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Functional characterization of mast cells and histamine in the immune system of gilthead seabream (*Sparus aurata* L.)

Caracterización funcional de las células cebadas y de la histamina en el sistema inmunitario de dorada (*Sparus aurata* L.)

Nuria Esther Gómez González

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LIST OF ABBREVIATIONS

11KT	11-ketotestosterone
Ab	antibody
AC	adenylate cyclase
actb	β-actin gene
Adaptor	adaptor protein
AG	acidophilic granulocyte
Akt	protein kinase B
AR	androgen receptor
ATP	adenosine triphosphate
BG4-	low fluorescence-labelled mast cells
BG4+	high fluorescence-labelled mast cells
BPA	bisphenol A
BSA	bovine serum albumin
c48/80 or Co 48/80	compound 48/80
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
cpa5	carboxypeptidase A5
CRP	C-reactive protein
csf1r	colony stimulating factor 1 receptor gene
dat	day after treatment
DDT	dichlorodiphenyltrichloroethane
DHR 123	dihydrorhodamine 1,2,3
DHT	dihydrotestosterone
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dot	day of treatment
dpb	day post-booster
dpp	day post-priming
E_2	17β-estradiol
EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
EDC	endocrine disruption chemical
EDTA	ethylenediaminetetraacetic acid
EE_2	17α-ethynylestradiol

EGC	eosinophilic granular cell
EGF	epidermal growth factor
EGFR	EGF receptor
ER	estrogen receptor
F	forward oligonucleotide
FBS	fetal bovine serum
FcεRI	high-affinity IgE receptor
FSC	Forward light scatter
FSH	follicle stimulating hormone
G7-	acidophilic granulocyte-lacking cell suspension or low fluorescence-labelled acidophilic granulocyte
G7+	acidophilic granulocyte-enriched cell suspension/fraction or or high fluorescence-labelled acidophilic granulocyte
GB10-	low fluorescence-labelled mast cells
GB10+	high fluorescence-labelled mast cells
GPER30 or GPER1	G protein-coupled estrogen receptor
HAT	hypoxanthine/aminopterin/thymidine
HB-EGF	pro-heparin-binding-EGF
HBSS	Hank's balanced salt solution
HDC	histidine decarboxylase
HK	head kidney
hkVa	heat-killed Vibrio anguillarum
HMC	human mast cell
HR	histamine receptor
hrh1	histamine receptor 1 gene
hrh2	histamine receptor 2 gene
hrh3	histamine receptor 3 gene
i.p.	intraperitoneal
ICI	fulvestrant
IFN	interferon
Ig	immunoglobulin
ighm	immunoglobulin M heavy chain gene
ight	immunoglobulin T heavy chain gene
IgM- B lymphocytes	IgM B lymphocytes-lacking cell suspension or low fluorescence-labelled

	IgM B lymphocytes
IgM+ B lymphocytes	IgM B lymphocytes-enriched cell suspension or high fluorescence-labelled IgM B lymphocytes
IL	interleukin
LH	luteinizing hormone
LPS	lipopolysaccharides
mAb	monoclonal antibody
MACS	magnetic-activated cell separation/sorting
MAPK	mitogen-activated protein kinase
MC	Mast cell
mER	ER at the plasma membrane
MFI	mean fluorescence intensity
MMP	matrix metalloproteinases
mpb	month post-booster
mRNA	messenger RNA
myd88	myeloid differentiation primary response gene 88
NADPH	nicotinamide adenine dinucleotide phosphate
NK	natural killer cell
NOS	nitric oxide synthase
NP	nonylphenol
P.CAS	caspase inhibitor
PAMP	pathogen-associeted molecular pattern
PBS	phosphate buffered saline
PCB 126	3,3',4,4',5-pentachlorobiphenyl
PCR	polymerase chain reaction
PE	peritoneal exudate
PEG	polyethylene glycol
PI	propidium iodide
PI-	non PI-labelled cells
PI+	PI-labelled peritoneal cells
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C

