08	< 0.001	8.22	ns	ns	ns	< 0.001	16.30	ns	0.043	3.97
Sialic acid in the epithelium	< 0.001	8.18	< 0.001	43.12	ns	ns	ns	< 0.001	6.26	ns	ns	ns



r_s	-0.444	-0.4553	-0.43	-0.548
P	<0.0001	<0.0001	<0.0001	<0.0001

Figure 8. *Enteromyxum leei* infecting *Sparus aurata*. Number (average + SEM) of goblet cells positive (GC+) for neutral (NEU), acidic (ACID) and carboxylic (CAR) mucins and sialic acid (SIAL) in the intestinal sections with no infection either from control (C) or recipient (R) fish (NON), or low (LO) or high (HI) intensity of infection in R fish. Data from both diet groups and the 3 intestinal sections have been combined. Within each type of carbohydrate residue, different letters indicate statistically significant differences ($p < 0.05$). Spearman correlation coefficients (r_S) and p-values are indicated below for each carbohydrate.

7.4 DISCUSSION

There is a growing interest in formulating diets with low levels of marine ingredients that are still capable of promoting the growth and health of farmed fish. The response to such plant-ingredient based fish diets is still far from being completed, especially with regard to health aspects (reviewed in Harikrishnan et al. 2011, Tacchi et al. 2011). The present study was focused on one aspect of gut health, the mucin pattern of the intestine of gilthead sea bream, because of the importance of mucins in many disease processes in which the interaction of epithelial cells and their surroundings has been altered (Gendler & Spicer 1995, Perez-Vilar & Hill 1999). Mucins are large, abundant, filamentous, highly glycosylated glycoproteins that consist of 80% carbohydrates, primarily *N*-acetylgalactosamine, *N*-acetylglucosamine, fucose, galactose, and sialic acid (*N*-acetylneuraminic acid) and traces of mannose and sulfate. Mucins are present in the piscine intestine very early in larval development (Leknes 2011), during which they may be involved in absorption and transport of macromolecules (Stroband et al. 1979) and may also exert an osmotic function, especially in marine species (Smith 1989). The glycoconjugate composition of mucous secretion in fish is remarkably different among species, and intraspecific (Domeneghini et al. 1998, Sarasquete et al. 2001) and age (Domeneghini et al. 1998, Parillo et al. 2002, Soffientino et al. 2006) variations also occur.

We have shown the effect of both the diet and *Enteromyxum leei* infection on the carbohydrate pattern of the intestine of gilthead sea bream. First, in C fish (those not exposed to the parasite), the 66VO diet produced a significant decrease of GC with neutral and acidic mucins at AI and MI and also of those with carboxylic mucins and sialic acid at MI. Remarkably, depletion effects were found in the intestinal sections that become infected later during the progression of the infection, and these sections had higher prevalence and intensity of infection in R-66VO fish than in FO ones (Estensoro et al. 2011a). There is a general consensus that acidic mucins, such as sialomucin and sulfomucin, play an important role in the protection of mucosa from infectious agents. This is illustrated by *Strongyloides venezuelensis* infections, in which sulphated glycoconjugates prevent the mucosal invasion by this nematode (Maruyama et al. 2000, 2002). Therefore, the suggestion that the higher levels of GC with such mucins in FO gilthead sea bream could somehow protect these intestinal sections from parasite invasion, or at least delay its entrance, is tempting.

Feeding habits seem to be correlated with the pattern of glycoconjugate glycosylation in different cyprinid fish (Fiertak & Kilarski 2002). However, there is almost no information on the specific effect of diet composition on mucin pattern in the intestine of fish, and most studies are focused on the effects on intestine morphometry (Escaffre et al. 2007), intestinal fatty acid uptake (Geurden et al. 2009) and digestive enzymes (Santigosa et al. 2008, Silva et al. 2010). The only remarkable findings are the increased number of cells secreting acidic mucins, associated with reduced gut bacterial translocation and improved resistance to *Vibrio alginolyticus* in European sea bass *Dicentrarchus labrax* fed with mannan oligosaccharides (MOS) (Torrecillas et al. 2007, 2011a,b). In other animal models, feeding an enzyme-supplemented diet led to changes in the mucin composition and carbohydrate expression of GC glycoconjugates, which were associated with a reduction in intestinal viscosity and decreased numbers of the bacteria *Campylobacter jejuni* in chicks (Fernández et al. 2000). However, broiler chickens receiving a plant-protein-based feed had significantly less intestinal colonization with this bacterium (Udayamputhoor et al. 2003). In weaned piglets fed a carboxymethylcellulose enriched diet, an increase in their ileal GC, mucin production and intestinal content viscosity was experienced, suggesting an improved protection against pathogens in the digestive tract (Piel et al. 2005). In the present study, there was no such stimulatory effect of the 66VO diet on GC in C fish, but rather the opposite effect was found. In any case, changes in digesta viscosity and their possible lubricant, digestive or protective effects, remain to be studied in gilthead sea bream. Other possible changes in the gut physiology induced by the 66VO diet also merit further study, similar to those already shown in trout fed vegetable oil-based diets, which displayed a higher paracellular leakage in the intestinal epithelium than fish fed the control diet (Geurden et al. 2009).

At the same time, the infection with *Enteromyxum leei* produced a significant decrease of GC+ for all the stains applied and for sialic acid at the apical part of enterocytes at PI, the target site of the parasite. The effect of the infection was also detected in FO fish in the AI for neutral mucins and for sialic acid in the BB of E-infected fish, and in the MI for neutral, acidic mucins and sialic acid in GC. In the affected intestinal areas, GC were not only less numerous but also smaller, and were even absent in heavily parasitised areas of the PI. This was also observed by Fleurance et al. (2008) in gilthead sea bream and turbot *Psetta maxima* with advanced *E. scophthalmi* infections, whereas an increase in GC occurred in mild infections (Bermúdez et al. 2009). Previous studies have shown that prevalence and intensity of infection were higher in the 66VO group than the FO one, with a higher percentage of fish harbouring the parasite in the entire intestinal tract and a faster establishment of the parasite (Estensoro et al. 2011a). This is in agreement with the fact that the lowest values of GC were mostly registered in early infected 66VO fish at PI and with the observed negative correlation between the intensity of infection and the number of GC with neutral, acidic and carboxylic mucins and sialic acid. Therefore, we can conclude that as time post-infection passed, the intensity of infection increased, the number of infected intestinal sections increased and the number of GC decreased.

Stimulation of the production of intestinal mucins has been widely shown for some nematode (Karlsson et al. 2000, Else 2005, Patel et al. 2009) and bacterial (Bergstrom et al. 2008) infections in mammalian models. Hyperplasia and hypertrophy of GC were evident in some enteric helminthiasis in brown trout *Salmo trutta*, with changes in the composition of the mucus and a significant increase in the number of GC staining positively for acid glycoconjugates, particularly close to the site of attachment (Bosi et al. 2005, Dezfuli et al. 2010). The number of GC was also raised in parasitised segments of eel *Anguilla anguilla* digestive tract, with an increase in the number of acid mucin-secreting cells (Dezfuli et al. 1997). Such increases are believed to contribute to the expulsion of enteric helminthes. However, the opposite GC depletion phenotype observed in *Enteromyxum leei*-infected fish could be due to the death or functional alteration of this cell type and implies a reduction of mucins released to the glycocalyx. The direct histopathological damage invoked by the myxosporean, which ends up occupying most of the mucosal intestinal surface, could explain such depletion. This GC reduction has also been reported in *Echinostoma caproni* infections (Fujino & Fried 1993) and in clinically important enteric pathogens, such as *Shigella* (Steinberg et al. 1975, Sachdev et al. 1993), *Campylobacter* (Lambert et al. 1979) and *Citrobacter rodentium* (Bergstrom et al. 2008).

Comparisons are difficult since *Enteromyxum leei* dwells in the paracelullar space of the intestinal epithelium and the above cited cases refer to pathogens inhabiting the intestinal lumen or attached to the epithelial surface. In any case, in *Citrobacter rodentium*, depletion of mucus-containing GC correlates with peak bacterial colonization, as happens in the current fish–parasite model with the highest intensity of infection. The biological consequences of the functional modulation of GC are unclear. Down-regulation of genes controlling GC-derived mucins could compromise the host defence when an animal is challenged with a bacterial pathogen. However, reducing mucin production might be important for reducing energy sources

for pathogenic bacteria that use carbohydrate-laden mucins as a food source (Bergstrom et al. 2008). In fact, the glycosylation pattern of isolated intestinal mucus was changed in gilthead sea bream parasitised by *E. leei* and bacterial adhesion to it was reduced (Estensoro et al. 2011b). Further studies should determine whether changes in the intestinal bacterial population occur in *E. leei*-parasitised gilthead sea bream. GC depletion in some enteropathogenic bacteria can also be mediated by components of the host immune system, such as some pro-inflammatroy cytokines and T-cells (Arnold et al. 1993, Bergstrom et al. 2008, Linden et al. 2008). The observed slight decrease in the number of GC in non-infected sections of fish that harbour the parasite in other sections could suggest certain immune modulation. In *Enteromyxum leei* chronic infections, several immune factors have been shown to be modulated. IL-1β and TNF-α expression were depleted in the intestine and some serum innate factors were significantly decreased in R fish (Sitjà-Bobadilla et al. 2008, Estensoro et al. 2011a), whereas others such as the respiratory burst in circulating leukocytes (Estensoro et al. 2011a) or the number of immunoglobin M (IgM) positive cells in the intestine (Estensoro et al. 2011c) were increased. Furthermore, in a global molecular profiling of *E. leei*-parasitised gilthead sea bream, a marked down-regulation of the host immune system was detected (Davey et al. 2011). This was suggested to be a mechanism of immune evasion, as described for other fish and mammalian parasites (see Sitjà-Bobadilla et al. 2008), but further studies are needed to determine the possible connection between such immunodepresion and GC depletion.

There were no significant differences in the number of GC for most of the stains applied among the 3 intestinal sections in C fish, regardless of the diet, except for sulphated mucins and sialic acid of C-66VO fish, which had lower values at AI than at MI and PI. Neutral and acidic mucins were common in GC, and carboxyilic mucins were the most abundant among acidic ones, followed by mixed and sulphated mucins. Among acidic mucins, a size gradient was observed in GC, in which carboxylic-positive GC were larger than mixed ones and sulphated the smallest ones. Generally, the mucin type in intestinal GC seems to be highly specific to each teleost species. Thus, in shi drum *Umbrina cirrosa*, GC are filled mainly with sulphated mucins (Pedini et al. 2001), whereas in common dentex *Dentex dentex* neutral mucosubstances dominate in the AI (Carrassón et al. 2006). In turbot, neutral mucins also dominate in the digestive tract and acidic mucins are not present (Redondo & Alvarez-Pellitero 2010a). The most common observation is that acidic and neutral mucins dominate and few acid mucopolysaccharides possess sulphate groups, while the majority are carboxylic, as in the current study (Scocco et al. 1997, Domeneghini et al. 1998, 2005, Fiertak & Kilarski 2002, Park et al. 2003, Leknes 2010). The coexistence of neutral and acid glycoconjugates probably reflects different ages or stages of differentiation for GC (Elbal & Agulleiro 1986, Murray et al. 1996, Leknes 2010). The same hypothesis could be applied for the different types of acidic mucins found in GC in the present study, and GC containing mixed carboxylic–sulphated mucins could be a transient stage from carboxylic to sulphated or vice versa. However, such results may also suggest a true cellular heterogeneity in the population of GC (see Leknes 2010).

In conclusion, changes in mucin composition and GC abundance in anterior and middle sections of the intestine of gilthead sea bream fed the 66VO diet appear to be one of the factors that make this diet as a predisposing cause that worsens the course of the disease when fish are exposed to *Enteromyxum leei*, the precipitating cause. These results together with the recent finding that some lectins inhibit the attachment and invasion of *E. scophthalmi* stages to the intestinal epithelium of turbot (Redondo & Álvarez-Pellitero 2010b) open the door to the development of diets potentially capable of inducing mucin changes of fish intestine that avoid parasite adhesion and penetration, and therefore could contribute to the control of enteromyxosis. In addition, future studies should focus on the expression of intestinal mucin genes in response to parasites and on additional changes in gut physiology induced by dietary vegetable oils that could facilitate parasite invasion and proliferation, such as membrane fluidity and paracellular permeability.

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08

Effects of *Enteromyxum leei* (Myxozoa) infection on gilthead sea bream (*Sparus aurata*) (Teleostei) intestinal mucus: glycoprotein profile and bacterial adhesion

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8.0 ABSTRACT

The intestinal myxosporean parasite *Enteromyxum leei* causes severe desquamative enteritis in gilthead sea bream (*Sparus aurata*) (Teleostei) that impairs nutrient absorption causing anorexia and cachexia. In fish, as in terrestrial vertebrates, intestinal goblet cells are responsible for the adherent mucus secretion overlying epithelial cells, which constitutes a first line of innate immune defense against offending microorganisms but serves also as substrate and nutrient source for the commensal microflora.

The secreted intestinal mucus of parasitized (n = 6) and unexposed (n = 8) gilthead sea bream was isolated, concentrated, and subjected to downward gel chromatography. Carbohydrate and protein contents (via PAS and Bradford stainings), terminal glycosylation (via lectin ELISA), and *Aeromonas hydrophila* and *Vibrio alginolyticus* adhesion were analyzed for the isolated intestinal mucins. Parasitized fish, compared with unexposed fish, presented intestinal mucus mucins with a lower glycoprotein content and glycosylation degree at the anterior and middle intestine, whereas both glycoprotein content and glycosylation degree increased at the posterior intestine section, though only significantly for the total carbohydrate content. Additionally, a slight molecular size increase was detected in the mucin glycoproteins of parasitized fish. Terminal glycosylation of the mucus glycoproteins in parasitized fish pointed to an immature mucin secretion (*N*-acetyl-α-D-galactosamine increase, α-L-Fucose and neuraminic-acid-α-2-6-galactose reduction). Bacterial adhesion to large-sized mucus glycoproteins (>2,000 kDa) of parasitized fish was significantly lower than in unexposed fish.

KEY WORDS:
Marine fish · Aquaculture · Intestinal parasite · Mucosal immunity · Mucin · Glycosylation

8.1 INTRODUCTION

Mucus acts as a first-line protective, physical barrier on exposed body surfaces, avoiding erosion and dehydration of the underlying epithelia as well as contact with offending pathogens. This biofilm is a mesh-like network of heavily *O*-glycosylated and densely packed mucin glycoproteins combined with other bioactive molecules, like immunoglobulins, lysozyme, and lectins in piscine mucus (Balebona et al. 1995; Huang et al. 2011; Magnadottir 2006). Goblet cells, present in intestinal epithelia of all vertebrate groups and in the epidermis of aquatic vertebrates, like fish and amphibians, synthetize and secrete these mucin polymers also in teleosts (Carrasson et al. 2006; Fleurance et al. 2008; Neuhaus et al. 2007a; Neuhaus et al. 2007b; Van der Marel et al. 2010; Leknes 2010; Redondo and Álvarez-Pellitero 2010; Losada et al. 2012; Estensoro et al. 2012a).

In mammals, the gastrointestinal mucus layer consists of two distinct sublayers, the inner, firmly adherent and virtually sterile, and the outer loosely adherent, which is associated with the commensal microflora (Bergstrom et al. 2010; Kim and Ho 2010). Adherence of pathogenic, enteric organisms from the luminal environment to the mucosal surface opens the door for colonization and initiation of infective diseases. Some light has been thrown on the specific interactions occurring during the colonization of mucus substrates by bacteria and parasites (Tse and Chadee 1991; Hicks et al. 2000; Theodoropoulos et al. 2001; Schroers et al. 2008; Redondo and Álvarez-Pellitero 2009; Álvarez-Pellitero 2011). Pathogenic and commensal microorganisms use the mucosal sugar moieties of the mucus glycoproteins as receptors for attachment. Primary host control of the pathogen burden in the outer mucus layer occurs through shedding and renewal of the mucus secretion. On a second line, host-mediated factors such as depth and viscosity of the mucus layer or mucin glycosylation pattern and polypeptide backbone structure, influence early host-pathogen interactions and therefore the course of infection (Bergstrom et al. 2010).

Quantitative changes of the fish mucus secretion during pathogenesis include increased mucus synthesis and secretion leading to an entrapping and expulsion effect (Lodemel et al. 2001; Neuhaus et al. 2007a; Schroers et al. 2009; Van der Marel et al. 2010; Dezfuli et al. 2010; Torrecillas et al. 2011) as well as depletion of goblet cells and thus reduced mucus secretion in other cases (Redondo and Álvarez-Pellitero 2010). An acute, massive mucus secretion followed by mucus depletion as detrimental side effect during chronic infections may occur (Kim and Ho 2010). Additionally, qualitative alterations of the mucus, mainly in its mucin glycosylation pattern, in response to parasite infections also occur (Roberts and Powell 2005; Neuhaus et al. 2007a; Schroers et al. 2009; Van der Marel et al. 2010; Redondo and Álvarez-Pellitero 2010; Álvarez-Pellitero 2011).

The myxosporean parasite *Enteromyxum leei* is responsible for high rates of morbidity and mortality in Mediterranean farmed sparids, such as gilthead sea bream (GSB) (*Sparus aurata*) (Palenzuela 2006; Rigos and Katharios 2010). This widespread parasite causes severe chronic enteritis by penetrating and invading the intestinal epithelium, which loses its palisade cellular organization suffering occasional desquamation (Álvarez-Pellitero et al. 2008; Fleurance et al. 2008; Cuadrado 2009; Estensoro et al. 2011). The impairment of nutrient absorption provokes anorexia leading to a reduced growth performance, followed by cachexia and even death in affected fish (Sitjà-Bobadilla et al. 2008; Estensoro et al. 2010). The lack of preventive and therapeutic treatments for this parasitosis together with its devastating effect on high-density stocking conditions due to its direct fish-to-fish transmission (Diamant 1997; Sitjà-Bobadilla et al. 2007) stresses the importance of understanding the mechanisms of this host-parasite interaction.

The aim of the present study was to characterize the biochemical alterations of the secreted intestinal mucus mucins of GSB in response to an *E. leei* infection and to determine the possible effect on microbial adhesion. Thus, we try to evaluate the effects of this parasitosis on the mucosal intestinal barrier and bacterial adhesion.

8.2 MATERIALS AND METHODS

8.2.1 Fish

Parasite-free and clinically healthy gilthead sea bream (*S. aurata*) from a commercial fish farm were kept in 5 µm-filtered and UV-irradiated sea water at 18° C. They were used as control (C) fish. Recipient (R) fish obtained from this stock were exposed to *E. leei*-contaminated effluent using the method already described (Sitjà-Bobadilla et al. 2007). After 137 days of exposure, C and R fish were starved for 2 days and then euthanized by overexposure to MS-222 (Sigma, St. Louis, MO, USA). The parasitological status of R fish was checked in fresh smears of the anterior, middle, and posterior intestine scrapings by light microscopy. In order to have a very homogeneous group of samples, only those R fish (n = 6) diagnosed with the highest intensity of infection at the posterior intestine (>100 parasite stages per microscope field at x250 magnification) were selected for the subsequent analyses (Table 1). According to our previous experience, the presence of the parasite in the gut scrapings correlates very well with the presence of the parasite in histological sections when the infection is well established. Samples from eight randomly chosen C fish were also taken. Body and intestinal weights of all the fish were registered.

Table 1. Body weight, intestine weight, and intensity of infection of the sampled fish.

FISH WEIGHT (g)	INTESTINE WEIGHT (g)	INTENSITY OF INFECTION		
		Ai	*Mi*	*Pi*
208	2.6	-	-	6+ (SP > DSB)
204	3.2	-	-	6+ (SP > DSB)
137	2.9	-	-	6+ (SP > DSB)
222	3.6	-	-	6+ (SP > DSB)
182	3.6	6+ (SP > DSB)	3+ (SP, DSB)	6+ (SP > DSB)
234	2.2	-	-	6+ (SP > DSB)
202	3.1	-	-	-
208	2.5	-	-	-
172	2.3	-	-	-
175	3	-	-	-
149	1.9	-	-	-
186	2.5	-	-	-
180	2.5	-	-	-
188	2.9	-	-	-

Intensity of infection evaluated on fresh smears of anterior (*Ai*), medium (*Mi*), and posterior intestine (*Pi*) is indicated according to a semiquantitative scale ranging from 1+ to 6+ depending on the parasite stages found per microscope field at 250 magnification (range, 1+ = 1-5; 2+ = 6-10; 3+ = 11-25; 4+ = 26-50; 5+ = 51-100; 6+ > 100). The observed parasite stages were spores (*SP*) and disporoblasts (*DSB*) and their relative abundance shown by *greater than sign. Minus sign* indicates absence of parasite.

8.2.2 Mucus isolation

Each intestine was subdivided into two samples processed individually, one containing the anterior and middle intestine sections (Ai and Mi) and the other containing the posterior intestine section (Pi). Isolation of secreted luminal mucus glycoproteins from the intestinal sections followed the procedure described in Neuhaus et al. (2007b) and Schroers et al. (2009). Briefly, intestines were opened lengthwise, cut into small 1-cm pieces, and then incubated in isolation buffer with protease inhibitors and antibiotics (phosphate-buffered saline (PBS) with 1 % dithiothreitol (Sigma), 1% sodium pyruvate (Sigma), 0.6 % HEPES (Gibco-Life technologies, Alcobendas, Madrid), 0.03 % amphotericin B (Sigma)) for 20 min at 37º C. Intestinal tissue was removed and the isolation buffer centrifuged at 13,500xg for 30 min. The particle-free supernatant was kept at -20º C until further processing. All samples were concentrated by ultrafiltration (Amicon, Beverly, MA/USA, exclusion limit 30 kDa) to a final volume of 2 ml. Concentrated mucus samples were subjected to downward gel chromatography on a Sepharose CL-4B column (Sigma, Munich, Germany; flow rate, 6 ml/h; fraction volume, 1.5 ml; 50 fractions). Pig gastric mucin (PGM; molecular weight > 2,000 kDa), thyroglobulin (molecular weight 670 kDa), and bovine serum albumin (BSA; molecular weight 69 kDa) were used as molecular weight standards for calibration (Sigma). Carbohydrate content of each fraction was determined by periodic-acid-Schiff (PAS) reaction at 550 nm and protein content by Bradford reaction at 580 nm in a microplate spectrophotometer (BMG Labtech, Offenbach, Germany). Results were expressed per milligram intestinal weight (Table 2). A 5 mg/ml lyophilised PGM solution was used via PAS reaction as a standard to obtain the calculated glycoprotein content (CGC) (Σ OD sample fractions/Σ OD PGM fractions). Results are expressed as mean value and standard error (Table 2).

After downward gel filtration and subsequent staining for carbohydrates and proteins, a biphasic elution profile was obtained for all the luminal mucus samples. For further analysis, fractions were pooled in two size areas (peak I: fractions 14-20 containing molecular size > 2,000 kDa, and peak II: fractions 28-40 of molecular size 69-670 kDa).

The protein/carbohydrate (PC) ratio was calculated in order to determine the glycosylation degree of the isolated mucus glycoproteins. A low PC ratio reflected a high glycosylation degree as the amount of carbohydrate side chains increases relative to the protein core.

Table 2. Luminal mucus glycoproteins (GP) of gilthead sea bream intestines.

FISH AND INTESTINAL PART	TOTAL GP				GP > 2,000 kDa				GP 69-670 kDa			
	GP mean values *(OD/g IW) ± STE*		CGC ((mg/ml) /g IW)	Glycosi-lation degree	GP mean values *(OD/g IW) ± STE*		CGC ((mg/ml) /g IW)	Glycosi-lation degree	GP mean values (OD/g IW) ± STE		CGC ((mg/ml) /g IW)	Glycosi-lation degree
	Carbo-hydrates[a]	Proteins[b]			Carbo-hydrates[a]	Proteins[b]			Carbo-hydrates[a]	Proteins[b]		
C fish Ai & Mi	0.14 ± 0.09	0.50 ± 0.22	3.28	3.48	0.29 ± 0.19	0.37 ± 0.13	0.13	1.29	0.18 ± 0.02	1.21 ± 0.09	0.08	6.85
R fish Ai & Mi	0.10 ± 0.04[a]	0.42 ± 0.17	2.29	4.16	0.17 ± 0.08	0.27 ± 0.12	0.08	1.59	0.11 ± 0.02	1.04 ± 0.14	0.05	9.66
C fish Pi	0.06 ± 0.02	0.14 ± 0.10	1.50	2.24	0.08 ± 0.00	0.12 ± 0.03	0.04	1.44	0.06 ± 0.01	0.30 ± 0.15	0.03	4.68
R fish Pi	0.07 ± 0.01[a]	0.14 ± 0.08	1.64	1.93	0.08 ± 0.01	0.10 ± 0.01	0.04	1.24	0.07 ± 0.01	0.32 ± 0.06	0.03	4.30

The carbohydrate and protein contents of control unexposed (*C*) and recipient (*R*) fish are given in mean OD per gram intestine weight (*IW*) of pooled anterior and middle (*Ai* and *Mi*) and posterior intestine (*Pi*) segments. Total GP stand for fractions 1-50, GP of molecular weight > 2,000 kDa stand for fractions 14-20 (peak I), and GP of 69-670 kDa stand for fractions 28-40 (peak II). Mean calculated GP contents (*CGC*) (mg/ml per g IW) were obtained by using a 5 mg/ml PGM standard. Glycosylation degree is the protein/carbohydrate ratio calculated from the mean ODs for protein and carbohydrate contents. *Different letters* indicate statistically significant differences ($p < 0.05$) between carbohydrate and protein contents for the different molecular weight fractions. Asterisk indicates statistically significant differences in R fish compared to C fish ($p < 0.05$). *STE* standard error.