PMA	phorbol myristate acetate
PRR	pattern recognition receptor
R	reverse oligonucleotide
RNA	ribonucelic acid
RNase	ribonuclease
RNS	reactive nitric species
ROS	reactive oxygen species
rps18	Ribosomal protein S18 gene
SAA	serum amyloid protein
sPBS	PBS supplemented with 0.35% NaCl
sRPMI	RPMI-1640 culture medium supplemented with 0.35% NaCl
SSC	side light scatter
T	testosterone
Tc	T cytotoxic cell
TF	transcriptional factor
Th	T helper cell
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
TRIS	Tris(hydroxymethyl)aminomethane
VaDNA	genomic DNA from Vibrio anguillarum
vtg	vitellogenin gene
WHO	Worlh Health Organization

SUMMARY

The immune system of teleost fish has been studied for decades but, despite the important advances made in our related knowledge, much is still unknown, especially concerning the presence and role of mast cells (MCs) and histamine. The gilthead seabream (*Sparus aurata* L.), a marine teleost fish of great commercial value in Mediterranean aquaculture, has been widely used for better understanding the fish immune response. Moreover, and due to the fact that the gilthead seabream is a hermaphrodite species, many studies have focused on the effects that the synthetic estrogen, 17α-ethinylestradiol (EE_2), widespread in the aquatic environment, exerts in it. However, no studies have been focused on peritoneal leukocytes. Within the above framework, the aim of this thesis is to advance our knowledge of the role of MCs and histamine in the immune response of the gilthead seabream.

The first chapter finishes with the development of a protocol to isolate peritoneal MCs of gilthead seabream. Using discontinuous density gradient centrifugations, cell culture, magnetic-activated cell sorting, and specific monoclonal antibodies (mAb), 95% pure MC fractions are obtained. Isolated peritoneal MCs possess histamine which is released by compound 48/80 (Co 48/80), the classical mast cell activator. Moreover, IL-1β and IL-8 gene expression was induced in these cells by stimulation with bacterial DNA. This MC isolation protocol will be useful for advancing our knowledge of how vertebrate inflammatory mechanisms evolved.

In the second chapter, we study the effect of histamine and compound 48/80 (Co 48/80) on the innate and adaptive immune response of gilthead seabream. For this purpose, histamine and Co 48/80 were intraperitoneally injected, alone or combined, with the pathogen, *Vibrio anguillarum*, and their effects on peritoneal exudate and head kidney were analyzed. Histamine and Co 48/80 were seen to alter the percentage of peritoneal exudate and head kidney immune cells, increase the production of ROS by peritoneal leukocytes and impair the humoral adaptive immune response by producing IgM specific to *V. anguillarum*. Moreover, both, histamine and Co 48/80 reduce the expression of the gene encoding histamine receptor 2 in peritoneal leukocytes. These results show that, although the systemic administration of histamine and Co 48/80 is safe, neither can be regarded as an efficient adjuvant for use in gilthead seabream intraperitoneal vaccination.

In the third chapter, we report the production and characterization of a mAb, GB10, which specifically recognizes gilthead seabream MCs. GB10 was seen to react with 20-30% of peritoneal leukocytes. The incubation of MCs with GB10 rapidly induced death, via oncosis, of gilthead seabream MCs, as demonstrated by flow cytometry and transmission

electron microscopy. *In vivo* assays, in which GB10 was intraperitoneally injected in gilthead seabream, will helps understand the evolutionary aspects of both MCs and histamine. Moreover, a second mAb, BG4, wich specifically recognizes MCs has been obtained.

Finally, in the fourth chapter, we analyze the effects of EE_2 in the peritoneal immune response of gilthead seabream. Juvenile fish were fed a pellet diet supplemented with EE_2 for 76 days, after which they were intraperitoneally injected with hemocyanin plus imject alum adjuvant, and then again injected after another 92 days. The dietary intake of EE_2 induced the expression of genes encoding for the nuclear estrogen receptor α and the G protein-coupled estrogen receptor 1 in the peritoneal leukocytes. Interestingly, the dietary intake of EE_2 induced an inflammatory response in the peritoneal exudate, which was largely maintained for several months after the cessation of the treatment and, besides, modulated histamine receptor gene expression. Taken together, the study provides fresh information about endocrine immune disruption, focusing on peritoneal leukocytes.