[a.] Statistically significant differences indicated in R fish compared with C fish ($p < 0.05$)

8.2.3 Terminal glycosylation

The terminal glycosylation pattern of the mucus glycoproteins was determined by lectin ELISA as described previously (Enss et al. 1995; Neuhaus et al. 2007b). Fraction pools (peak I, peak II) were used to coat 96-well-microtiter plates (Nunc Maxisorb, Wiesbaden, Germany) overnight at room temperature. After blocking with 1% BSA in PBS, plates were incubated with biotin-labelled lectins (10 µg/ml in PBS) for one hour. The employed lectins and their sugar specificities are summarized in Table 3 (DBA, RCA, UEA 1: Vector Laboratories, Burlingame, USA; ConA, SNA: Sigma). Lectin binding was visualized by subsequent incubation with streptavidin-horseradish-peroxidase for 30 min and orthophenilenediamine-peroxide (DAKO Chemicals, Hamburg, Germany) for 15 min. The enzymatic reaction was stopped by addition of 0.5 M sulphuric acid, and the OD was measured at 485 nm in a microplate spectrophotometer (BMG Labtech).

Table 3. Lectins used in ELISA, their acronym and sugar binding specificity.

LECTIN	ACRONYM	SUGAR BINDING SPECIFICITY
Concanavalia ensiformis-Agglutinin	ConA	α-D-mannose, α-D-glucose
Dolchios biflorus-Agglutinin	DBA	*N*-acetyl-α-D-galactosamine
Ricinus communis-Agglutinin	RCA	*N*-acetyl-β-D-galactosamine
Sambucus nigra-Agglutinin	SNA	Neuraminic-acid-α-2-6-galactose
Ulex europaeus-Agglutinin 1	UEA 1	α-L-Fucose

8.2.4 Bacterial adhesion

Adhesion of *Aeromonas hydrophila* (DSM 30187) and *Vibrio alginolyticus* (DSM 2171) to mucus-coated microtiter plates (Nunc Maxisorb) was studied as previously described (van der Marel et al. 2008). Bacterial suspensions were kindly offered by the Poultry Clinic of the Centre of Infectious Diseases of the Veterinary School of Hannover, Germany. After coating black microtiter plates overnight with the glycoprotein fraction pools (peak I, peak II) they were incubated with the fluorescent-labelled bacterial suspensions (10^9 bacteria/ml) in the dark for 30 min. Therefore, a 1:100 Syto 9 (Invitrogen, Darmstadt, Germany) dilution was used for the green fluorescent staining of the bacterial nucleic acid. Bacterial fluorescence was measured twice during the assay (470 nm excitation, 520 nm emission) in a microplate spectrophotometer (BMG Labtech). First measurement took place immediately after the incubation to record the 100 % of bacterial fluorescence. After plates were washed with 0.9 % NaCl and shaken off to remove non-adhered bacteria, the second fluorescence measurement was recorded. Results were expressed as percentage of adhered bacteria fluorescence.

8.2.5 Statistics

The Student´s *t* test for normal distributed data was used to analyze the possible differences between intestine segments, the two size elution areas (peak **I**, peak **II**) and C and R fish regarding mucus carbohydrate and protein contents, oligosaccharide terminal glycosylation, and bacterial adherence. Data which failed the normality test were analyzed with Mann-Whitney *U* sum test. Statistical analyses were performed using Sigma Stat software (SPSS Science, Chicago, IL, USA) at a significance level of $p < 0.05$.

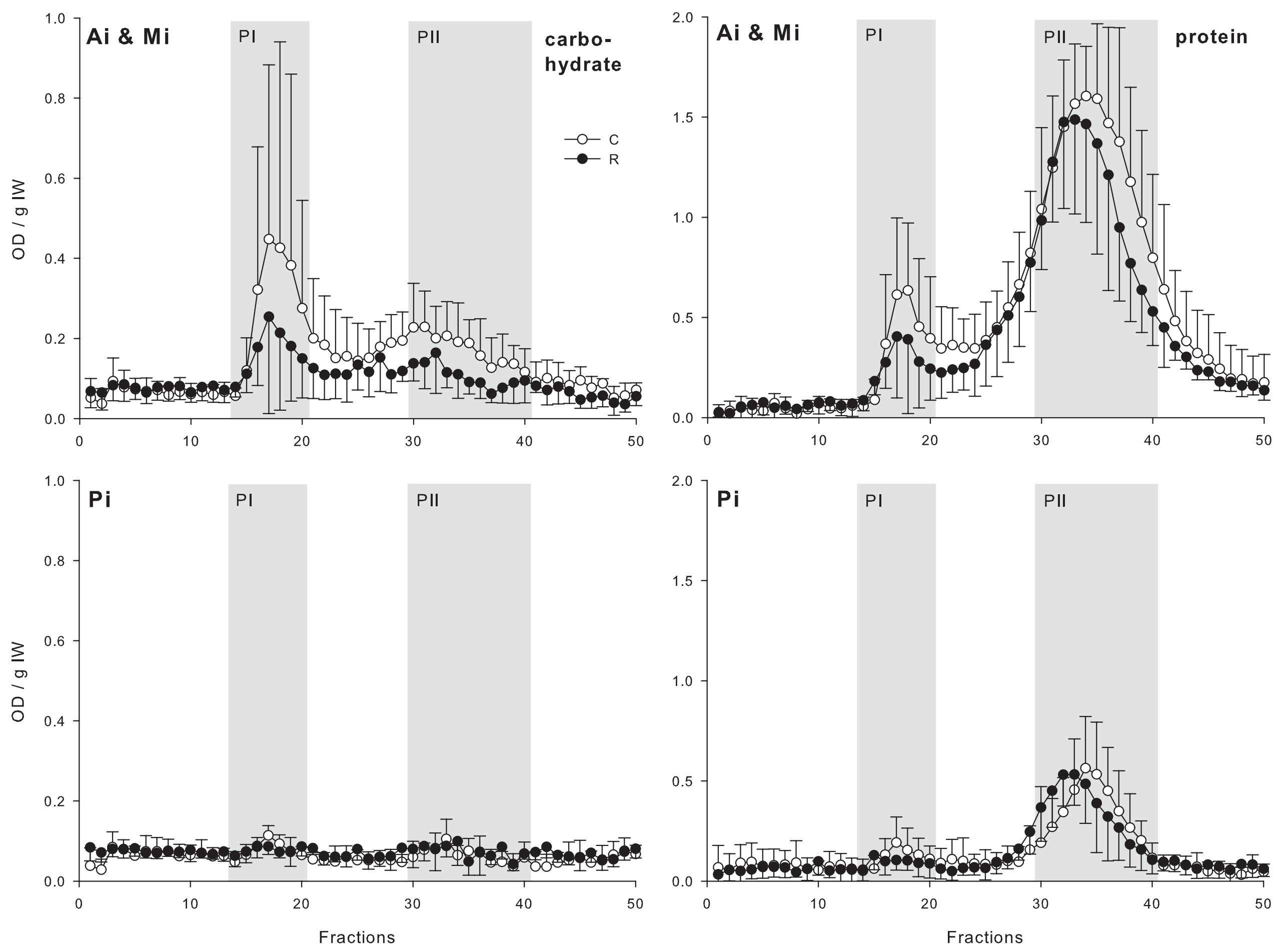

Figure 1. Intestinal carbohydrate (*left*) and protein (*right*) content of luminal mucus glycoproteins in gilthead sea bream. Elution profiles according to the OD per gram intestine weight (OD/g IW) of the anterior and middle intestine (Ai and Mi) (*upper row*) and of the posterior intestine (Pi) (*lower row*) in control unexposed (**C**) and recipient (**R**) parasitized fish. Mean values and standard deviations of 50 fractions for each group, C ($n = 8$) and R ($n = 6$) are shown. *PI*: peak I, glycoproteins > 2,000 kDa; *PII*: peak II, glycoproteins between 69 and 670 kDa

8.3 RESULTS

8.3.1 Mucus glycoproteins

The isolated luminal mucus showed a biphasic elution profile for the protein and carbohydrate contents when fractions from downward gel filtration were monitored (Fig. 1, Table 2). At the Pi, the carbohydrates were found in a low concentration and the biphasic profile almost disappeared. In all studied mucus samples, high-molecular-weight glycoproteins (>2,000 kDa) were eluted in a first peak (PI, fractions 14-20) and smaller glycoproteins (69-670 kDa) in a second peak (PII, fractions 28-40). The mean protein content of the isolated mucus glycoproteins was significantly higher than the mean carbohydrate content, regardless of the intestine section for C and R fish (Table 2). The CGC and the glycosylation degree were significantly higher in the glycoproteins of the PI fractions than in those of the PII fractions, which had lower carbohydrate content, for all C and R intestinal segments.

The isolated mucus glycoproteins varied in parasitized with respect to control fish along the intestine (Fig. 1, Table 2). Thus, the total CGC was lower in Ai and Mi and higher in Pi in R fish than in C fish, though the differences were not statistically significant. A similar variation occurred for the total carbohydrate content, both in Ai and Mi and in Pi, and the differences were statistically significant. The situation was also similar for the glycosylation degree of the isolated glycoproteins (lower in Ai and Mi and higher in Pi in R fish than in C fish for the total fraction collection and in the fractions PI and PII separately, with the only exception of the PI glycoproteins of C fish), but the differences were not statistically significant. The carbohydrate and protein profiles of R fish were eluted two to three fractions earlier than those of C fish, indicating a slight molecular size increase in all intestinal sections.

8.3.2 Terminal glycosylation

All the tested terminal monosaccharide residues were present in the isolated mucus glycoproteins of C and R fish (Fig. 2). *N*-acetyl-α-D-galactosamine (α-galNAc) was the most abundant sugar residue detected, followed by *N*-acetyl-β-D-galactosamine (β-galNAc), α-L-Fucose (α-L-Fuc), and neuraminic-acid-α-2-6-galactose (αNeuNAc(2→6)gal), whereas α-D-mannose (α-D-man) and α-D-glucose (α-D-glc) were the scarcest in both C and R fish. The presence of αNeuNAc(2→6)gal, α-L-Fuc, and α-D-man/α-D-glc predominated in glycoproteins of 69-670 kDa (PII), while α-galNAc and β-galNAc were more abundant in glycoproteins > 2,000 kDa (PI). The total content of terminal α-galNAc and α-D-man/α-D-glc was higher in R than in C fish, whereas the other sugar residues were scarcer in R fish. However, the terminal presence of the analyzed glucans was highly variable for the different intestinal sections, molecular size ranges, and infective status.

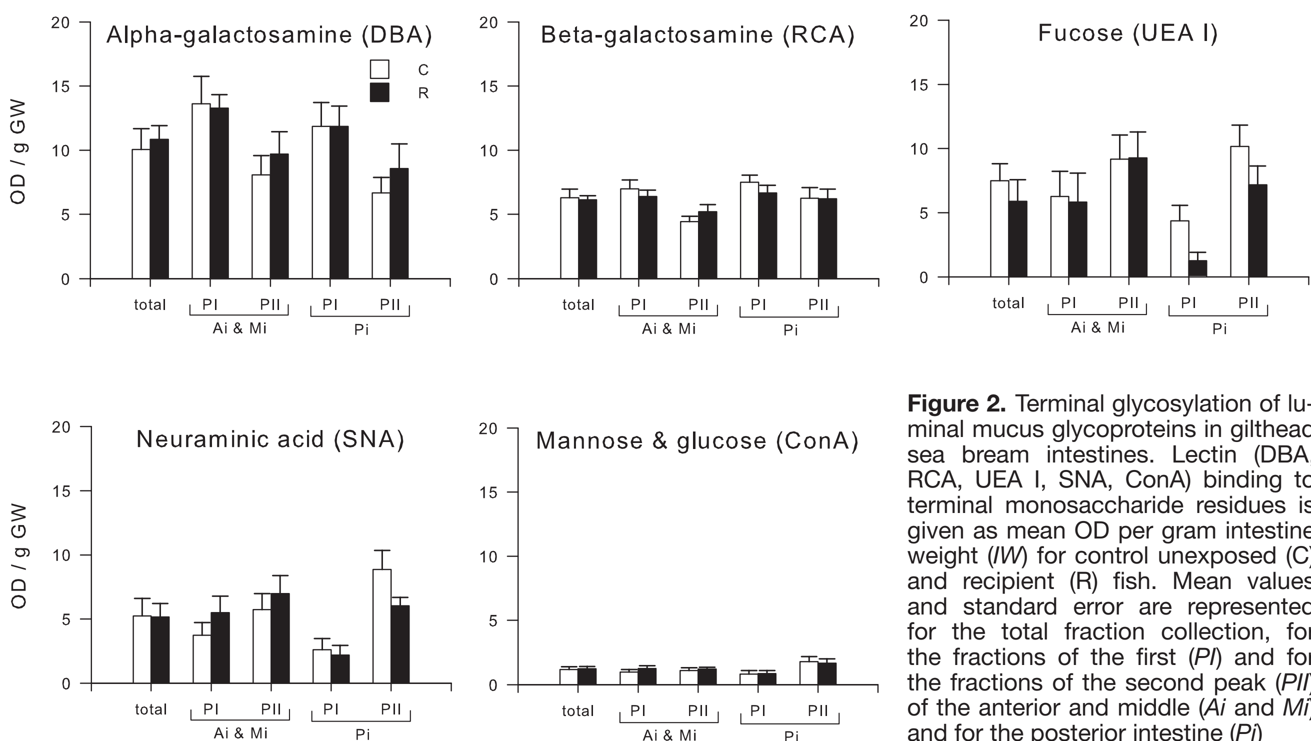

Figure 2. Terminal glycosylation of luminal mucus glycoproteins in gilthead sea bream intestines. Lectin (DBA, RCA, UEA I, SNA, ConA) binding to terminal monosaccharide residues is given as mean OD per gram intestine weight (*IW*) for control unexposed (C) and recipient (R) fish. Mean values and standard error are represented for the total fraction collection, for the fractions of the first (*PI*) and for the fractions of the second peak (*PII*) of the anterior and middle (*Ai* and *Mi*) and for the posterior intestine (*Pi*)

8.3.3 Bacterial adhesion to mucus

The adhesion ability of the two bacterial strains to the isolated mucus glycoproteins differed, ranging the mean percentage of adhesion of *A. hydrophila* from 0.54 % to 1.28 % and that of *V. alginolyticus* from 2.18 % to 3.17 %. Both bacterial strains adhered significantly stronger to the mucus glycoproteins of PII (69-670 kDa) than to those of PI (>2,000 kDa), regardless of the intestinal section for both C and R fish (Fig. 3). Bacterial adhesion was significantly lower in R than in C fish for the mucus molecules > 2,000 kDa (PI) in all intestinal sections. For the smaller-sized glycoproteins of PII, no significant differences in bacterial adhesion between C and R fish were recorded.

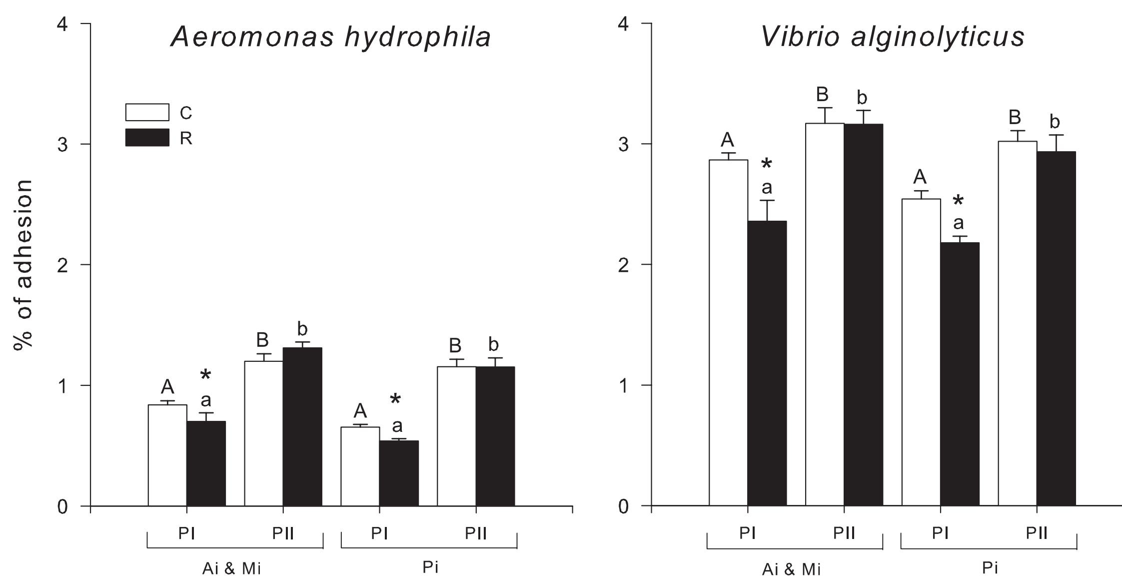

Figure 3. Bacterial adhesion to luminal intestinal mucus glycoproteins of gilthead sea bream. Means and standard error of the percentage of adhesion are given for the first (*PI*) and second peak (*PII*) eluted fractions of the anterior and middle (*Ai* and *Mi*) and posterior intestines (*Pi*) in control unexposed (C) and recipient fish (R). *Different upper* and *lower case* letters indicate statistically significant differences ($p < 0.05$) between PI and PII of C an R fish, respectively. Statistically significant differences between C and R groups are indicated by *asterisks* ($p < 0.05$)

8.4 DISCUSSION

Parasite outbreaks, together with those due to virus and bacteria, are a major thread for aquacultured fish, due to their rapid spread and devastating effects on growth performance and survival, as well as to their complex control strategies which have to consider also consumer safety issues. The mucosal biofilm is the first host-defence barrier that harmful luminal microorganisms encounter in the intestine. Consequently, the mucus interface constitutes an anchoring or entrapping surface for all the macromolecules that contact this mesh-like structure, mainly formed by mucins, highly *O*-glycosylated filamentous glycoproteins. Thus, given the importance of the secreted intestinal mucins in the host-parasite interaction during disease processes, this study focused on the effect of *E. leei* infection on gut mucins of GSB.

Biphasic elution profiles were obtained for the secreted intestinal mucins of almost all intestinal sections of GSB, both peaks representing two clearly distinct size ranges of mucins: > 2,000 kDa (PI) and 69 - 670 kDa (PII). As could be expected, large-size mucins of the PI presented significantly higher CGCs and glycosylation degrees than those of PII, regardless of the intestine section and the parasitic state of the fish. Therefore, the PI mucins seem to constitute the densely glycosylated mature mucins mainly contributing to the visco-elastic properties of the adherent mucous gel, while PII would correspond to smaller immature mucus molecules also discharged by goblet cells.

The mucous secretion of R fish showed important alterations depending on the intestinal section. Thus, the CGC and the glycosylation degree of the isolated mucin glycoproteins at the Ai and Mi were lower in R than in C fish, in contrast to the higher values detected at the Pi in R fish compared with C fish. Besides, the glycosylation degree of Pi mucin glycoproteins was in most cases higher than that at Ai and Mi. In this study, all R fish had the parasite established at the Pi, being the infection load homogeneous among fish, and only one of them harbored parasitic stages also in the Ai and Mi. This agrees with the known preference of *E. leei* for the rectum and Pi of GSB, its target organ, from which the infection follows a posterior-anterior progression (Cuadrado 2009; Estensoro et al. 2010; Estensoro et al. 2011; Sitjà-Bobadilla and Palenzuela 2012). Previous works dealing with *E. leei* infections in GSB showed differences in the mucosal immune response of the Pi compared with the other less affected intestine sections. Thus, intestinal plasma cells detected by immunohistochemistry during inflammation increased significantly only at the Pi, suggesting a mucosal immune response at local level (Estensoro et al. 2012b) and the presence of host apoptotic cells, detected by the anti-active caspase3 Pab, decreased significantly only at the Pi (Estensoro et al. 2009). These observed cellular, physiological, and immunological differences along the intestine of GSB could be related with the differential anterior-posterior mucous secretion.

Interestingly, previous in situ studies on carbohydrate histochemistry of the intestinal goblet cells showed a clear depletion phenotype of this cell type in a long-term *E. leei* infection (102 days post-exposure), which was significant at the Pi but not at the Ai for acid, acid-carboxylic and SNA-positive mucins (Estensoro et al. 2012a). In other studies, goblet cell depletion was also found in GSB intestinal segments affected by enteromyxosis (Fleurance et al. 2008; Redondo and Álvarez-Pellitero 2010). This goblet cell depletion contrasts with the observed increase of the CGC and glycosylation degree in the Pi mucous secretion of R fish. The present fish harbored a longer-lasting infection than that of the histologically studied fish, and they could exhibit a rebound effect with an increase in the mucus secretion. On the other hand, the decrease of the CGC and glycosylation degree in Ai and Mi mucous secretion in R fish correlated with the goblet cell depletion phenotype documented for this host-parasite model (Redondo and Álvarez-Pellitero 2010; Estensoro et al. 2012a). To our knowledge, other examples of similar mucus depletion do not exist in teleosts challenged with enteric infections, except for enteromyxosis. Mucus overproduction is the most common innate immune response in the piscine intestine aiming to entrap and eliminate offending microorganisms (Lodemel et al. 2001; Neuhaus et al. 2007a; Schroers et al. 2009; Dezfuli et al. 2010; Torrecillas et al. 2011), and therefore it seems paradoxical that the host response triggers a reduction of mucus. However, this has already been documented in mammalian models in which the involvement of a complex immune regulation was shown. Thus, the goblet cell depletion in *Citrobacter rodentium*-infected mice, associated to a downregulated gene expression of goblet cell-specific factors, was mediated by the host immune system since it did not occur in T and B cell-deficient mice (Bergstrom et al. 2008). Furthermore, mucins are involved in both innate and adaptive mucosal immunity at gut level in vertebrates, being regulated through inflammatory cytokines (such as interleukins IL-1β, IL-4, IL-6, IL-9, IL-13, interferons, or tumor necrosis factor (TNF)-α) (Álvarez-Pellitero 2011). Indeed, the expression of IL-1β and TNF-α in the intestine of GSB

was downregulated after *E. leei* infection (Sitjà-Bobadilla et al. 2008). Further studies on the GSB immune response are needed to elucidate this mucin-immune connection and to track the detailed kinetics of the variations in the intestinal mucous secretion.

Mucin oligosaccharides are widely considered to be a central element in host-pathogen interactions due to the molecular charge they confer (Pedini et al. 2002; Roberts and Powell 2005; Redondo and Álvarez-Pellitero 2010), the attachment and energy source they provide (Kim and Ho 2010), and for their involvement in specific recognition and binding phenomena (Roussel and Delmotte 2004; Cheng et al. 2010; Kim and Ho 2010). Moreover, mucin glycoconjugates are known to prevent apomucin degradation by microbial proteases (Ascencio et al. 1998; Sarasquete et al. 2001; Neuhaus et al. 2007b; Schroers et al. 2009; Redondo and Álvarez-Pellitero 2010). Thus, modulation of mucin glycosylation plays a decisive role in either pathogen expulsion or settlement and invasion. The current results agree with previous descriptions of terminal mucin glycosylation in GSB intestine (Domeneghini et al. 1998; Redondo and Álvarez-Pellitero 2010), confirming the presence of all five monosacharides: α-galNAc, β-galNAc, α-L-Fuc, αNeuNAc(2→6)gal, and α-D-man. No significant glycosylation differences were detected in the mucous secretion, though some patterns depending on intestine section, mucin size range, and parasitic status can be discerned. *N*-acetylgalactosamines are initial sugars of the oligosaccharide side chains binding directly to the apomucin (Pedini et al. 2002; Álvarez-Pellitero 2011) and are therefore considered an indicator of not fully mature mucins (Enss et al. 1995; Schroers et al. 2009). In the current work, α-galNAc was the most abundant glycoconjugate detected in PII glycoproteins, and it was more abundant in R fish mucins of this size range, suggesting a premature mucin secretion due to enteromyxosis. α-L-Fuc and αNeuNAc(2→6)gal were more abundant in PII than in PI glycoproteins. Both terminal monosaccharides were less abundant in the Pi of our R fish than in C fish. A fucose reduction was previously detected in the intestine of parasitized GSB (Redondo and Álvarez-Pellitero 2010), and neuroaminic acid also decreased in common carp intestinal mucus in response to LPS application (Neuhaus et al. 2007a). Fucose and neuroaminic acid typically terminate the oligosaccharide side chains since they are transferred to the glycoprotein late during synthesis (Enss et al. 1995; Pedini et al. 2002; Schroers et al. 2008; Álvarez-Pellitero 2011). Thus, incompletely glycosylated immature mucins are secreted in the *E. leei* infected intestine segment. Additionally, both fucose and neuraminic acid are considered to intervene in microorganism adhesion (Schroers et al. 2008; Schroers et al. 2009; Estensoro et al. 2012a) and mucosal protection (Neuhaus et al. 2007a; Redondo and Álvarez-Pellitero 2010; Álvarez-Pellitero 2011), and their reduction is related to enteric infections. α-D-man/α-D-glc residues were the scarcest among the monosaccharides tested in GSB, in accordance with published data for fish (Pedini et al. 2002; Redondo and Álvarez-Pellitero 2010). In fact, mannose binds *N*-glycosylically to the mucin only at the C-terminal cysteine knot domains (Pedini et al. 2002; Bansil and Turner 2006; Álvarez-Pellitero 2011).