INTRODUCTION

Overview

Aquatic products are one of the most important sources of animal protein in the world. According to the FAO, in recent years fish has accounted for 16.7% of the world's animal protein intake and 6.5% of all protein consumed, accounting for 20% of total protein consumed in developing countries and 15% in Europe and North America. Undoubtedly, the greatest challenge faced by humankind in the next decades, other than energy, is to feed the 9.6 billion people who will inhabit the planet earth by 2050. The challenge is complex, given the limited availability of natural resources and given the need to respect ecosystems (APROMAR, 2016).

Aquaculture and fisheries are two activities that, historically, have taken opposing sides in the face of the growing global demand for aquatic products as healthy and nutritious foods. Aquaculture is not a complement to fishing, but rather the natural evolution of the same, just as rearing livestock replaced hunting (APROMAR, 2016). As in every production system, the efficiency of aquaculture depends on many factors, such as reproduction and the viability of fish populations. Studies related with the reproduction systems of the species of greatest commercial value are increasingly more abundant. In turn, viability rates are directly related with the efficiency of the immune response. So a better understanding of fish reproduction systems and the capacity of fish species to overcome environmental challenges will be very helpful for increasing the efficiency of aquacultural production systems. The practice of aquaculture involves the confinement of a large number of individuals in a limited space, while more or less regular handling triggers stress in the animals. Such conditions lead to the development of infectious diseases that are responsible for substantial economic losses. Therefore, knowledge of the immune system of fish, in general, and of the species in question in particular, has become one of the primary objectives in aquaculture research.

The immune system of teleost fish has been studied for decades but, despite the important advances made in our related knowledge, much is still unknown. The gilthead seabream (*Sparus aurata* L.) is a marine, seasonally breeding, protandrous hermaphrodite teleost. This species has also been widely used as a model for better understanding the fish immune system. However, the lack of biotechnological tools for use with fish is one of the most important problems for furthering our insight into this subject. It is important to note that the basic research into lower vertebrates has been amply supported by methodologies related with, and knowledge obtained, from mammals. In this context, the finding of the presence of histamine in the mast cells (MCs) of the gilthead seabream was made possible due to the

commercial availability of a polyclonal anti-histamine antibody (Mulero et al., 2007a). However, little is known about the role of MCs and histamine in immunity in fish, in general, and in gilthead seabream, in particular. To remedy this situation, specific tools to identify and isolate piscine MCs are required.

There has been increasing concern in recent decades concerning the presence in the freshwater and marine environment of a large number of anthropic-released molecules, some of which have been identified as endocrine disruptor chemicals (EDCs). Indeed, the scientific community has demonstrated that some EDCs act as steroid receptor-agonists or -antagonists in a variety of cell types, not only in the reproductive system (Liney et al., 2006; Prait and Nelson, 2002), but also in the immune system (Casanova-Nakayama et al., 2011; Liney et al., 2006; Milla et al., 2011). Nonetheless, most of these studies have focused on head kidney phagocytic leukocytes or lymphocytes, while studies focusing on the effects of EDCs on fish MCs are scant. One of the most widely used EDCs in experimental assays in fish is 17α-ethynylestradiol (EE_2), a synthetic estrogen which is used in oral contraceptives and hormone replacement therapy, whose presence has been previously demonstrated as an aquatic contaminant. In our laboratory, the effect of EE_2 has been analyzed in *in vivo* and *in vitro* assays on acidophilic granulocytes (AGs), the equivalent functional cells to mammalian neutrophils, macrophages and lymphocytes (Cabas et al., 2012, 2013a; Rodenas et al., 2015, 2016). However, no studies have been conducted to determine the action of EDCs on MC functions of gilthead seabream.

This PhD thesis has been developed in the research group *Innate Immune System of Teleost Fish,* which has a long history in the study of the immune system of Mediterranean species such as gilthead seabream and seabass (*Dicentrarchus labrax* L.), species of great commercial value in the Mediterranean area.

Within the above framework, the aim of this thesis has been to advance our knowledge of the role of MCs and histamine in the immune system of the gilthead seabream. First of all, a protocol to isolate peritoneal MCs and to obtain a monoclonal antibody specific to gilthead seabream MCs is developed. Moreover, the role of histamine released by MCs in the innate and adaptive immunity is studied. Finally, the impact of EE_2 on the peritoneal leukocyte populations and on the histamine signaling pathways is analyzed.

1. Immune system

The main function of the immune system is defence against foreign substances including microorganisms, such as viruses, bacteria, fungi, protozoa and multicellular parasites, and macromolecules, such as proteins and polysaccharides. The immune system is classically divided into two types: i) innate, natural or non-specific, and ii) adaptive, acquired or specific.

The innate immune response, which is the oldest defence system from a phylogenetic point of view, is present in 98% of the pluricellular organisms, while adaptive immunity appeared later in more evolutionary advanced animal species. Innate immunity acts as the first response against infection until the adaptive response is triggered. The biochemical and cellular mechanisms of the innate response are present even before the organism is exposed to the pathogen and it acts in the same way each time that the infection appears. However, the adaptive immune response is highly specific for a particular pathogen and becomes more effective with each successive encounter with the same pathogen.

The main components of the innate immune system are: i) physical and chemical barriers, ii) phagocytic cells (neutrophils and macrophages), eosinophils and natural killer cells (NKs), iii) blood proteins such as complement and acute phase proteins, and iv) cytokines (Abbas et al., 2008). The innate immune mechanisms are specific for common structures in microbial groups but are unable to distinguish between small differences in exogenous pattern recognition receptors (PRRs), which include Toll-like receptors (TLRs), retinoic acid-inducible gene I-like receptors, nucleotide-binding oligomerization domain-like receptors, and C-type lectin receptors (Takeuchi and Akira, 2010). These receptors recognize the pathogen-associated molecular patterns (PAMPs) conserved in pathogenic organisms such as polysaccharides, lipopolysaccharides (LPS), bacterial DNA or viral RNA. These PRRs also recognize endogenous molecules released from damaged cells, known as damage-associated molecular patterns (Takeuchi and Akira, 2010).

The key features of the adaptive immune response are specificity and memory (Male and Roitt, 1996). Unlike the innate immunity response, the adaptive immune response is very specific and creates immunological memory after the first encounter with a specific pathogen, leading to an enhanced response to subsequent infections. The adaptive immunity response is able to discriminate between extremely small differences in microorganisms or molecules. Lymphocytes (B and T cells) and their products, mainly antibodies/immunoglobulins (Ab/Ig) (IgA, IgD, IgE, IgG and IgM) and some cytokines, are the main components of adaptive immunity and antigens are their targets (Abbas et al., 2008). B cells produce antibodies,

which recognize and combat extracellular pathogens and their products. However, T cells have a large number of activities: T helper (Th) cells regulate the development and production of antibodies by B cells or interact with phagocytic cells, helping them to destroy the pathogens that they have phagocytosed, while cytotoxic T (Tc) cells recognize and destroy virus-infected and tumor cells.

Moreover, most immune responses consist of a wide variety of innate and adaptive components. In the early stages of infection, the innate response predominates but lymphocytes, acting in an integrated and coordinated form, subsequently begin to generate the adaptive response (Male and Roitt, 1996).