As a whole, *E. leei* infection produced changes in the lectin-binding pattern to terminal carbohydrate residues. Furthermore, the mucosal modulation induced by the infection invoked a significant reduction of bacterial adhesion to the mucus. Adhesion of *A. hydrophila* and *V. alginolyticus* was studied as an initial step for bacterial colonization. Adhesion to mucus can be correlated with the virulence of the strain but is also a selection criterion for probiotic microorganisms (Vesterlund et al. 2005). *A. hydrophila,* a facultative pathogen but also part of the intestinal microflora in healthy fish, is commonly found in freshwater and marine fish (Rodríguez et al. 2008; Sahoo et al. 2008). *V. alginolyticus* is a frequent causative agent of bacterial diseases affecting farmed GSB (Balebona et al. 1995; Bordas et al. 2003). The greater adhesion of *V. alginolyticus* to the isolated mucus mucins might be the reason for its frequent disease participation, while the less adherent *A. hydrophila* is an opportunistic pathogen. Besides, other bacterial factors like motility, chemotactic response, glucolytic/proteolytic enzyme production and temperature or salinity preferences are probably involved in such differential adhesion. Both bacterial strains adhered significantly stronger to the PII mucus glycoproteins (69 – 670 kDa), than to larger-sized glycoproteins. The preference of bacteria to adhere to smaller glycoproteins is considered a strategy to access core region carbohydrates (Schroers et al. 2008). In fact, no differences in bacterial adhesion to PII glycoproteins were detected, regardless of the intestinal section and the parasitic state. Interestingly, a consistent significantly lower bacterial adhesion to PI glycoproteins (>2,000 kDa) occurred in R fish with respect to C fish for both strains in all intestine sections. Sugar-binding proteins, like lectins and adhesins, are employed by many pathogens to recognize and bind to host glycoconjugates (Enss et al. 1995; Álvarez-Pellitero 2011). Thus, the changes in mucin glycosylation described above, especially the reduced amounts of terminal fucose and neuroaminic acid residues, could be in part responsible for the observed reduction of bacterial adhesion.

In the current study, the less adherent mucus produced by GSB during enteromyxosis prevented the adhesion of some pathogens, but this could also imply a deficient microflora with a subsequent weakness of fish. Indeed, complex populations of commensal bacteria can exclude pathogen colonization and the severity of enteric infections increases during decreased mucus production (Bergstrom et al. 2010). Further studies on the commensal gut microflora of GSB, including experimental infections, would confirm whether enteromyxosis favors the adherence and entrance of certain bacteria to the intestine.

The current work proves numerous alterations in the intestinal mucous secretion of GSB in response to *E. leei* infection. The mucus secreted after the enteromyxosis had modified mucin patterns in which abundance, size, and glycosylation were altered. The presence of larger-sized mucin glycoproteins in R fish with respect to C fish could be due to a change in mucin gene expression, though a reduced degradation by bacteria cannot be discarded, since commensal microflora is often depleted during enteric infections (Bergstrom et al. 2010). Mucin gene expression patterns in GSB are currently studied aiming to decipher the underlying mechanisms that modulate such alterations.

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Modulation of the IgM gene expression and IgM immunoreactive cell distribution by the nutritional background in gilthead sea bream (*Sparus aurata*) challenged with *Enteromyxum leei* (Myxozoa)

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9.0 ABSTRACT

The aim of the present work was to determine if a plant protein-based diet containing vegetable oils (VO) as the major lipid source could alter the distribution of IgM immunoreactive cells (IRCs) and the IgM expression pattern in the intestine and haematopoietic tissues of gilthead sea bream (GSB) (*Sparus aurata*) challenged with the myxosporean *Enteromyxum leei*. In a first trial (T1), GSB fed for 9 months either a fish oil (FO) diet or a blend of VO at 66% of replacement (66VO diet) was challenged by exposure to parasite-contaminated water effluent. All fish were periodically and non-lethally sampled to know their infection status. After 102 days of exposure, samples of intestine and head kidney were obtained for IgM expression and immuno-histochemical detection (IHC). Additional samples of spleen were taken for IHC. Fish were categorized as control (C, not exposed), and early (E), or late (L) infected. The 66VO diet had no effect on the number of IgM-IRCs in any of the tissues or on IgM expression in C fish, whereas the infection with *E. leei* had a strong effect on the intestine. A combined time-diet effect was also observed, since the highest expression and IRCs values were registered in the posterior intestine (Pi) of E-66VO fish. A positive correlation was found between IgM expression and the presence of IgM-IRCs in the Pi. The effect of the time of infection was studied more in detail in a second trial (T2) in which samples of Pi were taken at 0, 24, 51, 91 and 133 days after exposure to the parasite. A significant increase of the IgM expression was detected only in parasitized fish, and very late after exposure. These results show that the duration of the exposure to the parasite is the most determinant factor for the observed intestinal IgM increased phenotype which gets magnified by the feeding of a high VO-based diet.

KEY WORDS:
Teleost · Vegetable replacement diet · Immune response · Intestine · Haematopoietic tissues

9.1 INTRODUCTION

Farmed fish are constantly exposed to different types of stressors such as high density culture conditions, inadequate diets or infections which might compromise their immune response. Besides viral and bacterial diseases, parasitoses have become one of the major threats for intensive fish aquaculture, as reported in recent cases of massive losses [1, 2].

Gilthead sea bream (GSB) (*Sparus aurata*) is the main cultured fish species in the Mediterranean, with a total annual production of more than 130,000 tonnes in 2010 [3]. *Enteromyxum leei* is a widespread enteric myxosporean parasite causing one of the most threatening parasitic diseases in Mediterranean sparid farms [4, 5]. In GSB, this parasite starts invading the posterior intestine (Pi) causing severe chronic enteritis with an intense inflammatory response [2]. The parasite spreads towards the anterior intestine (Ai), eventually occupying the entire intestinal tract [6-8]. During the slow progression of the infection, anorexia and cachexia are induced in the fish, leading to reduced growth performance and even death. *E. leei* outbreaks in high density stocking conditions lead to fatal consequences due to the direct fish-to-fish transmission of the parasite, either by cohabitation with infected fish or by exposure to a contaminated effluent [9, 10]. The lack of preventive and therapeutic measures to content this devastating enteromyxosis points out the urgent need for further understanding of the immune response of GSB to *E. leei*.

The increasing interest in replacing fish meal and fish oil by plant proteins and oils in aquafeeds has focused research on finding diets with optimum growth performance results without detrimental effects on fish immune status [11-14]. Previous studies on GSB with gradual levels of substitution of fish oil (FO) by vegetable oils (VO) in plant-protein based diets have demonstrated that it can be accomplished up to 66% without any negative side-effects for the fish [15, 16]. Nevertheless, fish fed the 66% substitution diet (66VO) and challenged with *E. leei* showed a higher disease outcome than fish fed the FO diet [6]. In an effort to understand the possible underlying mechanisms involved in the greater progression of the infection in 66VO fish, we undertook a series of detailed studies of gut immunology and fish immune response in fish fed such diets and confronted with this myxosporean. Production of specific antibodies has been described for several myxosporean infections [17] and circulating antibodies against *Enteromyxum scophthalmi* produced by turbot [18] are involved in resistance to re-infection to survivor fish [19]. Furthermore, in *E. scophthalmi*-experimentally infected turbot, an increase of Ig+ cells occurred in the intestine, whereas a decrease was observed in lymphohaematopoietic tissues [20]. Protection against piscine parasitoses by generation of specific antibodies has been broadly documented [21]. Moreover, the expression and *in situ* localization of IgM in the intestine of rainbow trout suggests its important role interconnecting the humoral and local-mucosal immune responses [22].

The current work tries to decipher some of the above mentioned aspects for the GSB-*E. leei* model. We integrate the results of a first trial in which the combined effect of the myxosporean *E. leei* and the nutritional background of fish on IgM expression and localization in intestine and lymphohaematopoietic tissues was studied, with those of the kinetics of the expression of IgM in the intestine of GSB in response to the this parasite in a subsequent more focused trial.

9.2 MATERIALS AND METHODS

9.2.1 Experimental set up

Two experimental trials were undertaken in which naïve pathogen-free GSB were challenged by exposure to an *E. leei*-contaminated effluent as previously described [10]. Fish were kept in 5 µm-filtered and UV-irradiated sea water (37.5‰ salinity), always at a temperature above 18 °C. Details on water temperature of both trials can be found in Table 1. Before the experimental infections started, GSB were also checked for the absence of the parasite by non-lethal PCR diagnosis as described in Estensoro et al. (2011) [6] and fish were starved for two days before each sampling.

In the first trial (T1), samples were obtained as previously described [6]. Briefly, GSB were fed during 9 months either an FO based diet or a diet containing a blend of VOs at 66% replacement (66VO) (Supplementary Table 1, Supplementary Table 2) After this period, fish from both diet groups were exposed to *E. leei*-effluent (recipient group, R-T1, *n* = 30) or kept unexposed (control group, C-T1, *n* = 30). All fish were individually tagged with passive integrated transponders and non-lethally sampled at three consecutive times for parasite diagnosis. R-T1 fish were classified according to their first infection-timing in two categories: early infected (E), being infected at 32 or 53 days post exposure (p.e.) and late infected (L), being infected at 88 days p.e.. A final lethal sampling was performed 102 days p.e., and portions of Ai and Pi, head kidney (Hk) and spleen (Sp) were taken for immunohistochemistry. Pi and Hk samples were also immediately frozen in liquid nitrogen and stored at -80 °C until further gene expression analyses. Parasite diagnosis was performed histologically from intestine samples.

In the second trial (T2), the control (C-T2, *n* = 50) group was also kept unexposed, and the recipient (R-T2, *n* = 40) group received the *E. leei*-effluent from a donor tank. Ten fish from each group were lethally sampled in four consecutive times, and an initial sampling of ten C-T2 fish was performed one day before the challenge. Tissue samples of intestine were fixed in 10% buffered formalin for routine histological parasite diagnosis, and only Pi portions were collected in ice cold RNAlater solution (Ambion, TX, USA) in view of the results obtained in T1. They were kept for 24 h at 4 °C and stored at -20 °C until gene expression analysis was performed.

All experiments were carried out in accordance with national (Royal Decree RD1201/2005, for the protection of animals used in scientific experiments) and institutional regulations (CSIC, IATS Review Board) and the current European Union legislation on handling experimental animals. In all lethal samplings, fish were euthanized under benzocaine anaesthesia (3-aminobenzoic acid ethyl ester, 100 mg/ml) (Sigma, St. Louis, MO, USA). Details of both trials are summarized in Table 1.

Table 1 (next page). Experimental and sampling details of the effluent transmission of *Enteromyxum leei* to *Sparus aurata* in two trials. Experimental groups are control unexposed (C) and exposed recipient (R) fish. In trial 1 fish were fed either the fish oil based diet (FO) or the 66% vegetable oil substitution diet (66VO). Parasite diagnosis was performed by non-lethal PCR (NL-PCR) or histology (HIS), the number between brackets indicates the number of fish examined in each sampling post exposure (p.e.). For each sampling, it is indicated which tissue samples (Ai = anterior intestine, Pi = posterior intestine, Hk = head kidney, Sp = spleen) were taken for which type of analysis (GE = gene expression, IHC = immunohistochemistry), and the number of fish analyzed between brackets.

TRIAL	1				2				
GROUPS	C-FO, C-66VO, R-FO, R-66VO				C, R				
INITIAL WEIGHT	224 g				214 g				
TEMPERATURE[1]	21.3 ± 0.25 (18.5-26)				18.6 ± 0.16 (15- 21.5)				
	PARASITE CHALLENGE		**SAMPLES FOR**		**PARASITE CHALLENGE**				**SAMPLES FOR**
	Days p.e.	Diagnosis[2]	GE	IHC	Days p.e.	Diagnosis	Prevalence[3] (%)	MI[3]	GE
	0	NL-PCR	-	-	0	NL-PCR (n = 10 C)	0	-	Pi (n = 8 C)
	32	NL-PCR	-	-	24	HIS (n = 10 C; n = 10 R)	0	-	Pi (n = 8 C; n = 8 R)
	53	NL-PCR	-	-	51	HIS (n = 10 C; n = 10 R)	50	3.2	Pi (n = 8 C; n = 8 R)
	88	NL-PCR	-	-	91	HIS (n = 10 C; n = 10 R)	80	5.2	Pi (n = 8 C; n = 10 R)
	102	HIS	Pi, Hk (n = 19 C; n = 25 R)	Ai, Pi, Hk, Sp (n = 13 C; n = 14 R)	133	HIS (n = 10 C; n = 9 R)	55.6	5.8	Pi (n = 8 C; n = 9 R)

[1] Mean water temperature (ºC) ± standard error; maximum and minimum values are indicated between brackets.
[2] Infection values for Trial 1 are available in Estensoro et al. (2011).
[3] Prevalence of infection and mean intensity (MI) of infection were calculated from the posterior intestine of R fish.

9.2.2 Immunohistochemical detection of IgM – Trial 1

Samples of Ai, Pi, Sp and Hk from T1 fish (C-FO *n* = 6; C-66VO *n* = 7; R-FO *n* = 7; R-66VO *n* = 7) were fixed in Bouin for 24 h, dehydrated in a graded ethanol series and embedded in paraffin. Sections (4 μm-thick) were collected on Super-Frost-plus microscope slides (Menzel-Glaser, Germany) and allowed to dry overnight. Slides were deparaffinised, hydrated and the endogenous peroxidase activity of the tissues was quenched by incubating in 0.3% (v/v) hydrogen peroxide for 30 min. Incubations were performed in a humid chamber at room temperature and all washing procedures consisted of successive 5 min immersions in TTBS (20 mM Tris-HCl, 0.5 M NaCl, 0.05% Tween 20, pH 7.2) and TBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.2). After washing, slides were blocked for 30 min with 1.5% normal goat serum (VECTOR Laboratories, Burlingame, CA, USA) and washed again. Sections were then incubated with a rabbit Pab anti-GSB IgM (1:60,000) obtained by Palenzuela et al. (1996) [23] for 1 h and washed. A biotinylated secondary goat anti-rabbit IgG antibody (1:200) was applied for one further hour. After washing, slides were incubated with the avidin-biotin-peroxidase complex (ABC, VECTOR Laboratories) for 1 h and washed. Finally, bound peroxidase was visualized by addition of DAB chromogen (3,3'-diaminobenzidine tetrahydrochloride) (Sigma) for 5 min. The reaction was stopped with deionized water, and the sections counterstained with Gill's haematoxylin, dehydrated and mounted in DPX (di-*N*-butyl-phthalate in xylene). Negative controls were carried out omitting the primary antibody, the secondary antibody and the avidin-biotin-peroxidase complex, respectively, and were found to be negative.

For the quantitative analysis of IgM IRCs, ten random digital fields from each tissue section were captured with an Olympus DP70 camera connected to a Leitz Dialux22 light microscope at x400 magnification. Immunoperoxidase stained cells were counted for each field.

9.2.3 IgM expression

IgM expression was measured from both Pi and Hk samples obtained in T1 and from Pi samples obtained in T2. Tissues from both trials were homogenized in guanidine-detergent lysis buffer at a 50 mg/ml concentration. After protease K digestion, total RNA extraction was carried out with the ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA). The vacuum-based wash and elution steps were performed according to the manufacturer's instructions. The RNA yield was 123 μg with absorbance measures of ($A_{260/280}$) 2.05-2.15, which were determined by spectrophotometry (Nanodrop 2000c, Thermo Scientific, Wilmington, DE, USA).

Reverse transcription (RT) was performed with 500 ng of the purified RNA (T1 and T2) using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) in a final volume of 100 μl. RT reaction conditions were set to 25°C for 10 min, followed by 37°C for 2 h and a final step of 5 sec at 85ºC to inactivate the reverse transcriptase.

Real-time PCR assays were carried out to quantify the abundance of intestinal and renal transcript levels of IgM, using an iCycler IQ Real-time Detection System (Bio-Rad, Hercules, CA, USA). The total PCR reaction volume of 25 μl included 7.5 μl of diluted cDNA and 17.5 μl of IQ SYBR Green Supermix (Bio-Rad) and specific primers at a final concentration of 0.3-0.9 μM to obtain amplicons of 51-134 bp in length (Table 2). Primers for GSB IgM were designed from a 1464 nucleotide sequence found by BLAST search on the transcriptome database of the Aquamax European Project (www.sigenae.org/iats). It comprises the short sequence (136 nt) present in GenBank (accession AM493677) and has been submitted to GenBank with accession JQ811851. β-actin was chosen as reference gene, and the amplification efficiency of PCR reactions of both β-actin and IgM genes varied between 95% and 98%, respectively.

The dynamic range of standard curves (serial dilutions of RT-PCR reactions) spanned five orders of magnitude, and the amount of product in a particular sample was determined by interpolation of the cycle threshold (Ct) value.

Three replicates of each reaction were performed and the fluorescence data obtained during the extension phase were normalized to β-actin by the delta-delta method [24]. β-actin expression did not change in response to dietary treatments or to infective status.

Table 2. Forward and reverse primers for intestinal and renal real-time PCR assays of IgM and β-actin used for the transcriptional analysis in T2.

GENE	GENBANK ACCESSION	PRIMER SEQUENCE	POSITION
IgM	*JQ811851*	F TCA GCG TCC TTC AGT GTT TAT GAT GCC	*990-1019*
		R CAG CGT CGTCGT CAA CAA GCC AAG C	*1123-1099*
β-actin	*X89920*	F TCC TGC GGA ATC CAT GAG A	*811-829*
		R GAC GTC GCA CTT CAT GAT GCT	*861-841*

9.2.4 Statistical analysis

One-way analyses of variance (ANOVA-I) followed by Student-Newman-Keuls test were performed to detect differences in GE and IHC values within each diet group in T1 and within each group at the different sampling times in T2. When the test of normality or equal variance failed, a Kruskal-Wallis ANOVA-I on ranks followed by Dunn's method was applied instead. Differences in GE between C and R fish within each sampling point in T2 and between FO and 66VO groups within each time of infection (C, E, L) in T1 were analysed *via* *t*-test. Two-way ANOVAs (ANOVA-II) were performed in order to check the significance of the two factors considered in T1, time of infection and diet, and for interactions between them. A Spearman correlation test was run aiming to detect a possible correlation between gene expression and immunohistochemistry data in Pi and Hk of T1, gathering the data from both diet groups. The significance level was set at $p < 0.05$ for all tests. The statistical analyses were performed using Sigma Stat software (SPSS Inc., IL).

9.3 RESULTS

9.3.1 Intestinal and renal IgM expression

Fig. 1 shows the IgM expression pattern in Pi and Hk obtained from GSB in T1. In the Pi, C-T1 groups had the lowest IgM expression values and no effect of the diet was observed. In R-T1groups, L-infected fish had slightly increased levels which did not differ significantly from their corresponding C groups, whereas E-infected fish presented a clearly up-regulated IgM expression, though only statistically significant for fish fed the 66VO diet, whose levels were fifteen-fold higher than those of C-T1-66VO. A significant effect of the diet was detected in both R-T1 fish, as the relative IgM expression of R-T1-E fish in the 66VO group was 3.0 times higher than in the respective FO group, and the expression of R-T1-L fish was 2.4 times higher for 66VO than for FO.

In the Hk, no effect of the diet was observed on the relative IgM expression for any of the groups. There was no statistically significant difference between R-T1 and their respective C-T1 group, though a slight but not significant IgM up-regulation was detected in R-T1-L-66VO fish.

The ANOVA-II allowed the analysis of the relationship between the factors involved in this study, i.e. the time of infection and the diet. In the Pi, both factors affected the IgM expression significantly ($p < 0.001$) and furthermore, a significant interaction between them was detected ($p < 0.001$). In the Hk, no significant effect of infection time or diet was detected.

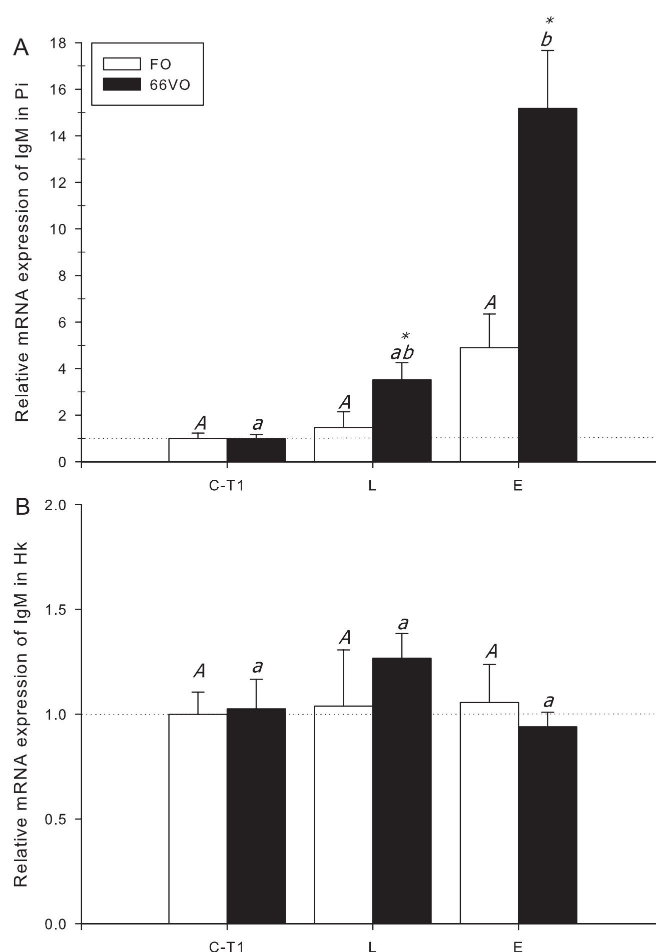

Figure 1. IgM transcript levels in posterior intestine (Pi) (**A**) and head kidney (Hk) (**B**) during Trial 1 (T1). Data of C-FO fish were used as arbitrary reference values in the normalization procedure (values >1 or <1 indicate decrease or increase according to the reference values). Upper and lower case letters indicate statistically significant differences between C, L and E fish of the FO and the 66VO diet, respectively ($p < 0.05$). Statistically significant differences between diet groups are indicated by * ($p < 0.05$).

9.3.2 Kinetics of IgM expression in intestine

As the results of T1 showed that IgM expression in the intestine was more prone than Hk to reflect changes induced by the infection and the diet, and that a time effect was also detected, the kinetics of the IgM expression in intestine were studied more in detail in T2 (Fig. 2). There was a gradual non-significant increase in the intestinal IgM expression in R-T2 fish at 24, 51 and 91 days p.e., which corresponded to a gradual increase in the percentage of fish with a fold change ≥1.5 in their transcription levels. Thus, at 24 days p.e., 25% of R-T2 fish had reached this fold change in their intestinal IgM expression, reaching 60 % at 91 days p.e. However, the IgM transcription of R-T2 fish was significantly and clearly up regulated at 133 days p.e. ($p < 0.001$) *vs.* C-T2, with values higher than those of R-T2 fish at 0, 24 and 51 days p.e. There were no statistically significant differences among C-T2 groups. When R-T2 fish were classified in parasitized and non-parasitized fish, regardless of their sampling time, the up-regulation of the IgM intestinal expression was statistically significant ($p < 0.05$) only for parasitized fish, though an increasing trend was observed for non-parasitized fish (inset, Fig. 2).

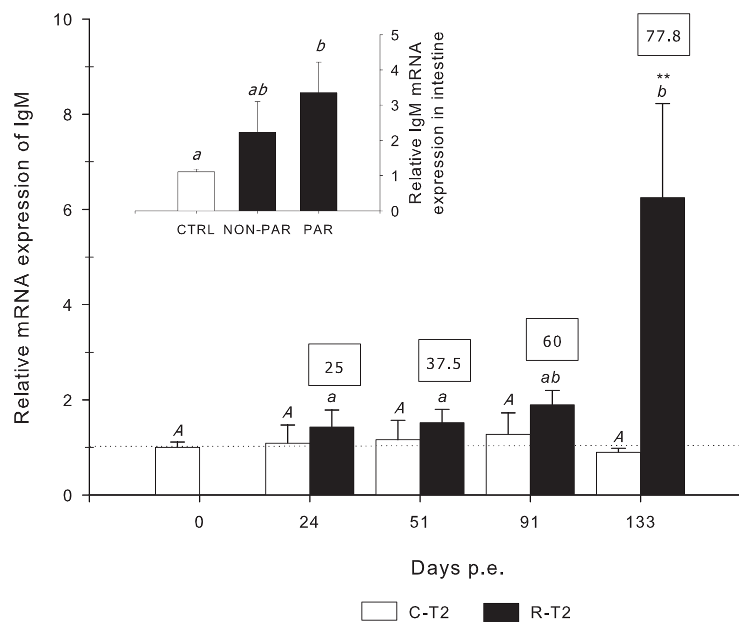

Figure 2. IgM transcript levels in the posterior intestine (Pi) of gilthead sea bream exposed to *Enteromyxum leei* by effluent in Trial 2 (T2) at different sampling times. Data of C fish at 0 days post exposure (p.e.) were used as arbitrary reference values in the normalization procedure (values >1 or <1 indicate decrease or increase according to the reference values). Numbers above each bar of R fish indicate the percentage of fish with a fold change ≥1.5. The inset graph shows the expression values after grouping all the sampling days and splitting the values of R fish in parasitized (PAR) and non-parasitized (NON-PAR) fish. Upper and lower case letters indicate statistically significant differences ($p < 0.05$) at different times among C and R groups, respectively. Significant differences between C and R groups are indicated by ** ($p < 0.001$).