2. Immune system of teleost fish

Teleost fish occupy a key position from the evolutionary point of view since they are the earliest class of vertebrates known to possess both innate and adaptive immunity response (Whyte, 2007). The immune system of teleost fish shows much similarity with that of higher vertebrates, presenting both innate and adaptive immune responses, besides small differences. Because fish are free-living organisms from the early embryonic stages of life and that they live in aquatic environments, they have developed a robust innate immune system for survival in the face of a variety of microorganisms (Rombout et al., 2005) mainly in low temperature conditions (fish are poikilothermic) since the adaptive immune response is dependent on temperature (Abruzzini et al., 1982; Avtalion, 1981; Clem et al., 1984, 1985, 1991a; Cuchens and Clem, 1977; Miller and Clem, 1984). Moreover, the immune response wild and farmed fish can be influenced by a variety of parameters, among which temperature, stress management, fish density, light, water quality, salinity, food or immunostimulants should be mentioned (Abruzzini et al., 1982; Avtalion, 1981; Clem et al., 1991b; Cuchens and Clem, 1977; Hoseinifar et al., 2015; Magnadottir, 2006, 2010; Van Muiswinkel and Vervoorn-Van del Wal, 2006).

The immune response is composed of physical and chemical barriers and cellular and humoral agents. In innate immunity, the epithelium and mucosal tissues represent the physical barriers, while phagocytes, non-specific cytotoxic cells and eosinophilic cells, including MCs, represent the cellular effectors, and a variety of molecules (the complement, acute phase proteins and cytokines) direct the humoral immune response. On the other hand, adaptive immunity is directed by B and T cells as the cellular components, and Ig, IgM, IgD and IgT/Z as the humoral components, with IgM being the most common form found in teleost species (Rubio-Godoy, 2010).

Side by side with the physical and chemical barriers, TLRs and the complement are the very first line of protection of the fish immunity. TLRs are considered the principal inducers of the innate immune response, while the complement stimulates the adaptive immune response. Moreover, the complement prevents the attachment, invasion or proliferation of pathogens (Bayne and Gerwick, 2001; Ellis, 2001; Whyte, 2007). When the PRRs recognize the presence of PAMPs, the TLRs initiate an intracellular signal transduction that ends with the expression of inflammatory genes, antiviral response and the maturation of dendritic cells (Whyte, 2007).

The head kidney acts as a lymphohematopoietic organ and is the equivalent of bone marrow in higher vertebrates, which is lacking in teleost fish. Head kidney is the anterior or cephalic part of the kidney, in which the middle and posterior part are mainly tubular, with scarce immune cells (Zapata et al., 1996). In general, lymphohematopoietic cells are scattered at random throughout a stroma of fibroblastic reticular cells and sinusoidal blood vessels (Zapata et al., 1996). Among other leukocyte components, precursor cells, macrophages, neutrophils/AGs and lymphocytes are present in the head kidney as has been described in gilthead seabream (Sepulcre et al., 2002). In gilthead seabream, AGs are the major cell type participating in innate host responses, the head kidney being the central immune organ that acts as the main source for this cell type (Sepulcre et al., 2002). As in most evolutionary advanced vertebrates, the lymphoid organs thymus and spleen are also present in teleost fish. The thymus suffers involution and produces T cells, while the absence of lymph nodes in fish has led it to be speculated that the spleen could be considered as an antigen-presenting organ that initiates the adaptive immune response (Chaves-Pozo et al., 2005a).

Although peritoneal exudate is not an organ *per se*, the high number of leukocytes that are found in peritoneal exudates should be mentioned. AGs, lymphocytes, macrophages and MCs are the main immune cells present in the teleost peritoneal exudate.

2.1. Innate immune system

The innate immune system of teleost fish is the first barrier of host against pathogens and it deals with any foreign material until the adaptive immune system is potent enough to take over (Dixon and Stet, 2001; Fearon and Locksley, 1996; Whyte, 2007). The innate immune response is thought to be essential in combating diseases in fish due to the limitations of the adaptive immune response by their poikilothermic nature and their limited antibody repertoire, affinity maturation and memory and relatively slow lymphocytes proliferation (Magnadottir, 2006; Uribe et al., 2011; Whyte, 2007).

The skin and the presence of scales or the secretion of toxic substances, as well as the mucus, gills and the gastrointestinal tract represent defensive mechanisms. Moreover, there is a wide variety of humoral effectors that act in the innate of fish (Alexander and Ingram, 1992).