9.3.3 Detection of intestinal, renal and splenic IgM-IRCs

IRCs for the anti-GSB IgM antibody were found in Ai, Pi, Hk and Sp (Figs. 3, 4). Most positive cells exhibited the typical morphology of plasma cells with ovoid shape, a variable amount of cytoplasm depending on their degree of differentiation, usually voluminous, and a round or oval nucleus (large cytoplasm/nucleus ratio) (Fig.5). Cells morphologically resembling lymphocytes, round with small cytoplasm (small cytoplasm/ nucleus ratio), were often present at the epithelial base and submucosa of R-T1 fish, though most of them were not immunoreactive (Figs.5E, 5F). The label obtained with the polyclonal anti-IgM antibody was strong and located in the cytoplasm and varying degrees of immunoreactivity were observed in positive cells (Figs. 5A, 5G). Another very scarce, morphological IgM-IRC type was detected in intestine as well as in lympho-haematopoietic tissues of both, C-T1 an R-T1 fish (Figs. 5I, 5K, 5L). These IgM-IRCs were round shaped, presented an eccentric nucleus and a voluminous cytoplasm with variable immunoreactivity, *i.e.* intense and homogeneous staining or weakly stained centre with a peripheral strongly stained fringe.

In intestinal as well as in lymphohaematopoietic tissues, the C-T1 fish presented the lowest numbers of IgM-IRCs, regardless of the diet. No statistically significant differences occurred between any of the diet groups, though R-T1-66VO fish presented the highest counts of IgM-IRCs, with the only exception of R-T1-E in Hk for FO fish (Fig. 3).

In intestinal sections, IgM-IRCs were located mainly in the lamina propria and submucosa (Figs. 4B, 4D). Occasionally, IgM-IRCs were detected in close vicinity to blood vessels or to parasite stages in the epithelium and sporadically forming cell clusters (Figs. 5D, 5G, 5H, 5J). Infiltrated plasma cells between intestinal enterocytes were often observed in parasitized epithelia (Fig. 5G), showing in some occasions cytoplasmatic protrusions (Fig. 5D) or being apparently in direct contact with the parasite even surrounding it (Figs. 5H, 5J).

At the Ai, R-T1-E fish presented the highest numbers of IgM-IRCs, regardless of the diet. Thus, the R-T1-E-66VO group exhibited a statistically significant increase of IRCs compared to the correspondent C-T1 group ($p < 0.05$) (Figs. 3A, 4C, 4D). A statistically significant effect of the time of infection on the IgM-IRC abundance ($p = 0.004$) was detected by the ANOVA-II in the Ai. At the Pi of R-T1, the main target site of *E. leei*, IgM-IRCs were more abundant than at the Ai. The same pattern of IRC distribution was observed, the number of IRCs being significantly higher in R-T1-E fish than in C-T1 in both dietary groups ($p < 0.001$ for FO; $p = 0.01$ for 66VO) (Figs. 3B, 4A, 4B). The time of infection had a statistically significant effect ($p < 0.001$) on the number of IRCs in the Pi.

IgM-IRCs in head kidney exhibited the morphology of plasma cells, as described above (Fig. 5B). They were scattered throughout the interstitial tissue, isolated or forming clusters, especially in the vicinity of ellipsoids. Occasionally, they were also found in close contact to melanomacrophage centres (MMCs). R-T1-E fish presented a statistically significant increase of IRCs compared to C-T1, only for animals fed the FO diet ($p < 0.05$) (Figs. 3C, 4E, 4F). A significant effect ($p < 0.009$) of the time of infection on the IRC abundance was detected by the ANOVA-II in this organ.

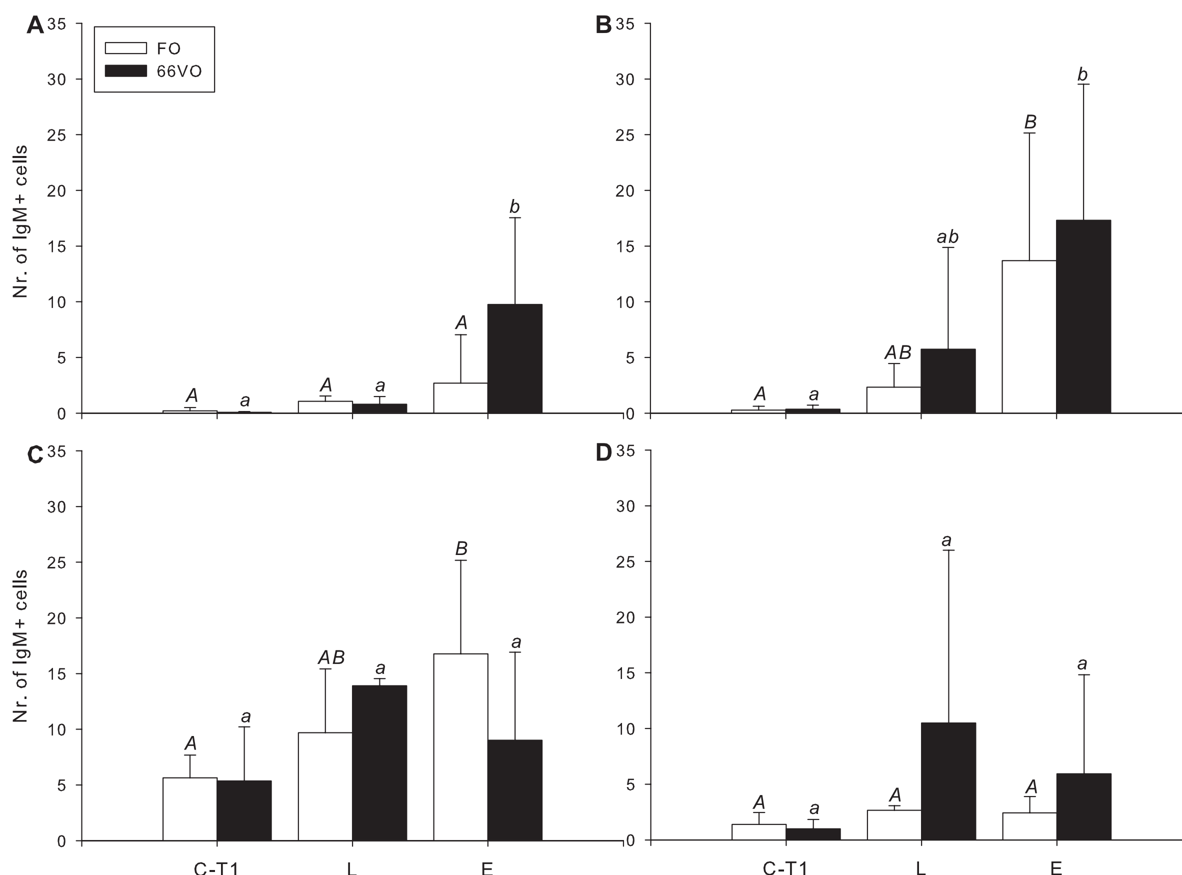

Figure 3. Average counts of IgM+ cells in anterior intestine (Ai) (**A**), posterior intestine (Pi) (**B**), head kidney (Hk) (**C**) and spleen (Sp) (**D**) of gilthead sea bream in Trial 1 (T1). Upper and lower case letters indicate statistically significant differences between C, L and E fish of the FO and the 66VO diet, respectively ($p < 0.05$).

C-T1 and R-T1 fish presented IgM-IRCs dispersed throughout the spleen parenchyma, isolated as well as clustered (Figs. 4G, 4H, 5C). These positive cells exhibited also the typical plasma cell morphology and were often found around blood vessels, occasionally close to MMCs. A non-significant increase of IRCs in the Sp of R-T1 was detected, which was stronger in the 66VO group (Fig. 3D). No statistically significant effect of the time of infection or the diet was observed.

No statistically significant interaction between both factors affecting the IgM-IRC abundance, *i.e.* the time of infection and the diet, was detected by the ANOVA-II in none of the analyzed tissues.

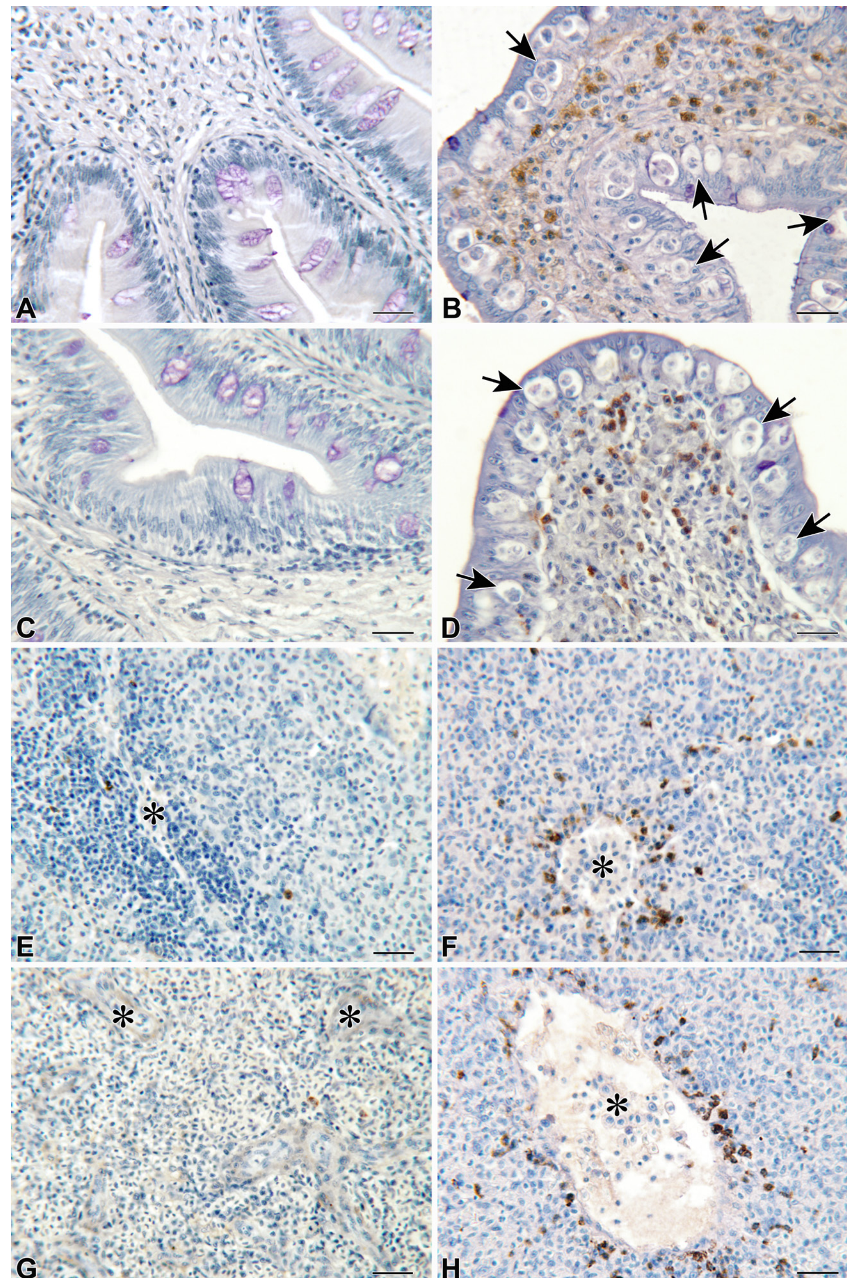

Figure 4. Photomicrographs of immunohistochemistry for IgM in control unexposed (**A, C, E, G**) and recipient (**B, D, F, H**) gilthead sea bream paraffin sections counterstained with heamatoxylin. **A-B**, Posterior intestine: **A**, fish oil diet (FO) fish; **B**, early infected, 66% replacement vegetable diet (66VO) fish. Note the epithelial parasite stages (arrows) and the abundant immunoreactive (IR) plasma cells in the submucosa. **C-D**, Anterior intestine: **C**, 66VO fish; **D**, early infected, 66VO fish. Note the epithelial parasite stages (arrows) and the abundant IR plasma cells in the submucosa. **E-F**, Head kidney: **E**, FO fish; **F**, early infected, FO fish. Note the gathering of IR plasma cells around blood ellipsoids (asterisks) in recipient fish. **G-H**, Spleen: **G**, 66VO fish; late infected 66VO fish. Note the gathering of IR plasma cells around blood vessels (asterisks) in recipient fish. Scale bar = 20 µm.

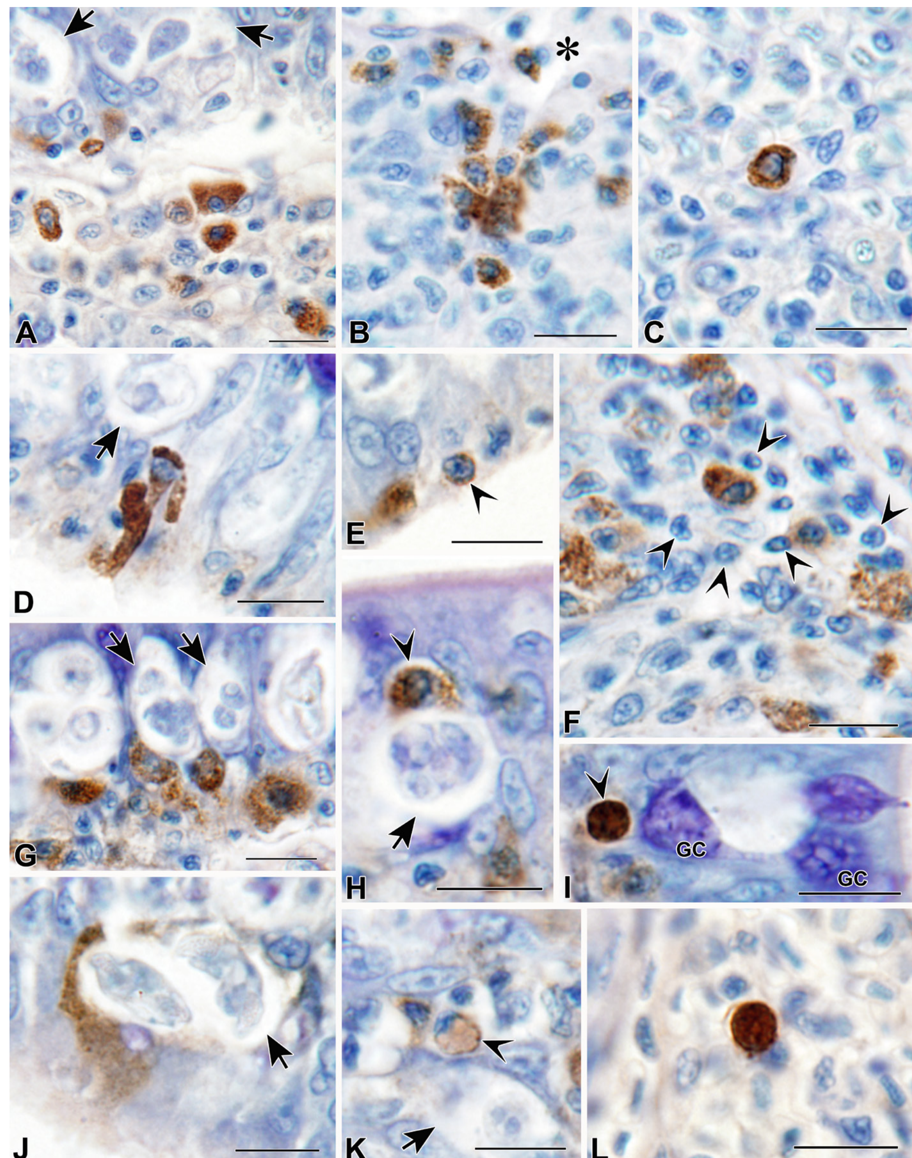

Figure 5. Photomicrographs of immunohistochemistry for IgM in recipient gilthead sea bream intestine (**A, D, E, F, G, H, I, J, K**), head kidney (**B**) and spleen (**C, L**) paraffin sections counterstained with heamatoxylin. Epithelial parasite stages are indicated with arrows. **A**, immunoreactive (IR) plasma cells presenting variable differentiation degrees in the submucosa. **B**, IR plasma cells forming a cluster close to a blood ellipsoid (asterisk). **C**, scattered IR plasma cell. **D**, IR plasma cell infiltrating in the epithelium. Note its cytoplasmatic protrusions. **E**, IR B-cell (arrowhead). **F**, IR plasma cells in the submucosa and numerous lymphocyte-like IgM negative cells (arrowheads). **G**, IR plasma cells infiltrated in the epithelial base. **H**, IR plasma cell (arrowhead) adjacent to an epithelial parasite stage. **I**, unidentified epithelial IR cell with a strong homogeneous staining in the cytoplasm (arrowhead) and goblet cells (GC). **J**, IR plasma cell surrounding parasite stage. **K**, unidentified epithelial IR cell (arrowhead) presenting a weakly stained central cytoplasm with a strongly stained external fringe and a peripheral nucleus. **L**, unidentified splenic IR cell with a strong homogeneous staining in the cytoplasm. Scale bar = 10 µm.

9.3.4 Correlation analysis: mRNA IgM transcripts vs. IgM-IRCs

The transcription of IgM in the Pi of T1 fish was positively correlated with the number of IgM-IRCs in this tissue ($p = 0.000$), with a correlation coefficient, $r_s = 0.612$. Thus, high IgM gene expression levels coincided with high amounts of IgM-IRCs in the Pi, regardless of the diet group or time of infection. In Hk, IgM transcription and the number of IgM-IRCs were not significantly correlated ($p = 0.194$).

9.4 DISCUSSION

There is a growing demand for new approaches combining nutritional and immunological studies to improve health management and animal welfare in aquaculture. The current study is the first one in focussing on the modulation of IgM by a myxosporean infection and by the nutritional background of GSB at both molecular and cellular levels, aiming to understand the local-systemic interaction. Immunohistochemistry demonstrated the presence of IgM-IRCs in all examined tissues, even in non-exposed fish, but IgM-IRCs were more abundant in the intestine than in lymphohaematopoietic organs in exposed fish. Most detected IgM-IRCs were compatible with plasma cells, followed by small amounts of IR-B-cells. Both IgM-bearing cell types had already been described in salmonids [25] and were also involved in the immune response of turbot to *E. scophthalmi* [20]. The IgM-IR macrophages containing strongly positive phagosomal-like structures observed in the latter infection model were not observed in the present study. Other Ig-containing cell types such as macrophages, neutrophils and non-specific cytotoxic cells have been found in several fish species [26, 27], probably due to Ig-binding to Fc receptors or due to phagocytized immune complexes or plasma cells. Additionally, a scarce and unidentified IgM-IRC type with a variable but consistent cytoplasmatic staining was observed, suggesting variable stages of differentiation. To our knowledge, this unidentified IgM-IRC type has only been described in another enteromyxosis [20], but its size in GSB was not even half as large (4.5 - 5 μm) as in turbot (11 - 13 μm). IgM negative lymphocyte-like cells were often detected at the epithelial base and submucosa of the R-T1 (parasitized fish) intestines. These were also described in *E. scophthalmi* infected turbot and interpreted as T-cells [20].

IgM-IRCs were scattered in the lymphohaematopoietic tissues of C-T1 (unexposed fish), being the basal levels higher in Hk than in the remaining studied organs. By contrast, IgM-IRCs formed outstanding clusters around blood vessels in R-T1 fish, sometimes in close contact to MMCs. The connective tissue surrounding arterioles in these organs might be the equivalent to peri-arteriolar lymphoid sheaths (PALS) of mammals, which are poorly developed and not always present in teleost fish [28-31]. In PALS, lymphocytes and plasma cells accumulate, and are related to antigen trapping and lymphocyte stimulation. Furthermore, plasma cell clusters are considered primitive germinal centres, especially those close to antigen retaining MMCs [20, 27, 31-33]. Thus, the higher clustering of IgM-IRCs in Hk and Sp of infected fish, together with the higher values in E-infected fish, would indicate a higher cell stimulation and differentiation and the key role of these tissues in the initiation of an adaptive immune response, for antigen trapping and presentation functions. Parasitized fish also exhibited hypertrophied submucosal intestinal areas with high density of plasma cells, in comparison with the low numbers of C-T1. This increase indicates clearly a mucosal immune response, either produced locally or derived from blood or other organs. These observations agree with the dispersed GALT structure of teleosts and the existence of a mucosal immune system responsible for the local synthesis of mucosal Ig in secretions [34].

The intestine of GSB, the target site for the parasite, also underwent the highest IgM up-regulation during the infection, reaching fifteen-fold and six-fold increases of IgM mRNA expression in the Pi of R-T1 and R-T2 fish, respectively. This increase correlated strongly with the highest levels of IgM-IRCs in this tissue. By contrast, no significant changes were observed in the expression levels in Hk, and therefore no correlation was found between IgM transcripts and IgM-IRCs. Studies integrating gene expression and *in situ* detection of Igs in fish are scarce, and they depict a great variability among fish species and pathogens. In Atlantic halibut lymphoid organs, a positive correlation was observed between IgM expression and ISH-IgM+ cells in Hk and Sp [35], whereas in European sea bass experimentally infected with betanodavirus, up-regulation of IgM in Hk occurred weeks before the number of B-cells from blood increased [36]. The ciliate *Ichthyophthirius multifilis* increased the IgM expression in the gills of rainbow trout, which was related to an active efflux of IgM-IRCs through the lamellar capillaries towards the site of infection [22]. In our study, no IgM-IRCs were detected in blood vessels, but they were found in high densities around them as a sign of influx and efflux from the blood stream. Thus, in the GSB-*E. leei* model, IgM also seems to be linking the systemic humoral immunity and the local response. The current results are in accordance with the central role of IgM in gut mucosal immune reactions [33, 37-39] in response to threatening situations (pathogens [36, 40-42], or environmental stress [43-45]). However, we cannot discard the possible action of another Ig isotype, IgT/IgZ, which seems to act exclusively in mucosal areas, and has been described in very few fish species, with outstanding results in a myxosporean infection [40, 42, 46, 47], but not yet found in GSB.

The intense IgM synthesis/mobilization accounted for plasma cells and B-cells, did not however, result in a substantial level of protection against *E. leei* infection, as a high prevalence and intensity of infection was achieved in both trials. Several examples exist of adaptive humoral immune responses that do not result in a substantial level of protection [37, 48]. Failure of the IgM defence against *E. leei* might be attributed to resistance mechanisms described for other parasites, such as suppression of antibody function or avoidance of recognition [49]. In fact, specific antibodies against *E. leei* in GSB were detected from 50 days on after exposure in a low number of fish, and high antibody titres were found only in fish from long-lasting exposures or survivors of epizootics [50]. In turbot, specific antibodies against *E. scophthalmi* also appeared late after infection and antibody mediated resistance to this parasite only occurs in some previously exposed fish [18, 19]. Similarly, binding of rainbow trout antibodies to *I. multifilis* was observed in gills and contributed to the exit of the parasite [40].

Previous studies revealed that the administration of the 66VO diet did not entail any harmful consequences for the fish growth or gut tissue integrity [15, 51-53], but was a predisposing factor that worsens the progression and severity of the disease in experimentally infected fish [6]. In the present study, the diet did not account for any detectable differences among the C-T1 fish, neither for the IgM mRNA expression nor for the IgM-IRC abundance. This agrees with previous data obtained from another trial in which no differences in total serum IgM were detected between FO and 66VO fish (unpublished results). The diet effect was only observed in the intestine when fish were exposed to the parasite (R-T1), as 66VO infected fish had increased intestinal IgM expression. Furthermore, 66VO fish bearing the infection for a longer time period (R-T1-E-66VO) underwent the strongest up-regulation. The effect of the diet on IgM was also evident at cellular level in both intestine sections, as the highest numbers of IgM-IRCs were observed in R-T1-66VO fish. Although modulation of immune related factors/genes by dietary factors is well recognized, understanding the underlying mechanisms of action is still a broad challenge. The 66VO diet contains lower levels of *n*-3 long-chain polyunsaturated fatty acids than the control FO diet (Supplementary Table 2) and this could enhance the synthesis of pro-inflammatory products *via* a high arachidonic:eicosapentanoic acid ratio [54]. This diet effect on the IgM profile only at the intestinal level points to a direct local action. Some mechanisms affecting membrane fluidity and therefore permeability [34, 55, 56] of the epithelial barrier might be responsible for the severe infection in 66VO fish, which in turn would induce an up-regulation of immune relevant genes and a stronger immune response.

The time of infection was a predominant factor in IgM dynamics, as evidenced by IgM expression in Pi and IgM-IRC abundance in Ai, Pi and even Hk, in T1. R-T1-E fish (early infected, and therefore with a well established infection) had increased numbers of IgM-IRCs in these three organs, but these differences were only significant at the Pi for both diet groups. The lower values of R-T1-E in Ai than in Pi were probably due to the also lower prevalence of infection at the Ai [6]. Thus, a pattern of IgM-IRC richness (E > L > C) can be drawn for intestinal segments and also for Hk of FO fish, which differed from that observed in the Hk and Sp of 66VO (L > E > C). In fact, the Hk of R-T1-L-66VO presented the highest number of IgM-IRCs among the organs of recently infected fish. This decreasing trend in the lymphohaematopoietic organs of early infected fish could indicate the beginning of plasma cell/B-cell depletion, but cannot be considered a significant depletion of Ig-IRCs, as occurred in *E. scophthalmi*-infected turbot [20], which could be explained by the higher pathogenicity of *E. scophthalmi* [57] that could account for a higher immunosuppressive effect.

In T2, the effect of the time of infection on IgM expression was confirmed. A progressive but slight increase was detected from 24 to 91 days p.e., which ended up with a very strong up-regulation of the IgM expression in the Pi of R-T2 fish at 133 days p.e., which coincided with a high prevalence and intensity of infection. Interestingly, non-parasitized R-T2 fish experienced a slight up-regulation, suggesting the on-set of a local immune response to confront the parasitic challenge, even before the parasite settlement takes place. Thus, a true parasite invasion was the main triggering factor of IgM mRNA expression in the Pi. Similar up-regulation of IgM has been documented in other pathogen (parasites, bacteria or virus) models, but with substantial differences in the timing [36, 37, 41]. It seems that both local and systemic adaptive immune responses are triggered against such pathogens, but in the GSB-*E. leei* model the local immune response seems to prevail and the increased IgM profile (expression and IRC) occurs remarkably later (>100 days p.e) than in the aforementioned studies. In fact, the higher IgM expression values of Pi in R-T2 fish (six-fold up-regulation, 133 days p.e.) than those of R-T1-E-FO fish (five-fold up-regulation, 102 days p.e.) could be due to the 31 days longer exposure in T2.

In conclusion, both the immunohistochemical and the gene expression studies showed that the increase of IgM is more pronounced at the local level where the parasite proliferates (intestine) than in lymphohaematopoietic organs. The time of exposure to the parasite (which determines the infection level) is the most determinant factor for the observed intestinal IgM increased phenotype, but gets magnified by long term feeding of a high VO diet which however did not affect growth or nutrient utilization.

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Appendix A. Supplementary material
Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsi.2012.05.029.

Supplementary Table 1 (p. 157).

Supplementary Table 2 (p. 158).

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SUPPLEMETARY MATERIAL

Supplementary Table 1. Fish Oil (FO) and 66% Vegetable Oil (66VO) diet ingredients.

INGREDIENT (%)	FO	66VO
Fish meal (CP 70%) [1]	15	15
CPSP 90 [2]	5	5
Corn gluten	40	40
Soybean meal	14.3	14.3
Extruded wheat	4	4
Fish oil [3]	15.15	5.15
Rapeseed oil	0	1.7
Linseed oil	0	5.8
Palm oil	0	2.5
Soya lecithin	1	1
Binder	1	1
Mineral premix [4]	1	1
Vitamin premix [5]	1	1
$CaHPO_4.2H_2O$ (18%P)	2	2
L-Lys	0.55	0.55
PROXIMATE COMPOSITION		
Dry matter (DM, %)	93.13	92.77
Protein (% DM)	53.2	52.62
Fat (% DM)	21.09	20.99
Ash (% DM)	6.52	6.57

[1] Fish meal (Scandinavian LT).
[2] Fish soluble protein concentrate (Sopropêche, France).
[3] Fish oil (Sopropêche, France).
[4] Supplied the following (mg / kg diet, except as noted): calcium carbonate (40% Ca) 2.15 g, magnesium hydroxide (60% Mg) 1.24 g, potassium chloride 0.9 g, ferric citrate 0.2 g, potassium iodine 4 mg, sodium chloride 0.4 g, calcium hydrogen phosphate 50 g, copper sulphate 0.3, zinc sulphate 40, cobalt sulphate 2, manganese sulphate 30, sodium selenite 0.3.
[5] Supplied the following (mg / kg diet): retinyl acetate 2.58, DL-cholecalciferol 0.037, DL-a tocopheryl acetate 30, menadione sodium bisulphite 2.5, thiamin 7.5, riboflavin 15, pyridoxine 7.5, nicotinic acid 87.5, folic acid 2.5, calcium pantothenate 2.5, vitamin B12 0.025, ascorbic acid 250, inositol 500, biotin 1.25 and choline chloride 500.

Supplementary Table 2. FA composition of the experimental diets (% of total fatty acid methyl esters).

FA %	FO	66VO
14 :0	7.18	2.7
15:0	0.12	tr
16:0	22.26	18.48
16:1n-7	7.06	2.62
16:2	0.47	0.15
16:3	1.66	0.46
16:3n-3	0.11	0.03
16:4	1.8	0.47
17:0	0.96	0.32
18:0	4.27	3.55
18:1n-9	12.49	24.59
18:1n-7	2.97	tr
18:2n-6	10.35	17.48
18:3 n-6	0.34	0.09
18:3n-3	0.81	17.33
18:4n-3	1.8	0.62
20:0	0.07	0.06
20:1n-9	0.92	1.03
20:2n-6	0.6	0.19
20:3 n-6	0.07	tr
20:4n-6	0.69	0.18
20:4n-3	0.3	0.15
20:5n-3	13.57	4.38
22:1n-11	0.97	0.76
22:5n-3	0.81	0.23
22:6n-3	4.78	1.88
TOTAL	97.83	97.81
SATURATES	34.86	25.11
MONOENES	24.41	29
n-3 LC-PUFA[1]	19.46	6.64
n-6 LC-PUFA[2]	1.36	0.37

tr = trace values
[1] Calculated excluding 16 C and 18 C.
[2] Calculated excluding 18 C.

10

Modulation of leukocytic populations of gilthead sea bream (*Sparus aurata*) by the intestinal parasite *Enteromyxum leei* (Myxozoa: Myxosporea)

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10.0 ABSTRACT

The cellular mucosal and systemic effectors during the acute immune response of gilthead sea bream (GSB) (*Sparus aurata*) to the intestinal parasite *Enteromyxum leei* were studied in GSB experimentally infected by anal route. In the intestinal inflammatory infiltrates and in lymphohaematopoietic organs (head kidney and spleen) of parasitized fish, the number of plasma cells, B cells (IgM immunoreactive) and mast cells (histamine immunoreactive) were significantly higher, whereas the number of acidophilic granulocytes (G7 immunoreactive) decreased, compared to non-parasitized and unexposed fish. These differences were stronger at the posterior intestine, the main target of the parasite, and no differences were found in the thymus. In non-parasitized GSB, the percentage of splenic surface occupied by melanomacrophage centres was significantly higher. These results suggest that the cellular response of GSB to *E. leei* includes proliferation of leukocytes in lymphohaematopoietic organs and recruitment into intestines *via* blood circulation involving elements of innate and adaptive immunity. Acidophilic granulocytes and mast cells, presented opposite patterns of response to the parasite infection, with an overall depletion of the former and an increased amount of the latter. Some differences between both cell types were also detected in regard to their granule density and cell morphology.

KEY WORDS:
Acidophilic granulocytes · Mast cells · Plasma cells · Eosinophilic granular cells · Inflammation · Gut associated lymphoid tissue · Mucosal infection

10.1 INTRODUCTION

Enteromyxosis in gilthead sea bream (GSB) (*Sparus aurata*) consists of severe catarrhal enteritis, which can cause a cachectic syndrome, including anaemia, leading to fish morbidity and mortality. The etiologic agent is the myxozoan parasite *Enteromyxum leei*, which penetrates and proliferates in the paracellular space between enterocytes following a posterior-anterior invasion pattern along the intestine. The parasite disrupts the epithelial organization and provokes epithelial desquamation, impairing nutrient absorption and triggering an intense local inflammatory response. In infected intestines, hypertrophied lamina propria-submucosa with inflammatory infiltrates is frequently found. In the intestinal lumen, desquamated epithelial cells together with parasite stages occur (Cuadrado, 2009; Cuadrado *et al.* 2008; Fleurance *et al.* 2008; Sitjà-Bobadilla and Palenzuela, 2012).

As fish live in direct contact with an environment that may be rich in pathogens, the cellular effectors of the innate immune response play a key role in the defence against pathogens *via* two main mechanisms: phagocytosis and cytotoxicity. In particular, at the mucosal barriers, lymphoid elements constitute the basis for local and systemic interactions of the immune system (Salinas *et al.* 2011). At the gastrointestinal level, the gut associated lymphoid tissue (GALT) involves a secretory component and two immune-cell compartments, namely the lamina propria-submucosa and the epithelium. In fish, these two compartments harbour dispersed leukocytic populations, mainly intraepithelial lymphocytes and submucosal granulocytes, plasma cells and macrophages (some of them with non-specific cytotoxic activity), which however, show variable distribution patterns along the distinct intestinal segments (Rombout *et al.* 2011). Parasite infections frequently trigger the recruitment of inflammatory cells at local or systemic sites. Their activation, with subsequent associated secretions, or induction of immune-relevant gene expression may affect parasite establishment and proliferation (Álvarez-Pellitero, 2008; Jones, 2001). In regard to piscine myxosporeans, especially the histozoic ones, severe inflammatory responses are often invoked leading to immunopathological condition, as occurs with the intestinal *Enteromyxum* spp. and *Ceratomyxa shasta* or with the branchial *Henneguya ictaluri* (Sitjà-Bobadilla and Palenzuela, 2013).

Previous studies on blood and head kidney leukocytic popupulations of GSB demonstrated the existence of plasma cells and B cells, neutrophilic, basophilic and acidophilic granulocytes (AGs), macrophages, melanomacrophages (MMΦs) and thrombocytes in this species (López-Ruiz *et al.* 1992; Meseguer *et al.* 1994*a*, *b*; Zuasti and Ferrer, 1988) and in other tissues, putative T cells and mast cells have been described (Mulero *et al.* 2007; Noya and Lamas, 1996). Among these cell types, AGs have been suggested to be the equivalent of mammalian neutrophils, being the most predominant cell type involved in the immune response against bacterial or viral pathogenes in GSB (Couso *et al.* 2001; Chaves-Pozo *et al.* 2005; Chaves-Pozo *et al.* 2004; Dezfuli *et al.* 2012*b*). Mast cells, showing functional and morphological similarities to mammalian mast cells, play a role in the mucosal immune response of GSB (Mulero *et al.* 2007) and other teleosts (Reite and Evensen, 2006). Both aforementioned cell types are eosinophilic, magenta-red with Giemsa and can be differentiated by the G7 surface epitope and the histamine (HIS) content of the granules; thus, AGs are $G7^{+}$ HIS^{-} and mast cells are $G7^{-}$ HIS^{+} (Mulero *et al.* 2007; Sepulcre *et al.* 2002). Besides, in the *E. leei* infected intestinal mucosa of GSB, a significant expression of immunoglobulin M (IgM) and recruitment of IgM bearing cells (mostly plasma cells and some B cells) has been reported, which increased late after parasite exposure (Estensoro *et al.* 2012a). In teleost lymphohaematopoietic organs, melanomacrophage centers (MMCs), which retain and process parasite-derived antigens during immune responses, are also associated to inflammation and their size and abundance can be modulated by myxosporean infections. Thus, head kidney and spleen, apart from their haematopoietic function, also serve as secondary lymphoid organs by scavenging foreign material and participating in the induction and elaboration of immune responses (Agius and Roberts, 2003; Álvarez-Pellitero, 2008).

In order to shed some light on the host immune defence against enteromyxosis, the local and systemic cell response was analysed in the current work. By means of light microscopy, histochemistry and immunohistochemistry, the distribution patterns of AGs, mast cells and plasma cells/B cells in intestinal and lymphohaematopoietic tissues, and of splenic MMCs, were studied in *E. leei* experimentally infected GSB. This study aims to understand the contribution of these leukocyte types to the acute immune response of GSB against this parasite.

9.2 MATERIALS AND METHODS

10.2.1 Fish, experimental set up and sampling procedure

Naïve gilthead sea bream (GSB) were obtained from a commercial fish farm with no previous records of enteromyxosis. They were checked for the absence of the parasite and, during two weeks, acclimated to the experimental conditions in fibre-glass tanks (UV-treated, 5 µm-filtered water at a mean temperature of 21.2 ± 0.25 °C and 37.5 ‰ salinity). Fish were fed a commercial dry pellet diet (BioMar, Palencia Spain) at 1% of body weight daily. At the beginning of the experiment, fish (average initial weight 130.5 g) were placed in two 200-L tanks. The infection was performed by anal intubation as previously described (Estensoro *et al.* 2010). Briefly, 20 GSB were intubated with 1 ml of *E. leei* infected-intestinal scrapings (recipient group, R) and 20 fish were intubated with the same volume of sterile phosphate buffer saline (control group, C) during two consecutive days. Seven fish from both the C and R groups were sampled at 15 (time point = t1) and 40 (time point = t2) days post intubation (dpi). They were starved for two days and killed by overexposure to benzocaine anaesthesia (3-aminobenzoic acid ethyl ester, 100 mg/ml) (Sigma, St. Louis, MO, USA). Tissue portions of anterior intestine (Ai), middle intestine (Mi), posterior intestine (Pi), head kidney (Hk), spleen (Sp) and thymus (Th) were taken, fixed in Bouin for 24 h and embedded in paraffin for histological processing, following standard histology procedures.
All efforts were made to minimize animal suffering and all experiments were carried out in accordance with national (Royal Decree RD1201/2005, for the protection of animals used in scientific experiments) and institutional regulations (CSIC, IATS Review Board), and the current European Union legislation on handling experimental animals. Minimum number of animals was used to produce statistically reproducible results.

10.2.2 Parasite diagnosis

Parasite prevalence and intensity of infection was evaluated by microscopic examination of 4 µm haematoxylin/eosin stained paraffin sections of the three intestinal segments. The intensity of infection of each intestinal segment was indicated according to a semi-quantitative scale ranging from 1+ to 6+ depending on the parasite stages found per microscope field at ×250 magnification (scaling, 0 = no parasite stages; 1+ = 1–5 parasite stages; 2+ = 6–10 parasite stages; 3+ = 11–25 parasite stages; 4+ = 26–50 parasite stages; 5+ = 51–100 parasite stages; 6+ > 100 parasite stages).

10.2.3. IgM, G7 and HIS immunohistochemistry

Paraffin sections (4 µm thick) of Ai, Pi, Hk, Sp and Th were collected on Super-Frost-plus microscope slides (Menzel-Gläser) and allowed to dry overnight. Slides were deparaffinised, hydrated and the endogenous peroxidase activity was blocked by incubation in hydrogen peroxide (0.3% (v/v) for 30 min). Incubations were performed in a humid chamber at room temperature and all washing procedures consisted of successive 5 min immersions in TTBS (20 mM Tris-HCl, 0.5 M NaCl, 0.05% Tween 20, pH 7.2) and TBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.2). Slides were washed, blocked for 30 min either with 1.5% normal goat serum or with normal horse serum (VECTOR Laboratories, Burlingame, CA, USA). After washing, they were then incubated with the primary antibody for 1 h and washed again. Detailed information can be found in Table 1. Slides were incubated with a biotinylated secondary antibody (1:200), either goat anti-rabbit or horse anti-mouse (VECTOR Labs.), for one further hour. They were washed and the avidin-biotin-peroxidase complex (ABC) (VECTOR Labs.) was applied for 1 h before washing slides again. Bound peroxidase was finally revealed by adding 3,3'-diaminobenzidine tetrahydrochloride chromogen (Sigma) for 5 min and the reaction was stopped with deionised water. Eventually, tissue sections were counterstained with Gill's haematoxylin, dehydrated and mounted with di-*N*-butyl phthalate in xylene. Incubation of tissue sections with ABC alone served as control to discard the presence of endogenous biotin-binding proteins. Negative controls omitting the primary antibodies, the secondary antibody and the ABC, respectively, were carried out and were negative.

In addition, four routine staining procedures were performed on some tissue sections, namely toluidine blue, Giemsa, alcian blue and PAS.

Table 1. Antibodies used for the detection of gilthead sea bream (GSB) leukocytes in histological sections.

ANTIBODY	TYPE (ORIGIN)	WORKING DILUTION	SOURCE
Anti-GSB G7	Monoclonal (mouse)	1:100	UM (Sepulcre *et al.* 2002)
Anti-GSB IgM	Polyclonal (rabbit)	1:60,000	IATS (Palenzuela *et al.* 1996)
Anti-histamine	Polyclonal (rabbit)	1:100	Sigma

10.2.4 Image analysis and statistics

For all C and R fish sampled, ten random digital fields from each tissue section were captured with an Olympus DP70 camera connected to a Leitz Dialux22 light microscope at x400 magnification. Immunoreactive cells (IRCs) were detected and quantified using ImageJ software (open-source Java-based imaging program). The high density of G7-IRCs found in Hk hampered their individual quantification and, thus, measurement and recording of the immunoreactive surface in renal sections was performed instead. In addition, splenic images at x100 magnification covering the entire organ section were used to quantify the amount of MMCs and their surface.

Statistically significant differences in the IRC number, the immunoreactive surface and the MMC values between C and R fish tissues at both sampling times were analysed by one-way analyses of variance (ANOVA-I) followed by Student-Newman-Keuls test. Data which failed the normality or equal variance test were analysed with Kruskal-Wallis ANOVA-I on ranks followed by Dunn's method. If the differences between C and R groups at t1 and at t2 were not significant, data were pooled into C and R and Student *t*-tests were performed to detect differences between the two groups or instead, Mann-Whitney *U* sum tests were applied for non-normal distributed data. ANOVA-I was also performed to compare three groups when data were pooled and categorized according to the fish infection status as C (not exposed), exposed and not infected R (non-parasitized), and exposed and infected R (parasitized).

10.3 RESULTS

10.3.1 *Enteromyxum leei* infection

At 15 (t1) and 40 (t2) dpi prevalence of infection reached 85.7% in R GSB. At both times, all sampled fish except one were infected with a high intensity (6+) at the Pi, and the observed parasite stages in all examined intestine sections ranged between proliferative stages with secondary cells and disporous sporoblasts with mature as well as immature spores. Only one R fish was parasitized also at the Ai at t2. No parasites were detected at the Mi at any sampling and no C fish was infected. There were no significant differences between biometrical data of the sampled fish (C vs R and t1 vs t2) (average weights: C-t1 = 131.6 g; C-t2 = 168.0 g; R-t1 = 149.4 g; R-t2 = 149.0 g).

10.3.2 Granulocyte histochemistry

In haematoxylin/eosin and Giemsa stained sections, fuchsia-red coloured eosinophil/acidophil granulocytes were observed in all examined organs (Fig. 1). In intestines they predominated in the lamina propria-submucosa in both C and R fish and degranulation close to intraepithelial parasite stages was best observed (Fig. 1 F). Weakly stained, large eosinophil/acidophil granulocytes and smaller eosinophil/acidophil granulocytes densely packed with cytoplasmatic granules were distinguishable (Fig. 1 D). Such cells were also found within blood vessels of the different organs. No metachromasy was observed in toluidine stained tissues, no granulocytes were stained with alcian blue, and no PAS positive granules were found (not shown).

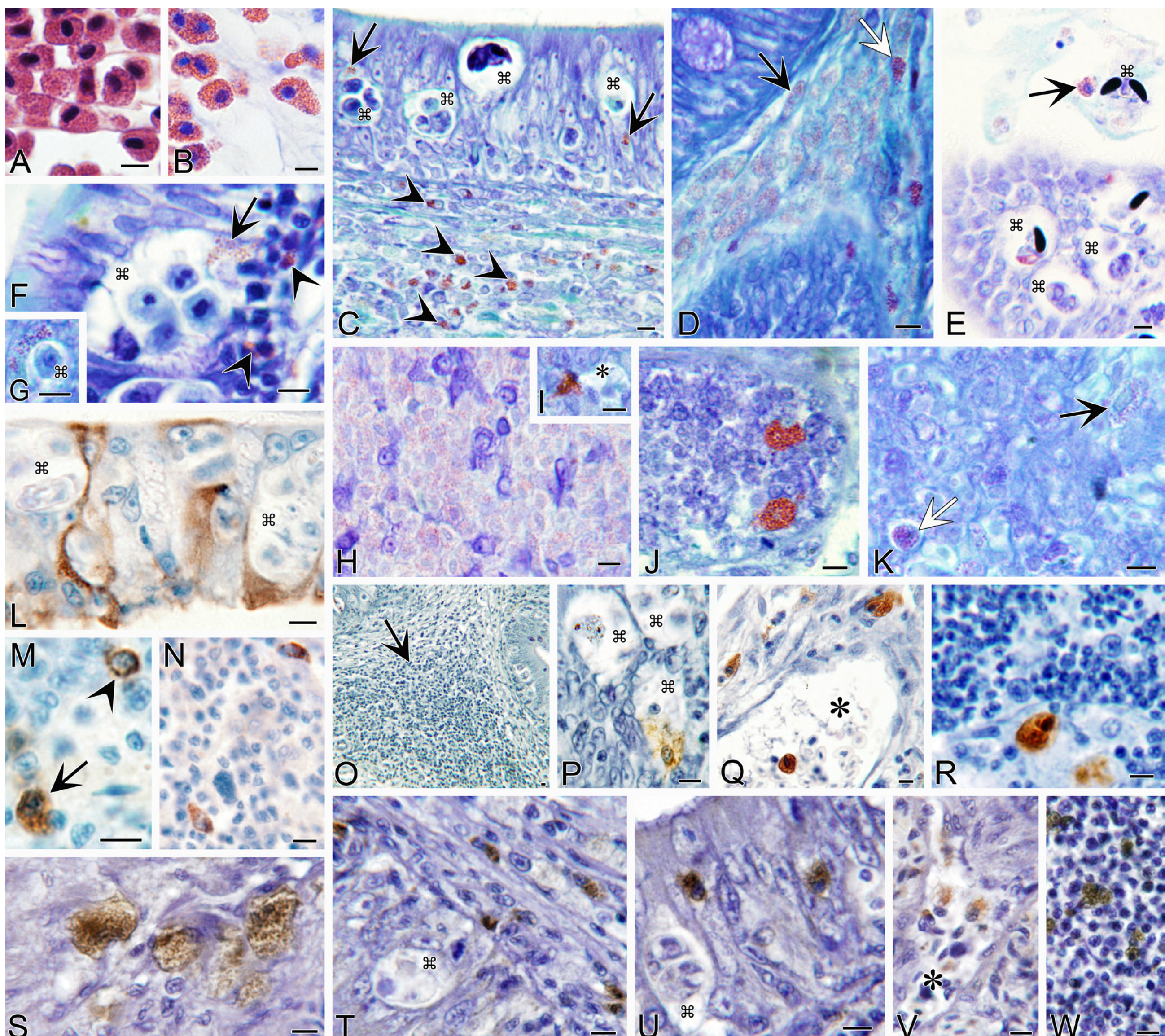

Figure 1 (previous page). Leukocyte histochemistry (**A**: haematoxylin & eosin, **B-K**: Giemsa) and immunohistochemistry (**L-W**: haematoxylin) of infected gilthead sea bream in different tissue sections. *Enteromyxum leei* stages are indicated by ⌘. (**A,B**) Eosinophil/acidophil granulocytes in the posterior intestine (Pi). (**C**) Small eosinophil/acidophil granulocytes in infected Pi. Note their abundance in the lamina propria-submucosa (arrowheads) and some infiltration in the epithelium (arrows). (**D**) Abundant, large eosinophil/acidophil granulocytes (black arrow) and scarce, small eosinophil/acidophil granulocytes (white arrow) in lamina propria-submucosa of Pi. Note the diffuse granule content in large ones and the densely packed granules in the small ones. (**E**) Desquamated cell material in the lumen of infected Pi containing an eosinophil/acidophil granulocyte (arrow) close to an *E. leei* spore. (**F**) Degranulation (arrow) into a proliferative parasite stage with numerous secondary daughter cells in the Pi. Several small eosinophil/acidophil granulocytes (arrowheads) are infiltrated in the epithelium. (**G**) Detail of eosinophil/acidophil granules surrounding an epithelial parasite stage in the Pi. (**H**) Head kidney parenchyma with numerous large eosinophil/acidophil granulocytes. (**I**) One small eosinophil/acidophil granulocyte close to a blood vessel (*****) in head kidney. (**J**) Two eosinophil/acidophil granulocytes in the thymus. (**K**) Scarce eosinophil/acidophil granulocytes in the splenic parenchyma showing different granule densities: disperse granules (black arrow), dense granules (white arrow). (**L**) IgM immunoreactive cells (IRCs) and immunoreactive secretion between parasite stages in the Pi epithelium. (**M**) IgM immunoreactive plasma cell (arrow) and **B** cell (arrowhead). (**N**) IgM IRCs in the thymus. (**O**) Hypertrophied lamina propria-submucosa in infected Pi with numerous IgM negative lymphocytes (arrow). (**P**) G7 IRC apparently degranulating on a parasite. (**Q**) G7 IRCs in a blood vessel (*) and in the lamina propria-submucosa of the Pi. (**R**) G7 IRCs in the thymus. (**S**) Large histamine IRCs in the lamina propria-submucosa of the anterior intestine. (**T**) Small histamine IRCs in the lamina propria-submucosa of an infected Pi. (**U**) Small histamine IRCs in the infected epithelium of Pi. (**V**) Histamine IRCs in a splenic blood vessel (*). (**W**) Histamine IRCs in the thymus. Scale bars = 5 µm.