When innate immune leukocytes sense the presence of pathogens or tissue damage, inflammation appears as the innate immune response to avoid pathogen replication or infection and to initiate the process of tissue repair. Inflammation provokes redness, heat (both due to blood flow and core temperature), swelling (accumulation of fluid), pain (release of chemicals) and loss of function (multiple causes) in the inflammatory site due to the high recruitment of leukocytes and the changes in the vascular components. Although each type of leukocyte, i.e., monocytes/macrophages, neutrophils/AGs and MCs, has a different role, they work together coordinately.

The main mechanisms of the innate immunity developed in teleost fish to destroy harmful stimuli include: degranulation, chemotaxis, phagocytosis, oxidative burst, and opsonic and haemolityc serum activities.

Degranulation involves the release of a wide battery of immune regulatory mediators and antimicrobial cytotoxic molecules, such as histamine, from the granules present in MCs and neutrophils/AGs. These molecules subsequently induce *chemotaxis* or leukocyte extravasation to the site of infection in order to recruit other leukocytes and eliminate pathogens.

Phagocytosis is a biological process in which cells (phagocytes) engulfs solid particles or pathogens. In the process, recruited phagocytes come into contact with microbes at the inflamed tissue site. The PRRs present at the cell-surface recognize PAMPs and then form a PAMP-PRR complex. Subsequently, in a rearrangement of the actin-myosin cytoskeleton, the PAMP-PRR complex is endocytosed and directed to the lysosomes to produce a phagolysosome that kills the microbes inside the phagocytes. Phagocytosis in fish is mainly mediated by macrophages and neutrophils (Secombes and Fletcher, 1992; Verburg van-Kemenade et al., 1994). MC-mediated phagocytosis is a very controversial issue even in higher vertebrates, although some authors have considered the MCs as phagocytic cells (Feger et al., 2002; Urb and Sheppard, 2012), previous studies performed in our laboratory indicated that gilthead seabream MCs do not possess phagocytic capacity (data not published).

The *oxidative burst* takes part in degranulation and chemotaxis processes since the release of reactive oxygen and nitric species (ROS/RNS) induce the recruitment of

leukocytes, but also in phagocytosis since it helps in the elimination of the phagolysosomes. Some authors consider that this activity is only present in macrophages and neutrophils (Swindle et al., 2002); however, some studies have demonstrated that ROS/RNS are also produced by MCs (Brooks et al., 1999; Urb and Sheppard, 2012). In this sense, studies performed in our laboratory have failed to detect ROS/RNS production by gilthead seabream MCs (unpublished data).

Opsonization is the molecular mechanism whereby molecules, microbes or apoptotic cells are chemically modified to enhance their interaction with cell surface receptors on phagocytes or nonspecific cytotoxic cells. Moreover, it has been described that the complement is able to neutralize viruses by enveloping them (Whyte, 2007). Opsonization also helps phagocytic efficacy through the plasma derived complement C3b (Ellis, 1999, 2001; Matsuyama et al., 1992; Sakai, 1984).

The complement is also responsible for the *haemolytic activity* of serum that produces the lysis of foreign cells and pathogens to avoid infection or pathogen replication (Whyte, 2007). In fish, haemolytic activity is more active and heat labile than in mammals (Whyte, 2007).

2.1.1. Cell types

The innate leukocyte populations of fish are constituted by non-specific cytotoxic cells (equivalent to mammalian NK lymphocytes), monocytes/macrophages, neutrophils/AGs, MCs and others nuclear leukocytes.

Non-specific cytotoxic cells are agranular and small cells (Uribe et al., 2011). They have been found in peripheral blood and fluid, thymus, spleen and kidney (Rubio-Godoy, 2010). They present cytotoxic activity against normal or transformed cell lines, virus-infected cells or parasitic protozoa (Manning, 1998; Utke et al., 2007). They release a variety of cytokines to eliminate the pathogens (Evans and Jaso-Friedmann, 1992).