10.3.3 IgM-IRC distribution pattern

IRCs for the polyclonal antibody (Pab) anti-GSB IgM (Pab-IgM) were detected in intestines as well as in lymphohaematopoietic tissues of C and R fish (Fig. 1, 2, 3). Their morphology was in most cases compatible with plasma cells for their voluminous cytoplasm (large cytoplasm/nucleus ratio) and in some occasions with B cells for their round shape and thin cytoplasm rim (low cytoplasm/nucleus ratio) (Fig. 1 M). In both cell types, the staining was located in the cytoplasm and different intensities of immunoreactivity were observed depending on the degree of cell differentiation. In intestines, IgM-IRCs were mostly located in the lamina propria-submucosa and less frequently infiltrated in the epithelium in close contact to the parasite (Fig. 1 L; Fig. 2 D, J). On the epithelial basal lamina and in the hypertrophied lamina propria-submucosa of R intestines, high amounts of cells morphologically resembling lymphocytes were present, which were not immunoreactive (Fig. 1 O). In lymphohaematopoietic tissues, IgM-IRCs were found in the renal and thymic parenchymas and in the splenic white pulp (Fig. 1 M, N), often close to blood vessels (Fig. 2 D) and MMCs. In C tissues, IgM-IRCs were more abundant in lymphohaematopoietic tissues than in intestines and Hk presented the highest IgM-IRC counts.

At t1, no statistically significant differences in IgM-IRCs between R and C fish were observed (Fig. 4). By contrast, at t2, a statistically significant increase in IgM-IRCs occurred in Pi and Hk of R fish compared to C, though more pronounced at the Pi, in which a six-fold increase was detected. In R fish Sp, the IgM-IRCs number was significantly higher at t2 than at t1. Though no significant differences in IgM-IRCs were detected at the Ai for none of the two sampling times individually, when data were pooled into C and R groups, regardless of the time of infection, the number of IRCs was significantly higher in R than in C fish (inset Fig. 4). Interestingly, at t1, a pattern of IgM-IRC increase was recorded for both intestinal segments of R fish compared to their C group, in contrast with the decreasing trend observed in the lymphohaematopoietic tissues (Hk, Sp and Th).

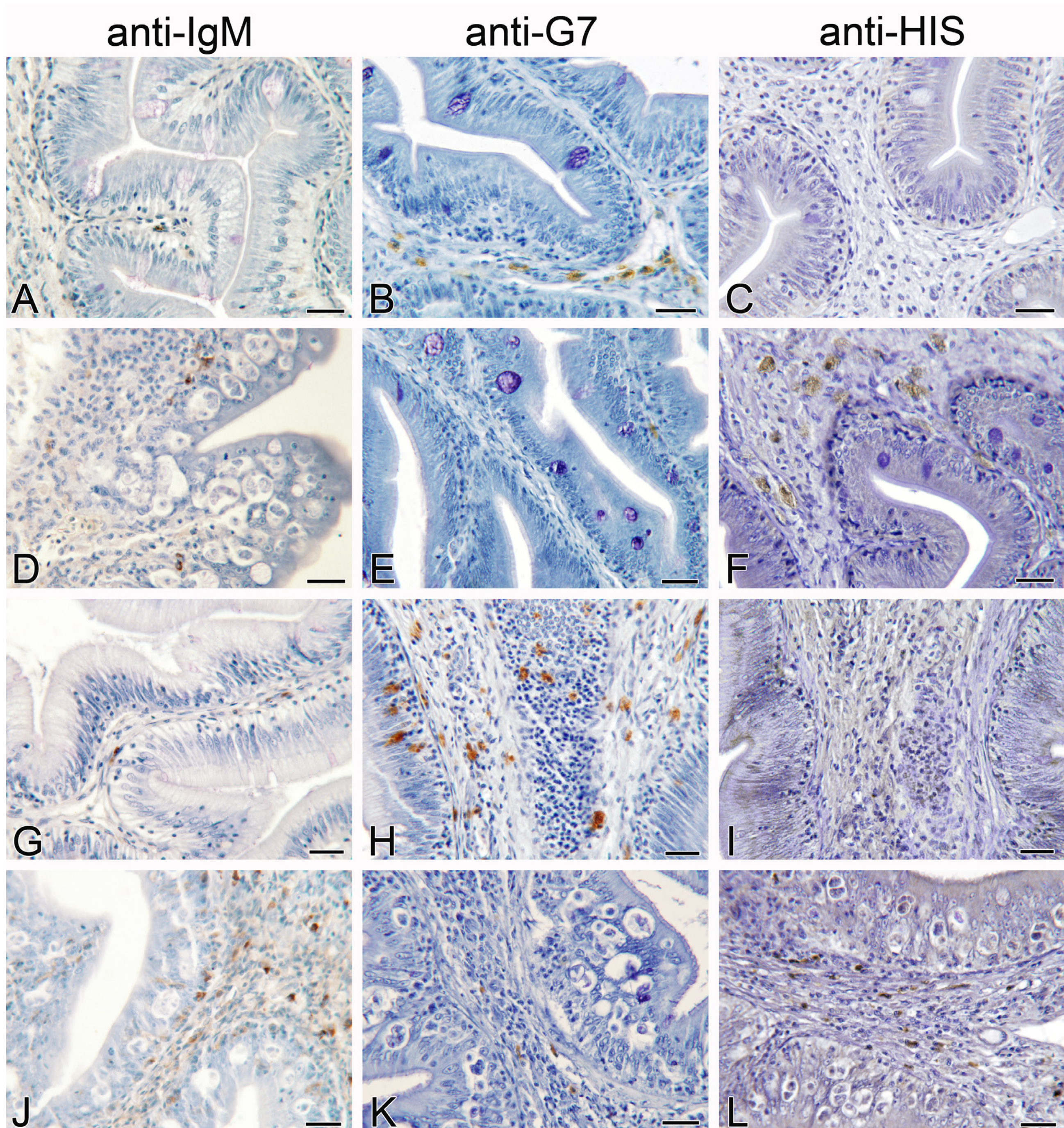

Figure 2. Leukocyte immunohistochemistry of gilthead sea bream intestinal sections counterstained with haematoxylin. Anterior intestine (**A-F**) and posterior intestine (**G-L**) sections of control, unexposed fish (**A-C** and **G-I**) and of *Enteromyxum leei*-exposed fish (**D-F** and **J-L**) were immunolabelled with the anti-IgM polyclonal antibody (Pab), with the anti-G7 monoclonal antibody or with the anti-histamine (anti-HIS) polyclonal antibody, which detect plasma cells/B cells, acidophilic granulocytes and mast cells, respectively. Scale bars = 20 µm.

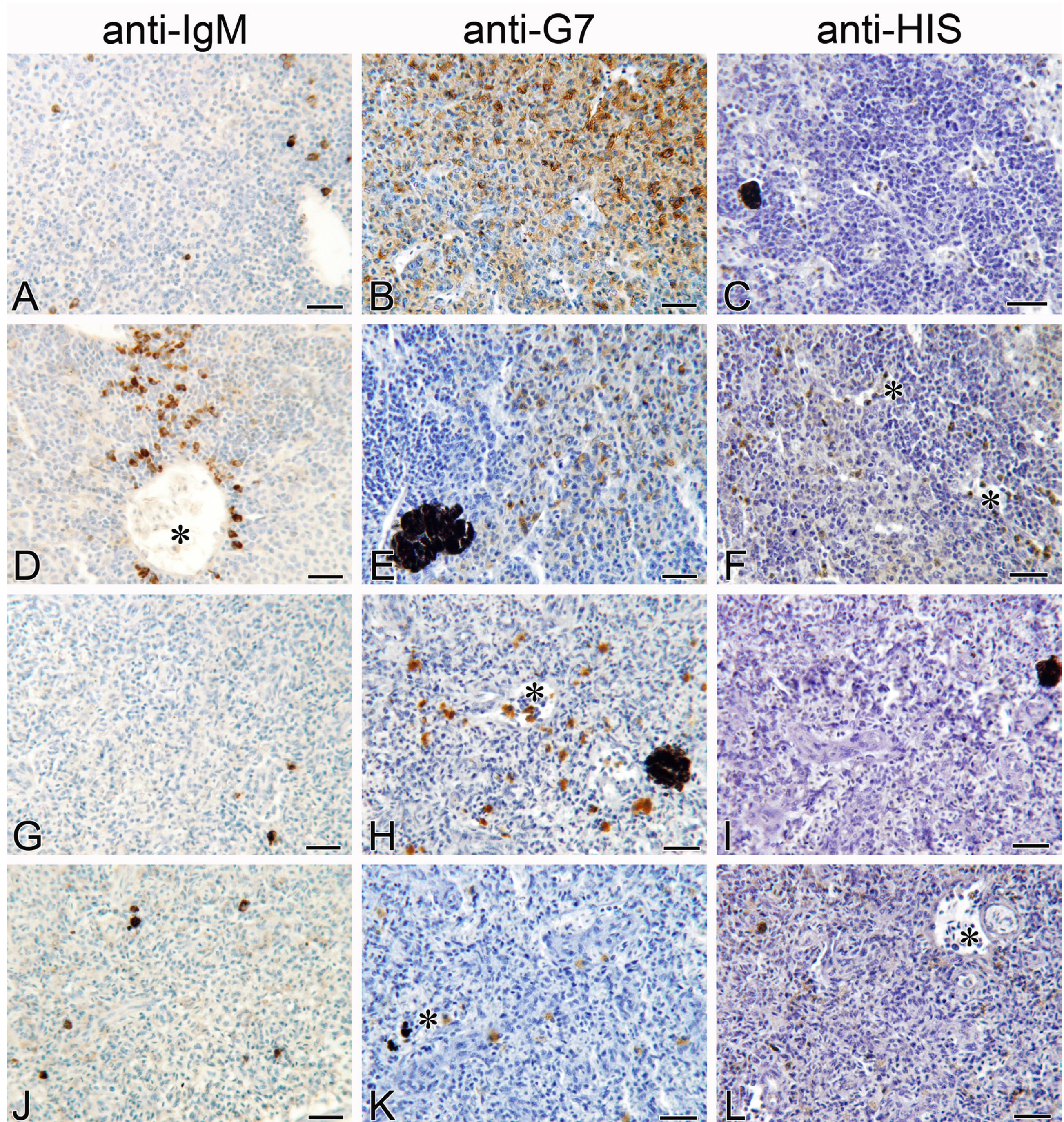

Figure 3. Leukocyte immunohistochemistry of gilthead sea bream sections of lymphohaematopoietic organs counterstained with haematoxylin. Head kidney (**A-F**) and spleen (**G-L**) sections of control, unexposed fish (**A-C** and **G-I**) and of *Enteromyxum leei*-exposed fish (**D-F** and **J-L**) were immunolabelled with the anti-IgM polyclonal antibody (Pab), with the anti-G7 monoclonal antibody or with the anti-histamine (anti-HIS) polyclonal antibody, which detect plasma cells/B cells, acidophilic granulocytes and mast cells, respectively. Note the presence of immunoreactive cells in and around blood vessels (*). Scale bars = 20 µm.

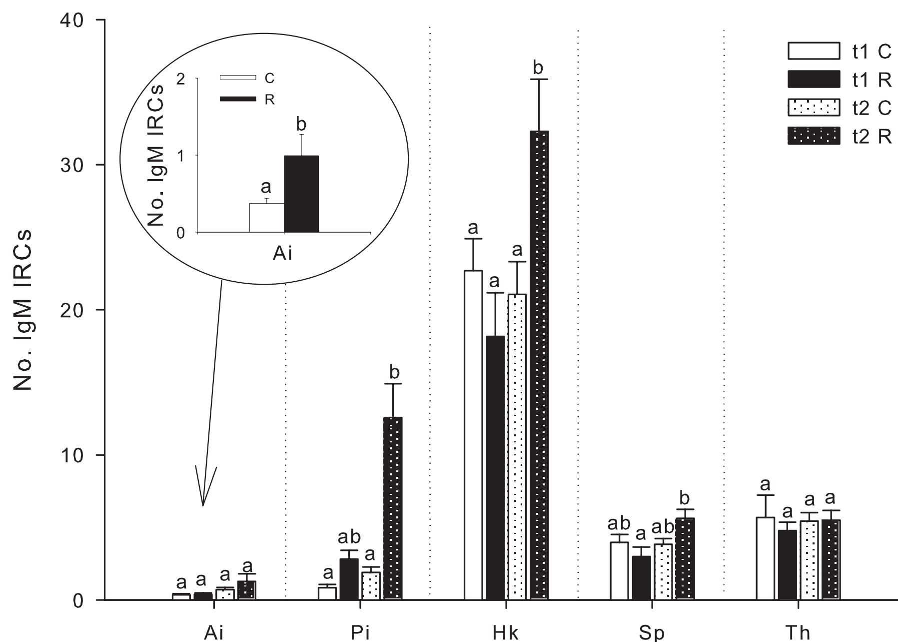

Figure 4. Mean + SEM of IgM immunoreactive cells (IRCs) in tissue sections of recipient (R) and control (C) gilthead sea bream at two sampling times: t1, 15 days post *Enteromyxum leei* intubation (dpi) and t2, 40 dpi. Pooled Ai data of t1 and t2 for C and R fish, respectively, are represented in the inset. For each tissue, letters indicate statistically significant differences ($p < 0.05$) between groups. Anterior intestine (Ai), posterior intestine (Pi), head kidney (Hk), spleen (Sp), thymus (Th).

10.3.4 G7-IRC distribution pattern

The monoclonal antibody (Mab) against the G7 molecule (Mab-G7) labeled AGs in all the examined intestinal and lymphohaematopoietic tissues of C and R GSB (Fig. 1, 2, 3). These leukocytes had a rounded eccentric nucleus and a voluminous granular cytoplasm (large cytoplasm/nucleus ratio), in which the immunolabel was observed by light microscopy. Their shape was spherical in the Sp (Fig. 3 H), while in the other tissues they presented heterogeneous morphologies (Fig. 2 P, Q, R). Different degrees of immunoreactivity were mainly observed in Hk (Fig. 3 B). In intestinal sections, AGs predominated in the lamina propria-submucosa but also infiltrated in the epithelium (Fig. 1 P; Fig. 2 H). In Hk, the highest amounts of AGs were found forming large clusters that covered a broad surface of the parenchyma (Fig. 3 B). By contrast, AGs in Sp were scattered throughout the organ (Fig. 3 H) as in the thymic parenchyma, in which scattered AGs were scarcely found (Fig. 1 R).

Statistically significant differences in the numbers of G7 immunoreactive granulocytes between R and C fish were only found at t2, when a decrease was detected in Pi, Hk and Sp (Fig. 5) However, such a decreasing trend of the AGs was, in any case, found in all tissues at both infection times. Though at the Ai no significant differences were observed for none of the two samplings, a significantly lower AG number was detected in R fish with respect to C fish for pooled data of both samplings (inset Fig. 5).

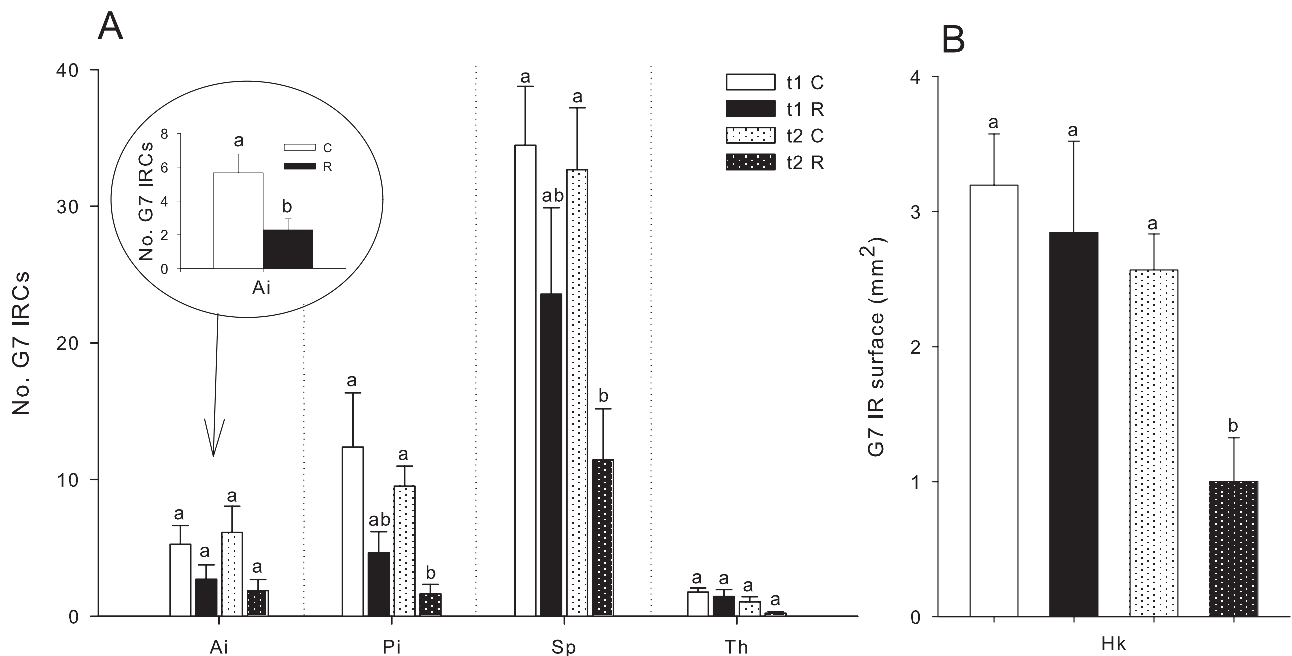

Figure 5. Mean + SEM of G7 immunoreactive cells (IRCs) (**A**) or immunoreactive surface (**B**) in tissue sections of recipient (R) and control (C) gilthead sea bream at two sampling times: t1, 15 days post *Enteromyxum leei* intubation (dpi) and t2, 40 dpi. Pooled Ai data of t1 and t2 for C and R fish, respectively, are represented in the inset. For each tissue, letters indicate statistically significant differences ($p < 0.05$) between groups. Anterior intestine (Ai), posterior intestine (Pi), head kidney (Hk), spleen (Sp), thymus (Th).

10.3.5 HIS-IRC distribution pattern

Immunoreactive mast cells for the Pab against HIS (Pab-HIS) were present in all the studied tissues of C and R fish (Fig. 1, 2). Two morphological types of mast cells were immunolabelled by the Pab-HIS, regarding their size. The small-sized type (4-7 μm) showed a round to oval eccentric nucleus and a small cytoplasm, which was densely immunolabelled (Fig 1 T, U). The large-sized type presented a similar nuclear appearance, but an extremely larger cytoplasm (10-15 μm cell size), in which a more dispersed and granular immunolabel was observed (Fig. 1 S). Intermediate sizes of immunoreactive mast cells could be observed mainly in lymphohaematopoietic tissues (Fig. 1 V, W; Fig. 3 G, F, I, L). Notably, almost only the large mast cell type was observed at the Ai (Fig. 2 F). At the Pi, by contrast, both morphological types coexisted with similar abundance, and scarce intermediate stages were found.

A similar pattern of mast cell increase in R compared to C fish was detected in all tissues at the two sampling times, though the increase was statistically significant only at t2 in Pi and Sp (Fig. 6). The increase of HIS-IRCs in Hk of R fish at t2 was only significant compared to R fish at t1, but not with respect to C. At the Ai, a significant increase in large-sized mast cells was detected in R compared to C, after pooling data of both infection times. At the Pi, the tissue with the highest amount of mast cells, the two size types were quantified separately. No variations in the number of the large type were found due to infection, but there was significant increase in the small type in R fish at t2.

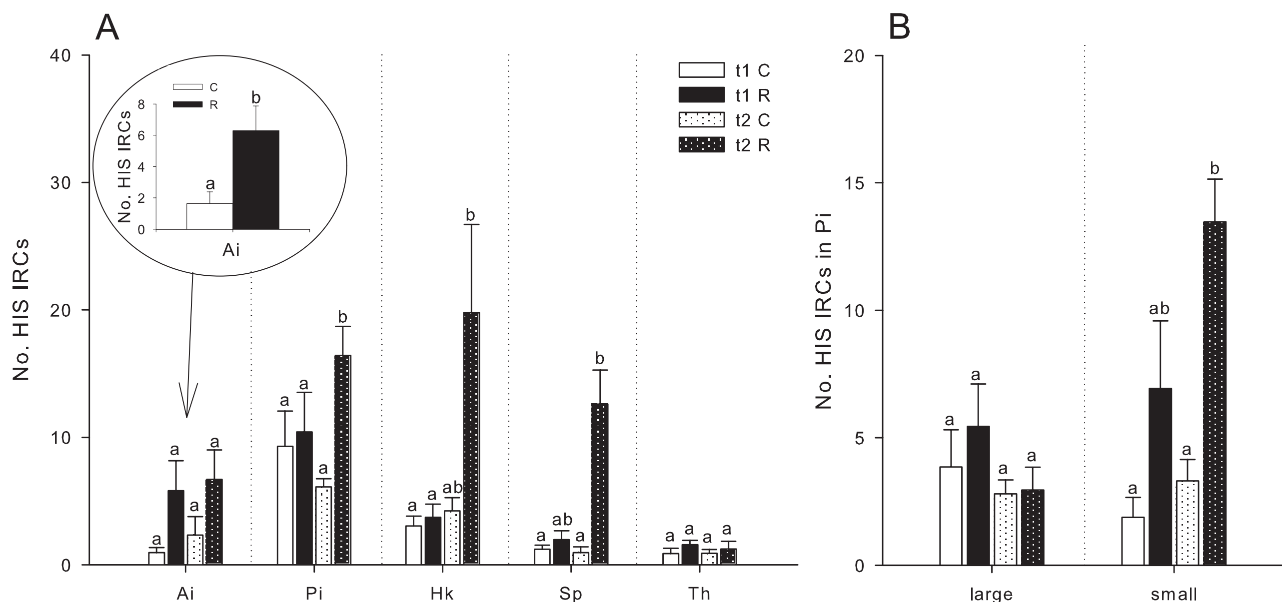

Figure 6. Mean + SEM of histamine (HIS) immunoreactive cells (IRCs) in tissue sections of recipient (R) and control (C) gilthead sea bream at two sampling times: t1, 15 days post *Enteromyxum leei* intubation (dpi) and t2, 40 dpi. (**A**) Pooled Ai data of t1 and t2 for C and R fish, respectively, are represented in the inset. Anterior intestine (Ai), posterior intestine (Pi), head kidney (Hk), spleen (Sp), thymus (Th) (**B**) HIS-IRC types by their size at the Pi. For each tissue and cell type, letters indicate statistically significant differences ($p < 0.05$) between groups.

10.3.6 MMCs distribution pattern

The size of the MMCs was analysed in splenic sections and no significant differences between C and R fish groups were found (Fig. 7, 8). The same happened for the measurements on the number of MMCs per mm^2 and for the percentage of splenic surface occupied by MMCs.

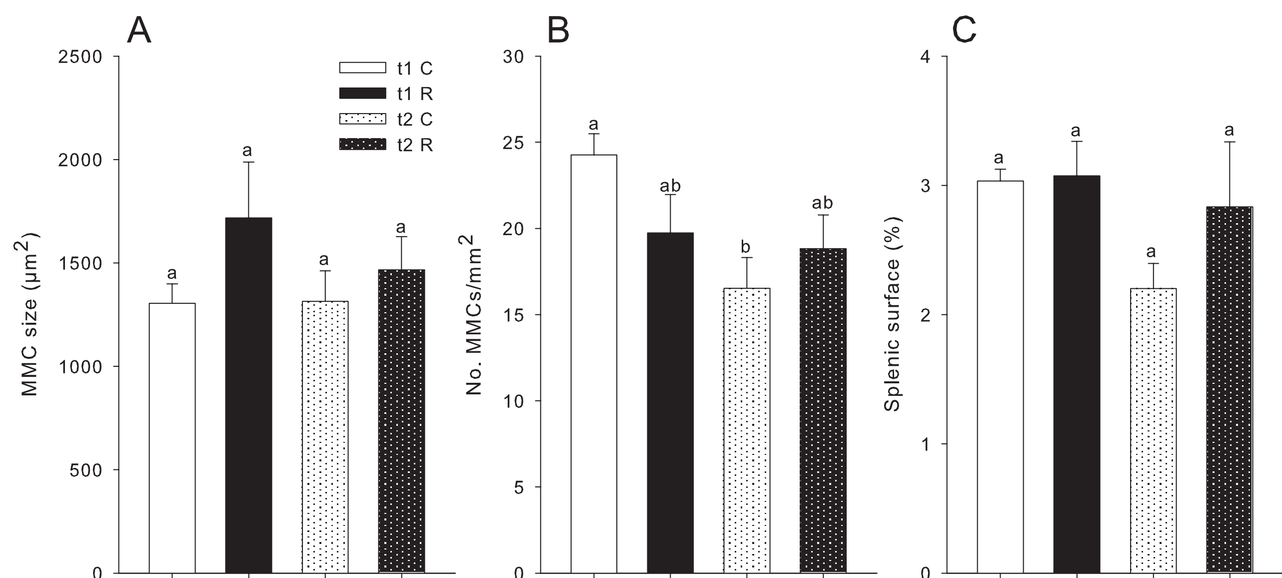

Figure 7. Mean + SEM of melanomacrophage centres (MMCs) in splenic sections of recipient (R) and control (C) gilthead sea bream at two sampling times: t1, 15 days post *Enteromyxum leei* intubation (dpi) and t2, 40 dpi. (**A**) MMC size in μm^2. (**B**) Average count of splenic MMCs per mm^2. (**C**) Percentage of splenic surface occupied by MMCs. Different letters indicate statistically significant differences ($p < 0.05$) between groups.

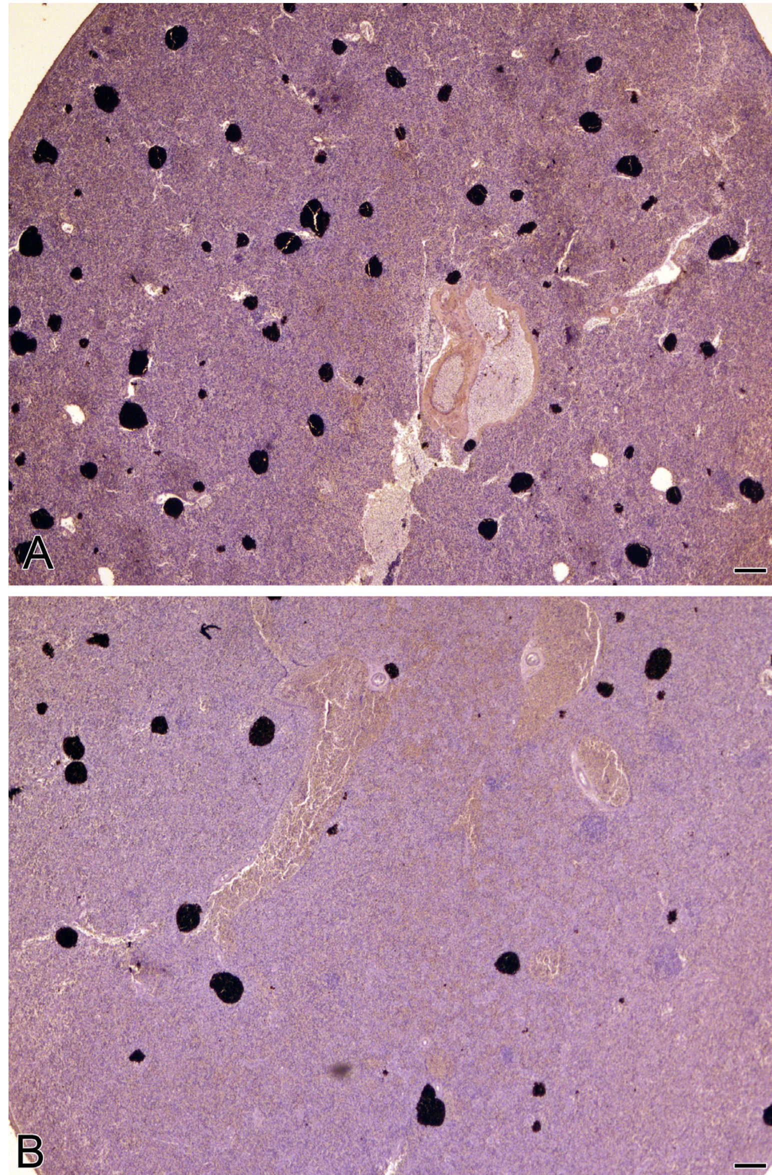

Figure 8. Representative spleen sections of gilthead sea bream used for the measurement of melanomacrophage centres parameters. (**A**) Non-parasitized fish exposed to *Enteromyxum leei*. (**B**) Control, unexposed fish. Scale bar = 100 µm, staining: haematoxylin.

10.3.7 Leukocyte populations vs parasitic status

When data for AGs in intestinal segments were pooled into C, NON-PAR and PAR, no difference between NON-PAR and PAR groups was found, but both groups showed a significant lower number of AGs compared to C fish (Fig. 9). Intestinal plasma cells/B cells, by contrast, were significantly higher in PAR than in C and NON-PAR intestines (Fig. 9). The number of intestinal mast cells was significantly higher only in PAR tissues compared to C counts, though certain increase of mast cells was also found in NON-PAR (Fig. 9). A significant increase of the splenic surface occupied by MMCs was detected in NON-PAR fish compared to C and PAR, when fish were pooled by the infective status at Pi (Fig. 10).

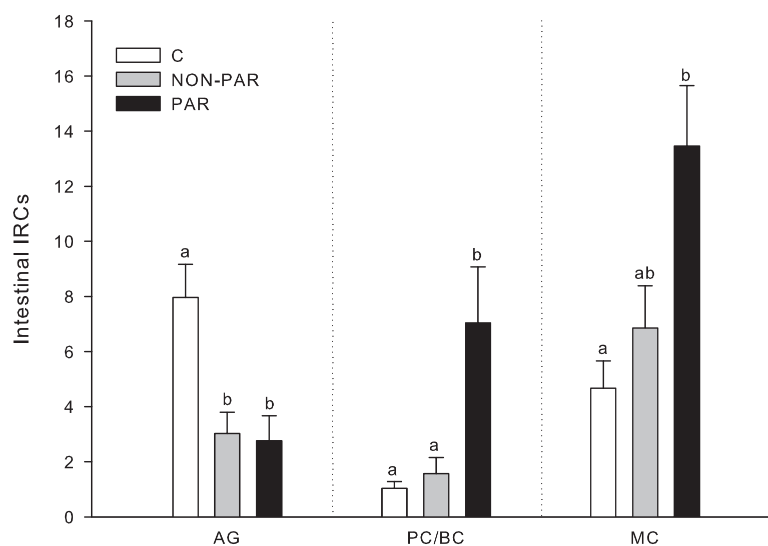

Figure 9. Mean + SEM of immunoreactive cells (IRCs) (acidophilic granulocytes (AG), plasma cells and B cells (PC/BC) and mast cells (MC) in intestinal sections of control (C) unexposed gilthead sea bream and recipient gilthead sea bream inoculated with *Enteromyxum leei*. Data of anterior and posterior intestines of two samplings (15 and 40 days post intubation) are pooled and recipient fish, split into parasitized (PAR) and non parasitized (NON-PAR) intestinal segments. Different letters indicate statistically significant differences between groups ($p < 0.05$).

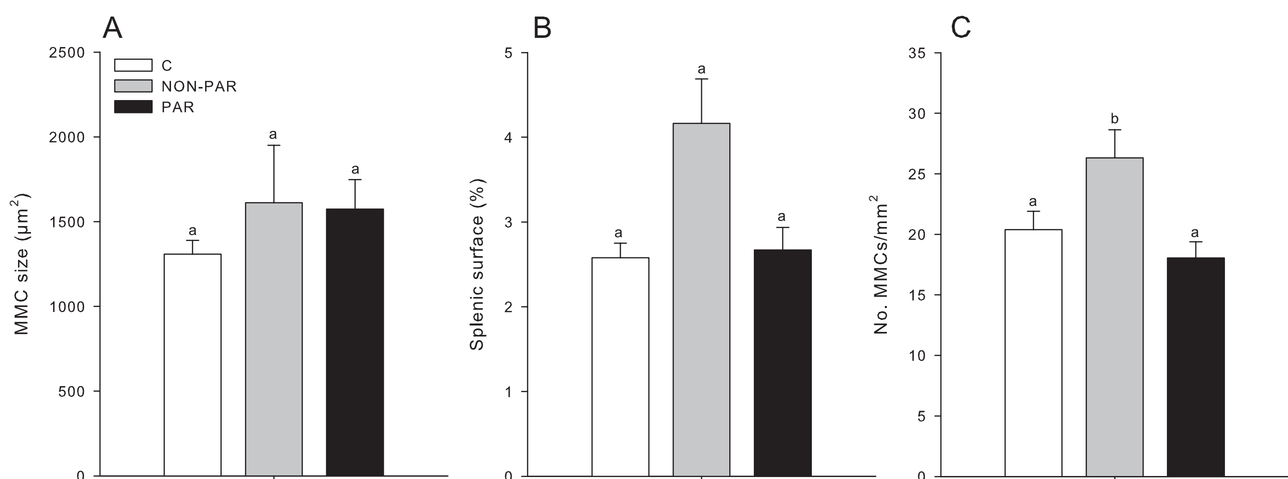

Figure 10. Mean + SEM of melanomacrophage centres (MMCs) in splenic sections of control (C) and recipient gilthead sea bream inoculated with *Enteromyxum leei*. Data of two samplings (15 and 40 days post inoculum) are pooled and recipient fish, split into parasitized (PAR) and non parasitized (NON-PAR) fish at the posterior intestine. (**A**) MMC size in µm2. (**B**) Average count of splenic MMCs per mm². (**C**) Percentage of splenic surface occupied by MMCs. Different letters indicate statistically significant differences between groups ($p < 0.05$).

10.4 DISCUSSION

Enteromyxum spp. entail a thread for several aquaculture reared fish species and the lack of available therapeutic treatments and vaccines point out the need to broaden our knowledge on the immune factors that may participate in host protection (Sitjà-Bobadilla and Palenzuela, 2012). Most available data on GSB immune response to enteromyxosis have been gathered from long-term experimental infections by cohabitation with *E. leei*-infected fish or exposure to *E. leei*-contaminated effluent (> 100 days exposure) (Cuesta *et al.* 2006*b*; Cuesta *et al.* 2006*c*; Davey *et al.* 2011; Estensoro *et al.* 2011; Estensoro *et al.* 2012*a*; Estensoro *et al.* 2013; Estensoro *et al.* 2012*b*; Sitjà-Bobadilla *et al.* 2008; Sitjà-Bobadilla *et al.* 2007). In the current study, the relatively fast progression of the *E. leei* infection by the anal route resulted in high prevalence and intensity of infection as soon as at 15 dpi, confirming previous findings on this experimental infection route (Estensoro *et al.* 2010). However, the disease outcome was not yet apparent, since no mortalities nor macroscopical signs of the disease were recorded, but fish harboured a well-established infection of proliferative as well as sporogonic parasite stages.

The infection route not only influenced the infection level (intensity and prevalence), but it also determined the on-set of host the immune response. This becomes particularly evident in regard to the IgM local response, when the current results are compared with data from a previous trial, in which GSB were exposed to the parasite by effluent (Estensoro *et al.* 2012*a*). In that previous study, a detailed analysis of IgM dynamics was performed, showing a positive correlation between IgM transcription levels and IgM-IRC abundance, and only after 133 days of exposure to the parasite, a significant increase of IgM expression occurred at the Pi. By contrast, the current experimental infection triggered a significant recruitment of plasma cells/B cells in PAR fish much earlier (40 dpi), when a six-fold IgM-IRC increase was recorded at the target site of the parasite, the Pi, and also weaker at the Ai. Such higher number of intestinal plasma cells/B cells, either from local synthesis, blood derived or both, contributed to the mucosal inflammatory response. At this time point (t2), renal and splenic plasma cells/B cells had significantly increased, probably as a sign of haematopoietic proliferation and of the maturation and initiation of the adaptive immune mechanisms.

The initial decreasing trend in lymphohaematopoietic tissues at t1, may suggest an early mobilization of IgM-IRCs from the systemic reservoirs to the local infection site, where such a cell type increased at this time point, but not significantly. Interestingly, the faster infection course due to the route employed in the current study seems to approach infection dynamics and immune response of the GSB-*E. leei* model to the ones of the more pathogenic turbot-*E. scophthalmi* model. In this species, an IgM-IRC increase in Sp and Hk of R fish at 20 and 40 dpi was associated with the initiation of a humoral immune response, which was followed by a subsequent migration of IgM-IRCs to the infected intestines and finally, by lymphohaematopoietic depletion owing to immunodepression (Bermúdez *et al.* 2006). In regard to IgM as well as to the mucosal IgT, the participation of mucosal-systemic and cellular-humoral interactions in the immune response of fish has been reported previously (Álvarez-Pellitero, 2008; Jorgensen *et al.* 2011; Olsen *et al.* 2011; Ordas *et al.* 2012; Salinas *et al.* 2011). Though the contribution of natural antibodies before the induction of an adaptive immune response has been recognized in teleosts, the involvement of innate-like lymphocytes is still far from being understood (Álvarez-Pellitero, 2008; Gómez and Balcázar, 2008; Hu *et al.* 2011; Magnadottir *et al.* 2009; Whyte, 2007). Remarkable is, however, the phagocytic capacity detected for B cells in some fish species suggesting their evolutionary relationship with macrophages and their possible role in innate immune functions (Li *et al.* 2006; Overland *et al.* 2010; Sunyer, 2012). However, the possible contribution of IgM-IRCs to the innate immune response of GSB against enteromyxosis deserves further studies.

The involvement of innate defence mechanisms on the immune response of fish to parasite infections has been studied in several host-parasite models (Álvarez-Pellitero, 2008). In GSB, lymphocytes, monocyte/macrophages and acidophilic granulocytes are being considered non-specific cytotoxic cells (NCCs) (Cuesta *et al.* 2005; Meseguer *et al.* 1996). In particular, an important role is attributed to the cellular effectors involved in the intestinal inflammatory response against *E. leei* (Cuadrado, 2009; Estensoro *et al.* 2010; Fleurance *et al.* 2008) and responsible for the variations detected in the cytotoxic, phagocytosis and respiratory burst activities and peroxidase content during enteromyxosis (Cuesta *et al.* 2006*b*; Cuesta *et al.* 2006*c*; Estensoro *et al.* 2011; Sitjà-Bobadilla *et al.* 2008). Such innate mechanisms were also engaged in the response of GSB to bacterial and viral threads (Chaves-Pozo *et al.* 2012; Dezfuli *et al.* 2012*b*; Reyes-Becerril *et al.* 2011*b*) as well as to bioactive compounds (Águila *et al.* 2013; Cabas *et al.* 2012; Cuesta *et al.* 2008; Reyes-Becerril *et al.* 2011*a*). Indeed, AGs are professional phagocytes of GSB (together with macrophages)

and are considered the functional equivalents of mammalian neutrophils as they can be rapidly recruited from Hk and are the most abundant circulating granulocytes (Chaves-Pozo *et al.* 2005; Sepulcre *et al.* 2002). They contribute to the respiratory burst activity in tissues and serum and contain in their granules reactive oxygen intermediates (Couso *et al.* 2001; Sepulcre *et al.* 2002), antimicrobial peptides (piscidin 3) (Mulero *et al.* 2008) and lysosomal enzymes (peroxidase and acid phosphatase) (Meseguer *et al.* 1994a), which can all be intracellularly (into phagosomes) or extracellularly secreted to degrade pathogens. AGs also express the pro-inflammatory cytokine interleukin-1β, the inflammatory prostanoid COX-2 (Chaves-Pozo *et al.* 2004; Sepulcre *et al.* 2007), and antigen presenting major histocompatibility complex II alpha chain (Cuesta *et al.* 2006a), thus suggesting that AGs participate in the regulation of inflammation and link innate and adaptive immune mechanisms. Our results showed that this paramount leukocyte for the defence of GSB decreased in all examined tissues of R fish (PAR and NON-PAR, except in Th), which may explain at least part of the limited protection of this species against the *E. leei.* In accordance, a decrease of the AG percentage (and consequent decrease in respiratory burst, phagocytic activity and leukocyte peroxidase) in Hk of *E. leei*-infected GSB was detected (Cuesta et al. 2006c). On the other hand, the high serum peroxidase content found after *E. leei* exposure supports leukocyte mobilization into the circulatory system (Cuesta *et al.* 2006b). Nevertheless, immunosupressive effects of some fish parasites included the inhibition of NCC proliferation, of their phagocytic activity and the induction of NCC apoptosis. Moreover, immunomodulation by some parasites interfere cytokine transcription to facilitate their survival (Sitjà-Bobadilla, 2008). Whatever the cause, the observed significant and almost ubiquitous decrease of AGs deserves further investigation.

Fish mast cells are mainly eosinophilic granulocytes actively involved in bacterial and parasite clearance at mucosal sites through degranulation of antimicrobial peptides and enzymes like lysozyme (Corrales *et al.* 2010; Murray *et al.* 2007; Reite, 1998). The cytoplasmatic granules of GSB mast cells participating in the innate mucosal defence may contain HIS, piscidin 3 and likely piscidins 1 and 2 (Mulero *et al.* 2008; Mulero *et al.* 2007). Our results show HIS containing mast cells with different morphologies among the granular infiltrates in the intestines of GSB but also in lymphohaematopoietic tissues, and these leukocytes presented an opposite distribution pattern to the distribution of AGs during the trial. Even if HIS containing granules are present in mature mast cells, different sizes of HIS-IRCs might be associated to different developmental cell stages, thus being the small ones the "young" cells proliferating in response to the parasite thread and being recruited from lymphohaematopoietic tissues into infection sites. The large-sized mast cells would therefore correspond to a more constant tissue resident population of "older" mast cells. Furthermore, Giemsa staining also revealed the existence of two types of acidophilic granular cells by their size and granule density. Generally speaking, fish mast cells are considered a heterogeneous cell population. It is accepted that mast cell precursors leave haematopoietic organs and, *via* blood circulation, reach mucosal sites in which maturation takes place locally (Reite, 1996, 1998). The presence of eosinophilic granular cells (EGCs), likely mast cells, among the intestinal inflammatory infiltrates of *E. leei*-infected species has been previously reported (Álvarez-Pellitero *et al.* 2008; Estensoro *et al.* 2010; Fleurance *et al.* 2008; Katharios *et al.* 2011; Sitjà-Bobadilla *et al.* 2007), as well as in response to other parasite diseases (Álvarez-Pellitero, 2008). Many authors have claimed EGCs to be mammalian mast cell equivalents for their functional and morphological similarities (Dezfuli *et al.* 2011*b*; Dezfuli *et al.* 2012*b*; Gómez and Balcázar, 2008; Mulero *et al.* 2007; Reite, 1996; Reite and Evensen, 2006). Apparently, different EGC1 and EGC2 types were involved in the response of both sparids GSB and *Diplodus puntazzo* to *E. leei*, though in the current study neither PAS positive nor different sized granules according to this description could be found (Álvarez-Pellitero *et al.* 2008; Estensoro *et al.* 2010). In the current results, the absence of PAS, but also toluidine and alcian blue positive granules reported for mast cells from other teleosts, may have been influenced by the different fixative we used, by the staining procedure itself, or by interspecific differences (Noya and Lamas, 1996; Reite, 1996, 1998; Rocha and Chiarini-Garcia, 2007). However, the number of EGC1 decreased whereas the number of EGC2 increased in R *D. puntazzo* (Álvarez-Pellitero *et al.* 2008), in coincidence with the detected AG decrease and mast cell increase in R GSB. While the term EGC refers to the staining properties of mononuclear granule-containing cells, it encompasses functionally different cell types, at least AGs and mast cells. In any case, a remarkable intraspecific heterogeneity among the EGCs of GSB seems obvious.

Nonetheless, the large EGC type with disperse granules seems to correspond to AGs, the predominant type in Hk, but also to some intestinal mast cells, whereas the small and densely packed EGC type seems to correspond exclusively to the mast cell population being recruited into the parasitized intestine. Both types of granular cells studied were found infiltrated in the intestinal epithelium in close contact with the parasite

and degranulation could be observed. Interestingly, epithelial AGs, mainly present in unexposed GSB, have not been observed in previous studies. Zones of extracellular IgM immunostaining and plasma cells surrounding parasite stages were also observed. Thus, the involvement of all three leukocyte populations in the GALT inflammatory reaction against the *E. leei* seems evident and, furthermore, this is apparently supported at systemic level since the distribution patterns of these leukocytes at the Pi and at the Hk show parallel changes. Circulating granulocytes observed in tissue vessels supports this. Accordingly, the perivascular position of many leukocyte types is associated to their regulatory function during inflammatory responses (Dezfuli *et al.* 2012*a*; Mekori, 2004; Mulero *et al.* 2007). Some examples are documented of cellular innate responses to piscine myxosporeans, such as the neutrophil and eosinophil-like infiltration during gill inflammation in channel catfish infected with *Henneguya* spp., whose degranulation within lesions was associated with further host tissue damage (Lovy *et al.* 2011), or the time-depending succession of granulocytes and lymphocytes in the edemata surrounding *Myxobolus pendula* branchial cysts in creek chub (Martyn *et al.* 2002). Other studies on leukocyte contribution during piscine parasitoses involve tapeworms and acanthocephalans (Dezfuli *et al.* 2011*a*; Dezfuli *et al.* 2012a; Dezfuli *et al.* 2011*b*; Dezfuli *et al.* 2012*c*; Dezfuli *et al.* 2013; Hansen *et al.* 2011). It should be noted that HIS may have an ambivalent effect on respiratory burst activity of professional fish phagocytes, thus suggesting a role for mast cells in regulating phagocyte activity and that mast cells may also regulate the inflammatory response by inducing vasodilatation and leukocyte chemotaxis and activation (Matsuyama and Iida, 1999, 2001; Mulero *et al.* 2007).

Our results show for the first time the presence of AGs and mast cells in Th of GSB. It is worth mentioning the absence of changes in the distribution patterns of the studied leukocytes in this organ during the whole trial. In agreement with the main role of this organ in T cell proliferation and maturation (Bowden *et al.* 2005), it does not seem to play a relevant role in granulocyte or plasma cell kinetics during the mucosal response. Nevertheless, low granulocyte content was found in the Th along with a relatively high and constant IgM-IRC presence, which was similar to the IgM-IRC content in Sp and higher than in intestines of C fish (about 4 times Pi levels and 10 times Ai levels). IgM-producing cells in the teleost Th have led several authors to suggest the involvement of this organ in humoral immunity of fish (Tian *et al.* 2009). Alternatively, thymic macrophages also participate in the clearance of immature self-reactive thymocytes during the development of functional T cells (Bowden *et al.* 2005), and therefore, IgM-bearing macrophages might be the IRCs we detected. At this point, a remark should be made on the absence of specific markers for GSB T cells as occurs for several teleosts. IgM negative, lymphocyte resembling cells in *E. leei*-infected intestines of GSB were previously considered putative T cells (Estensoro *et al.* 2012*a*), agreeing with observations made in *E. scophthalmi*-infected turbot intestines (Bermúdez *et al.* 2006). In the latter species, cross-reactivity with a heterologous anti-human CD3ε was found (Vigliano *et al.* 2011), which unfortunately did not cross-react with GSB in spite of its wide interspecific cross-reactivity (unpublished data).

The role of MMCs in the fish immune response as primitive germinal centres is related to antigen retention and processing, and thus involved in maintaining humoral memory (Vigliano *et al.* 2006). In regard to MMC abundance in the Sp, a significant increase was detected in the percentage of splenic surface they occupy in NON-PAR fish *vs* PAR and C fish. Such increase seemed to be related to greater MMC number rather than to their size increase. An increase of MMCs was already reported in the Sp of *E. leei*-infected GSB (Fleurance *et al.* 2008), of *E. scophthalmi*-infected turbot (Ronza *et al.* 2013; Sitjà-Bobadilla *et al.* 2006) and in the intestine of *E. fugu*-infected tiger puffer (Tun *et al.* 2002). This increase was associated with the inflammatory response and in some cases with the presence of engulfed parasite stages in MMΦs. Focal development of MMCs was also detected in response to several infections, including myxosporean infections (Agius and Roberts, 2003). It is tempting to suggest that a fast MMΦ proliferation and activation in the Sp may protect GSB from parasite invasion, but little evidence is still available.

The current results suggest the involvement of AGs, mast cells, plasma cells, B cells and MMΦs in the acute immune response of GSB to enteromyxosis at both lymphohaematopoietic and intestinal levels. Some morphological differences were detected between AGs and mast cells, which presented an opposite response to the parasite infection. Nevertheless, several links have been drawn between humoral and cellular mechanisms (leukocyte derived humoral factors like serum peroxidase) as well as between innate and adaptive immune responses (antigen presenting phagocytic and cytotoxic AGs; mucosal-systemic cooperation), in which such cell types participate, outlining once more the artificial nature of such boundaries (Álvarez-Pellitero, 2011; Criscitiello and de Figueiredo, 2013). In addition, immune evasive, immunosuppressive or even

immunomodulatory effects of the parasite on such cellular effectors are poorly understood and definitively deserve further studies.

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CONCLUSIONS

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1. A new method of experimental infection of gilthead sea bream with *Enteromyxum leei* by anal intubation was achieved. It provokes a faster and more uniform infection than other experimental transmission routes and methods.

2. The prevalence of infection by *Enteromyxum leei* in gilthead sea bream increases with water temperature and the parasite becomes latent and undetectable at the intestine at low temperatures (<18 °C), re-appearing when water temperature rises.

3. Rabbit polyclonal antisera were obtained against *Enteromyxum* spp., which are able to immunodetect the parasites in immunohistochemistry, ELISA and Western blot.

4. *Enteromyxum leei* seems to share common antigens with *Sphaerospora* spp. and with *Myxobolus pendula*.

5. Mannose and glucose are the main carbohydrate moieties in *Enteromyxum leei* extracts and are present in three antigenic glycoproteins identified with molecular weights of 15 kDa, 34 kDa and 165 kDa.

6. The 165 kDa antigenic glycoprotein also contains galactose, *N*-acetyl-galactosamine and *N*-acetyl-glucosasamine and is possibly located on spore valves of *Enteromyxum leei*.

7. Long-term feeding with a plant protein-based diet containing a blend of vegetable oils as the major lipid source at 66% replacement is a predisposing factor that worsens enteromyxosis in experimentally infected gilthead sea bream.

8. The decrease of intestinal goblet cells with neutral, acidic and carboxylic mucins, and with sialic acid at the anterior and middle intestine sections, in gilthead sea bream fed the 66% vegetable oil replacement diet, correlates with their higher prevalence and intensity of infection in these intestinal sections.

9. The goblet cell distribution pattern is modulated in *Enteromyxum leei*-infected gilthead sea bream provoking goblet cell depletion, which might be parasite-induced or host-mediated.

10. Abundance, size and glycosylation of the mucins of the intestinal mucous secretion are altered in *Enteromyxum leei*-infected gilthead sea bream, entailing a reduction of bacterial adhesion.

11. The immune response of *Enteromyxum leei*-infected gilthead sea bream at the posterior intestine segment includes the up-regulation of IgM gene expression, which is correlated positively with higher numbers of IgM immunoreactive cells, and is magnified in fish fed the 66% vegetable oil replacement diet.

12. The local IgM response to enteromyxosis is supported at systemic, lymphohaematopoietic level in the head kidney in gilthead sea bream fed a commercial diet.

13. The 66% vegetable oil replacement diet does not alter the biometrical, haematological and IgM profiles of gilthead sea bream.

14. The acute, local response of gilthead sea bream to enteromyxosis includes intestinal recruitment of plasma cells, B cells and mast cells and depletion of acidophilic granulocytes, which was supported at systemic level in the head kidney and the spleen.

15. The percentage of splenic surface occupied by melanomacrophage centres is higher in non-parasitized, parasite-exposed gilthead sea bream during acute response to enteromyxosis.

16. The eosinophilic/acidophilic granulocytes of gilthead sea bream, which are involved in response to enteromyxosis and correspond to mast cells and acidophilic granulocytes, present morphological heterogeneity and opposite distribution patterns.

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13

SUMMARY / RESUMEN

13.1 SUMMARY

The current thesis includes several aspects of enteromyxosis in gilthead sea bream, dealing mainly with the parasite transmission and characterization, with factors that affect parasite establishment and host-parasite interaction, and with the immune response elicited by the parasite.

First of all, a new method of transmission of *E. leei,* that improved the previous available ones, was established. The parasite was successfully transmitted to gilthead sea bream by peranal intubation with intestinal scrapings of infected fish. By the anal route, the progression of the infection was faster than by effluent and cohabitation, and more effective and uniform than by the oral route. In terms of infection intensity and parasite maturation, the progression of the infection followed a posterior-anterior intestinal gradient. The histopathological damage caused by the parasite included the usual disruption of the mucosa integrity and epithelial desquamation and the inflammatory host reaction involved lymphocyte and eosinophilic granulocyte infiltration, all at earlier time points than by the other infection routes. By anal route, the achieved prevalence and intensity of infection were therefore comparable with the infection level of *E. leei* in the more susceptible species sharpsnout sea bream *Diplodus puntazzo* and of the more pathogenic *Enteromyxum scophthalmi* in turbot *Psetta maxima.* Peranal intubation of gilthead sea bream is a fast and effective method for the experimental transmission of *E. leei*, assuring for each individual simultaneously a uniform infective dose to the main target site for the parasite.

In regard to the effectiveness of the parasite transmission, water temperature was shown to play an important role, having a clear relationship prevalence and water temperature during experimental infections. The inhibitory effect of the low temperatures on the development of enteromyxosis was confirmed since infection prevalence decreased gradually from high summer temperatures (22-25 °C), to autumn temperatures (19-22 °C) and under a constant temperature of 18 °C. Interestingly, under low winter temperature (11-12°C) no fish was infected, but infection appeared later on when water temperature increased in spring. This re-emergence of *E. leei* indicates that the parasite is capable of remaining latent and undetectable during cooler periods, thus false negatives become reservoirs for the parasite entailing epidemiological consequences.

In order to develop immunoassays that allow the detection of the parasite, its localization and functional characterization of antigens, rabbit polyclonal antibodies were raised against *E. leei* (aPab-Eleei) and *E. scophthalmi* (aPab-Escoph). Their sensitivity and specificity were characterized by ELISA and immunohistochemistry. After adsorption with non-infected intestinal scrapings of the corresponding host, both had high specificity for the corresponding proliferative (primary and secondary cells) and sporogonic parasite stages (sporoblasts, spore valves) and presented different titres in ELISA (aPab-Eleei 1:32,000; aPab-Escoph 1:16,000) and immunohistochemistry (aPab-Eleei 1:8,000; aPab-Escoph 1:16,000). The presence of more diverse parasitic stages in *E. leei* immunogens injected to the rabbits, probably accounted for the cross-reactivity detected with aPab-Eleei, which immunolabeled also *Sphaerospora dicentrarchi* and *S. testicularis,* whereas aPab-Escoph did not cross-react with any of the additionally tested myxozoans. Cross-reactivity among myxozoans is attributed to the share of antigenic epitopes, particularly carbohydrate moieties. These antisera enable *Enteromyxum* spp. detection at any phase of their life cycle.

For the antigenic characterization of the parasite, partially purified *E. leei* extracts were obtained, which contained large amounts of spores together with some disporous sporoblasts. Parasite extracts were separated by SDS-PAGE and compared with healthy intestinal extracts, leading to the visualization of the *E. leei* protein profile in Coomassie Brilliant blue stained gels (six reduced and denatured antigenic bands ranging from 10 to 49 kDa). In Western blots, the aPab-Eleei and the heterologous polyclonal antibody raised against the polar filament of *Myxobolus pendula* (PabMPPF) detected proteic epitopes (ranging from 15 to 165 kDa with aPab-Eleei; from 15 to >209 kDa with PabMPPF) on five *E. leei* antigenic bands and on an immunoreactive smear. These antigens were compared with results obtained in Lectin blots, and particularly, the 15 kDa and the 165 kDa glycoproteic bands were detected with both antibodies, suggesting shared antigens among myxozoans. In addition, both contained glucose and mannose moieties, a common trait of myxozoans. The 165 kDa parasite antigen also presented galactose, *N*-acetyl-galactosamine and *N*-acetyl-glucosamine residues pointing to its possible origin on chitin-built spore valves. The 15 kDa glycoproteic parasite antigen would match for its molecular weight with the minicollagen-homologue found in myxozoans. A 34 kDa glycoproteic antigen was also detected by aPab-Eleei (positive for glucose and

mannose moieties), which might be a further common antigen between *E. leei* and *M. pendula*. In zymographies of *E. leei* extracts, several functional gelatinolytic proteases were detected (ranging between 43 and 245 kDa), which may have a potential role in the parasite's pathogenesis.

The effect of dietary factors on gilthead sea bream enteromyxosis was studied in fish fed during 9 months a plant protein-based diet containing a blend of vegetable oils as major lipid source (at 66% of replacement) (66VO diet) and then exposed to *E. leei* by effluent, and compared with gilthead sea bream fed a fish oil (FO) commercial diet. The 66VO (*vs* FO) fish presented higher progression of enteromyxosis (higher prevalence and intensity of infection, broader and faster intestinal invasion) and severer disease signs (growth, condition factor, specific growth rate and haematocrit were all lower). However, body mass loss in recipient fish was mainly due to anorexia and probably enhanced by impairment of nutrient absorption due to the parasite-derived intestinal damage, osmoregulatory failure and metabolic cost of the host's immune response. The nutritional background by itself did not produce any detrimental effect on the fish biometric and haematological parameters since the 66VO control, unexposed group did not exhibit any harmful effect. However, this group presented significantly lower values of serum nitric oxide and lysozyme, whereas the alternative complement pathway of serum (ACH_{50}) was significantly enhanced. In any case, for recipient fish of both diet groups, the intensity of infection was negatively correlated with growth parameters and haematocrit. Thus, the 66VO replacement in gilthead sea bream can be a predisposing factor for enteromyxosis.

This was also suggested by the significant decrease of goblet cells with neutral and acidic mucins at the anterior and middle intestine sections, and of those with carboxylic mucins and sialic acid at the middle intestine in 66VO control fish, since these intestinal sections presented higher prevalence of infection in recipient fish of this diet group (compared to FO). The lower content of such mucin types could therefore be responsible for a deficient protective mucous barrier in 66VO fish. However, in FO fed fish, enteromyxosis accounted for a significant decrease of most goblet cell types, *i.e.* neutral, acidic, carboxylic and sialic acid containing ones, since their decrease was stronger at the posterior intestine (*vs* anterior and middle intestine) and in early infected fish, harbouring the parasite for a longer time (*vs* late infected). Goblet cells in infected intestinal areas were not only less numerous, but also smaller. This goblet cell depletion phenotype in *E. leei*-infected gilthead sea bream can be related to the direct histopathological damage caused by the epithelial invasion by the parasite, but the involvement of a host mediated or parasite-induced immune modulation cannot be discarded.

The observed changes in the goblet cell phenotype prompted us to further study the intestinal mucus composition in parasitized fish. Secreted mucins isolated from *E. leei*-infected gilthead sea bream intestinal mucus had a lower glycoprotein content and glycosylation degree at the anterior and middle intestine sections, and higher at the posterior intestine, compared with the mucus secretion of healthy fish. These mucins, regardless of the intestinal section and the parasitic state of the fish, presented two distinct size ranges: the large-size mucins (>2,000 kDa) with higher glycoprotein content and glycosylation degree, considered mature mucins, and the small-sized ones (69-670 kDa) that were immature mucins. Changes in terminal glycosylation of the secreted intestinal mucins pointed to an immature mucin secretion in the posterior intestine of parasitized fish through *N*-acetyl-galactosamine increase and fucose and neuraminic-acid reduction. The involvement of such terminal monosaccharides in microorganism recognition and binding was suggested since *Aeromonas hydrophila* and *Vibrio alginolyticus* adhesion to the large-size mucins was reduced in infected compared to unexposed fish, regardless of the intestinal section. Thus, biochemical modulation of the intestinal mucous secretion of gilthead sea bream in response to enteromyxosis was demonstrated and probably has further consequences on the interaction with commensal and pathogenic microorganisms.

The immune response elicited by *E. leei* in gilthead sea bream and the effect of feeding the 66VO diet were further analyzed at local and systemic levels. In this regard, after chronic exposure to the parasite by effluent, a late and significant up-regulation of IgM gene expression occurred at the posterior intestine of infected fish (*vs* control fish), which correlated with the increase of IgM immunoreactive cells, mainly plasma cells and B cells. This mucosal response to the parasite was magnified in fish fed the 66VO diet, suggesting its local pro-inflammatory effect, whereas no effect of the diet on the IgM profile was detected

in control, unexposed fish. With respect to lymphohaematopoietic tissues, an increase in plasma cells and B cells was only observed in the head kidney of FO fed, early infected fish, harbouring the parasite for a longer time. Clustering of plasma and B cells was observed around melanomacrophage centres and blood vessels of head kidney and spleen, which suggested the initiation of an adaptive immune response linking the systemic humoral immunity and the local response. The most determinant factor for the IgM increased phenotype was the time of exposure to the parasite (which determines the infection level) and this phenotype was more pronounced at local intestinal level and in the 66VO fed fish.

The modulation by *E. leei* of other leukocyte populations was analyzed in peranal experimentally infected gilthead sea bream after shorter exposure times (fed a commercial FO diet). In the intestinal inflammatory infiltrates and lymphohaematopoietic organs (head kidney and spleen) of parasitized fish, the number of plasma cells, B cells and mast cells were significantly higher, whereas the number of acidophilic granulocytes was lower, compared to non-parasitized and unexposed fish. This response was stronger at the posterior intestine, absent in the thymus and appeared earlier at local and systemic sites, with respect to the long-lasting infections by effluent transmission. The recruitment from systemic reservoirs into the local infection site seemed to happen *via* blood circulation and the apparent on-set of adaptive immunity was suggested by the higher percentage of splenic surface occupied by melanomacrophage centres in non-parasitized fish, compared to parasitized and unexposed ones. Strikingly, mast cells and acidophilic granulocytes, both acidophilic/eosinophilic cells, presented opposite patterns of response to enteromyxosis and a possible immunosuppressive effect of *E. leei* on the paramount acidophilic granulocytes may underlie. In addition, the heterogeneity among acidophilic/eosinophilic cells in gilthead sea bream was highlighted by the different cell morphologies and granule densities found.

13.2 RESUMEN

La presente tesis doctoral estudia diversos aspectos de la enteromixosis de la dorada, relacionados principalmente con la transmisión y la caracterización del parásito, con los factores que afectan a su establecimiento en el hospedador y a la interacción parásito-hospedador, y con la respuesta inmunitaria inducida por el parásito.

En primer lugar, se desarrolló un nuevo método de transmisión de *E. leei*, que mejora los resultados obtenidos por los hasta ahora disponibles. El parásito se transmitió con éxito a doradas mediante intubación por vía anal de raspados intestinales de peces infectados. Con este método, se consiguió una infección más rápida y extensa que por efluente y por cohabitación, y más eficaz, uniforme y repetitiva que por vía oral. En términos de intensidad de infección y de maduración del parásito, la progresión de la infección siguió un gradiente posterior-anterior en el intestino. El daño histopatológico causado por el parásito por esta vía fue similar al de otras formas de infección, con la consabida alteración de la integridad de la mucosa intestinal junto con descamación epitelial. Así mismo, la reacción inflamatoria local del hospedador consistió en la infiltración de linfocitos y granulocitos eosinófilos, todo ello en tiempos más tempranos que los registrados mediante las otras vías de infección. Por lo tanto, la prevalencia y la intensidad de la infección obtenidas en dorada por vía anal son comparables a los niveles de infección de *E. leei* alcanzados en el sargo picudo *Diplodus puntazzo*, especie muy susceptible a *E. leei*, y a los niveles de infección de *Enteromyxum scophthalmi*, cuya patogenicidad en rodaballo, *Psetta maxima,* es mayor. La intubación anal de la dorada es, por tanto, un método rápido y eficaz para la transmisión experimental de *E. leei*, que aporta simultáneamente para cada individuo una dosis infectiva uniforme aplicada directamente en el principal tejido diana del parásito.

En referencia a los factores que afectan a la transmisión de *E. leei*, se demostró inequívocamente que la temperatura del agua juega un papel importante, constatándose una evidente relación entre temperatura y prevalencia. La prevalencia de infección en las infecciones experimentales disminuyó gradualmente de las temperaturas registradas en periodos estivales (22-25 °C), a las temperaturas de otoño (19-22 °C) y a una temperatura constante de 18 °C. Se confirmó el efecto inhibidor de las bajas temperaturas sobre el desarrollo de la enteromixosis, ya que a las bajas temperaturas invernales (11-12 °C) los peces no se infectaron, pero la infección reapareció al aumentar la temperatura de nuevo en primavera. Esta reaparición de *E. leei* indica que el parásito es capaz de permanecer latente e indetectable durante los períodos más fríos, convirtiéndose los animales clasificados como falsos negativos en reservorios del parásito, con las consiguientes implicaciones epidemiológicas.

Con el fin de desarrollar inmunoensayos que permitan la detección del parásito, su localización y la caracterización funcional de sus antígenos, se obtuvieron anticuerpos policlonales de conejo contra *E. leei* (aPab-Eleei) y *E. scophthalmi* (aPab-Escoph). Se caracterizó su sensibilidad y especificidad mediante las técnicas de ELISA e inmunohistoquímica. Tras su adsorción con raspados intestinales no infectados del hospedador correspondiente, los anticuerpos mostraron una alta especificidad de unión a los estadios proliferativos del respectivo parásito (células primarias y secundarias) y a los estadios esporogónicos correspondientes (esporoblastos, valvas de las esporas). El título de los anticuerpos fue diferente en ELISA (aPab-Eleei 1:32.000; aPab-Escoph 1:16.000) que en inmunohistoquímica (aPab-Eleei 1:8.000; aPab-Escoph 1:16.000). El immunógeno de *E. leei* inyectado a los conejos tenía más diversidad de estadios parasitariosy probablemente por ello aPab-Eleei presentó reacción cruzada con otros mixosporidios (*Sphaerospora dicentrarchi* y *S. testicularis*), mientras que aPab-Escoph no detectó ninguna otra especie de mixozoo. La reactividad cruzada entre mixozoos se atribuye a la existencia de epítopos antigénicos compartidos, especialmente residuos de carbohidratos. Ambos anticuerpos permiten la detección de *Enteromyxum* spp. en cualquier fase de su ciclo vital.

Para la caracterización antigénica del parásito, se obtuvieron extractos parcialmente purificados de *E. leei*, que contenían grandes cantidades de esporas además de algunos esporoblastos diespóricos. Los extractos de parásito se separaron mediante SDS-PAGE y se compararon con extractos intestinales sanos. El perfil proteico de *E. leei* en geles teñidos con azul Coomassie brillante consistió en seis bandas antigénicas reducidas y desnaturalizadas con un peso molecular entre 10 y 49 kDa. En Western blots, aPab-Eleei y el anticuerpo policlonal heterólogo dirigido contra el filamento polar de *Myxobolus pendula* (PabMPPF) detectaron epítopos proteicos (de 15 a 165 kDa con aPab-Eleei; de 15 a >209 kDa con PabMPPF) en cinco

bandas antigénicas y en una amplia zona inmunorreactiva. Estos antígenos se compararon con las bandas obtenidas al aplicar lectinas en blots, resultando que ambos anticuerpos detectaron bandas glicoprotéicas de 15 kDa y de 165 kDa, lo que sugiere que podría tratarse de antígenos comunes entre mixozoos. Estas dos glicoproteínas tenían residuos glicosídicos de glucosa y de manosa, un rasgo común entre los mixozoos. El antígeno de 165 kDa también presentó residuos de galactosa, de *N*-acetil-glucosamina y de *N*-acetil-galactosamina, lo que apunta a un posible origen en las valvas de quitina de las esporas. El antígeno glicoprotéico de 15 kDa podría corresponder por su peso molecular con el homólogo de minicolágeno encontrado en otros mixozoos. También se detectó un antígeno glicoprotéico de 34 kDa mediante aPab-Eleei (positivo para residuos glicosídicos de glucosa y manosa) que podría corresponder con otro antígeno común de *E. leei* y *M. pendula*. En las zimografías realizadas con los extractos de *E. leei*, se detectaron varias proteasas gelatinolíticas funcionales (entre 43 y 245 kDa), que podrían tener un papel importante en la patogénesis del parásito.

Se estudió el efecto de algunos factores nutricionales sobre la enteromixosis de la dorada. Para ello, se alimentaron juveniles de dorada durante 9 meses con una dieta a base de proteína vegetal que contenía una mezcla de aceites vegetales como principal fuente de lípidos (al 66% de sustitución) (dieta 66VO), en oposición a doradas alimentadas con una dieta en la que la fuente de lípidos era exclusivamente de aceite de pescado (FO). Posteriormente ambos grupos se expusieron a *E. leei* mediante efluente. Las doradas 66VO (*vs* las FO) presentaron una mayor progresión de la enteromixosis (mayor prevalencia e intensidad de infección, invasión más extensa y más rápida de los distintos tramos intestinales) y signos de la enfermedad más severos (menor crecimiento, factor de condición, la tasa específica de crecimiento y hematocrito). La pérdida de masa corporal en los peces receptores se debió principalmente a la anorexia y probablemente se agravó por la deficiente absorción de nutrientes debido al daño intestinal ejercido por el parásito, la insuficiencia osmorreguladora y el coste metabólico de la respuesta inmune del propio hospedador. Los antecedentes nutricionales por sí mismos no produjeron ningún efecto perjudicial sobre los parámetros biométricos y hematológicos de las doradas en el grupo control, no expuesto 66VO. Sin embargo, este grupo presentó valores significativamente más bajos de óxido nítrico y lisozima séricos, mientras que la vía alternativa del complemento sérico (ACH_{50}) aumentó significativamente. En cualquier caso, la intensidad de la infección se correlacionó negativamente con los parámetros de crecimiento y de hematocrito para los peces receptores de ambas dietas. Por lo tanto, la sustitución 66VO en la alimentación de la dorada puede constituir un factor que predispone para la enteromixosis.

Este posible efecto de la dieta se podría deducir también de la significativa disminución de las células goblet positivas para mucinas neutras y ácidas en las secciones del intestino anterior y medio, y de las positivas para mucinas carboxílicas y para ácido siálico en el intestino medio de las doradas control 66VO, ya que estas secciones intestinales presentaron una mayor prevalencia de infección en el grupo de peces receptores de esta dieta (en comparación con el grupo FO). Este menor contenido de tales tipos de mucinas, podría provocar algún tipo de deficiencia en la barrera mucosa protectora de los peces 66VO. Sin embargo, la enteromixosis conllevó en las dos dietas una disminución significativa de la mayoría de tipos de células goblets, es decir, neutras, ácidas, carboxílicas y con ácido siálico, y su disminución fue más intensa en el intestino posterior (*vs* anterior y medio) y en peces infectados tempranamente, que albergaron el parásito durante más tiempo (*vs* tardíamente infectados). Las células goblets en las zonas intestinales infectadas no sólo fueron menos numerosas, sino también de menor tamaño. Este fenotipo de reducción de células goblet en doradas infectadas por *E. leei* puede estar relacionado con el daño histopatológico directamente causado por la invasión epitelial del parásito, aunque no se puede descartar la implicación de cierta inmunomodulación inducida por el parásito o mediada por el propio hospedador.

Los cambios observados en el fenotipo de células goblet nos llevaron a estudiar en mayor profundidad la composición del mucus intestinal en doradas parasitadas. Las mucinas intestinales secretadas, aisladas de doradas infectadas por *E. leei*, tuvieron un menor contenido glicoproteíco y un menor grado de glicosilación en las secciones intestinales anterior y media, y mayores en el intestino posterior, en comparación con la secreción mucosa de doradas sanas. Independientemente del tramo intestinal y del estado parasitario de los peces, estas mucinas presentaron dos rangos distintos de tamaño: las de tamaño mayor (> 2.000 kDa), con mayor contenido glicoproteíco y grado de glicosilación, se consideraron las mucinas maduras y las que de tamaño menor (69-670 kDa) se consideraron mucinas inmaduras. La variación de la glicosilación terminal de las mucinas intestinales secretadas apuntó a una secreción de mucinas inmaduras en el intestino posterior de los peces parasitados debido al aumento de residuos de *N*-acetil-galactosamina y a la

reducción de fucosa y ácido neuramínico. Estos monosacáridos terminales aparentemente participan en el reconocimiento y la unión de microorganismos al observarse una reducción de la adhesión *Aeromonas hydrophila* y *Vibrio alginolyticus* a las mucinas de mayor tamaño en doradas infectadas, comparadas con las no expuestas, independientemente del tramo intestinal. Por lo tanto, se demostró la modulación bioquímica de la secreción de mucosa intestinal en dorada en respuesta a la enteromixosis, probablemente con consecuencias sobre la interacción con microorganismos tanto patógenos como comensales.

Se analizó a nivel local y sistémico la respuesta inmunitaria inducida por *E. leei* en la dorada y el efecto de la dieta 66VO. Se detectó un aumento significativo pero tardío de la expresión génica de la IgM en el intestino posterior de los peces infectados crónicamente por efluente, (*vs* peces control), que se correlacionó con el aumento de células IgM inmunorreactivas, principalmente plasmacitos y linfocitos B. Esta respuesta local frente al parásito se magnificó en los peces alimentados con la dieta 66VO, lo que sugiere un efecto pro-inflamatorio a nivel local, mientras que no se detectó ningún efecto de la dieta sobre el perfil de IgM en las doradas control, sin exponer al parásito. Con respecto a los tejidos linfohematopoyéticos, únicamente se observó un aumento de plasmacitos y linfocitos B en el riñón anterior de las doradas alimentadas con la dieta FO e infectadas tempranamente (que llevaban más tiempo infectadas). Se observó una agregación de plasmacitos y linfocitos B alrededor de los centros melanomacrofágicos y de los vasos sanguíneos tanto del riñón anterior como del bazo, lo que sugiere el comienzo de una respuesta inmunitaria adaptativa, interconectando la inmunidad humoral sistémica con la respuesta local. El factor que resultó ser más determinante para el fenotipo de aumento de la IgM fue el tiempo de exposición al parásito (que a su vez determina el nivel de infección) y este fenotipo fue más pronunciado a nivel intestinal local en los peces alimentados con 66VO.

Se analizó el efecto modulador ejercido por *E. leei* sobre otras poblaciones de leucocitos en doradas infectadas experimentalmente por vía anal tras tiempos de exposición más cortos (alimentadas únicamente con una dieta comercial FO). En los infiltrados inflamatorios intestinales y órganos linfohematopoyéticos (riñón anterior y bazo) de peces parasitados, el número de plasmacitos, linfocitos B y mastocitos fue significativamente mayor, mientras que los granulocitos acidófilos disminuyeron, en comparación con peces no parasitados y con no expuestos. Esta respuesta fue más intensa en el intestino posterior, no se detectó en el timo y, en su conjunto, apareció antes, tanto a nivel local como sistémico, que en las infecciones de larga duración por efluente. El reclutamiento desde los reservorios sistémicos al lugar de la infección pareció ocurrir a través de la circulación sanguínea. El aparente comienzo de una respuesta inmunitaria adaptativa estuvo asociado al mayor porcentaje de superficie esplénica ocupada por los centros melanomacrofágicos en los peces no parasitados, en comparación con los parasitados y con los no expuestos. Sorprendentemente, los mastocitos y granulocitos acidófilos, ambos células acidófilas/eosinófilas, presentaron patrones opuestos de respuesta a la enteromixosis. No se descarta un posible efecto inmunosupresor de *E. leei* sobre los granulocitos acidófilos, que se consideran el principal tipo de granulocito en la dorada. Además, la heterogeneidad de las células acidófilas/eosinófilas de la dorada se manifestó por las distintas morfologías celulares y densidades de gránulos observadas.