Monocytes/macrophages are large leukocytes that can present different body shapes, but are mainly round or horseshoe shaped. They are found in head kidney, connective tissue, spleen, peritoneal exudate, gills and thymus (Bodammer, 1986; Lieschke et al., 2001; Olivier et al., 1986, 1992; Page and Rowley, 1984; Sakai, 1984; Suzuki, 1986; Temkin and McMillan, 1986). In some teleost species dark coloured-macrophages form clusters in the lymphohematopoietic tissues; those clusters are called melanomacrophage centres (Agius, 1980; Herraez and Zapata, 1991). They phagocyte pathogens and present oxidative burst in order to combat infection (Uribe et al., 2011) and to release a variety of cytokines (Ellis,

1998). In gilthead seabream, macrophages have been found to migrate to the injury site, to phagocyte pathogens after cytokine stimulation, to produce ROS/RNS against PAMPs and to possess bactericidal activity (Mulero and Meseguer, 1998).

The neutrophils/AGs have a round shape and a large content of granular vesicles. They are mainly found in the hematopoietic tissues and the peritoneal exudate, but also in blood, intestinal submucosa, skin, gill and gonads (Bullock, 1963; Chaves-Pozo et al., 2003; Ezeasor and Stooe, 1980; Mulero et al., 2007a). Together with macrophages, neutrophils/AGs are responsible for phagocytosis in teleost fish (Sepulcre et al., 2002). They can also produce ROS as a defence against pathogens. More specifically, studies in gilthead seabream have described AGs as the most active and abundant phagocytic cell type of the species (Sepulcre et al., 2002), and are considered as functionally equivalent to mammalian neutrophils (Sepulcre et al., 2002). Moreover, AGs show strong phagocytic and ROS production capabilities (Sepulcre et al., 2007; Sepulcre et al., 2002), produce cytokines in response to several immunological stimuli (Chaves-Pozo et al., 2004; Sepulcre et al., 2007) and express a broad range of TLRs (Sepulcre et al., 2007).

2.1.1.1. Mast cells

Fish MCs, also called eosinophilic granular cells (EGCs), are very large round cells with a high granular content and have been shown to be present throughout evolution in all vertebrates. Some authors have suggested that kidney (Da'as et al., 2011), spleen (Prykhozhij and Berman, 2014), gastrointestinal-associated haematopoietic tissue (Temkin and McMillan, 1986), epidermis (Roberts, 1973) and intestinal epithelium (Bergeron and Woodward, 1982) are the main sources of MCs in fish. In general, they reside close to the surface in contact with the external environment and possible pathogens, but, depending on the species, they may also be found in many tissues: for example, peritoneal exudate (Meseguer et al., 1993; Prykhozhij and Berman, 2014), gastrointestinal tract (Baccari et al., 2011; Crivellato et al., 2015; Mulero et al., 2007a; Prykhozhij and Berman, 2014), gills (Crivellato et al., 2015; Mulero et al., 2007a; Prykhozhij and Berman, 2014), skin (Baccari et al., 2011; Crivellato et al., 2015; Prykhozhij and Berman, 2014), brain (Baccari et al., 2011), liver, kidney (Baccari et al., 2011; Prykhozhij and Berman, 2014), heart, blood, haematopoietic tissue, meninges, nerves, pancreas, mesentery, gonads, olfactory mucosa and swim bladder (Prykhozhij and Berman, 2014). In gilthead seabream, MCs have been found in the peritoneal exudate (Meseguer et al., 1993), gills and intestine (Mulero et al., 2007a). Interestingly, MCs are

present in a high percentage in the peritoneal exudate of gilthead seabream (Meseguer et al., 1993).

Different MC subtypes have been described in vertebrate species (Dvorak, 2005) and it is though that in the same species, each type of MC is found in different tissues presenting different functional profiles (Crivellato et al., 2015). The commonly found term "MCs heterogeneity" is related to significantly differences in cell dimension, granule number, granular chemical content, differential sensitivity to fixatives, unequal response to drugs and distinctive substructural pattern (Crivellato et al., 2015; Reite, 1998; Reite and Evensen, 2006). In humans, three MC subtypes has been identified according to their protease content (Crivellato et al., 2015) and in avian, reptile and amphibian, histamine content and chymotrypsin-like and trypsin-like activity differ among MC subtypes (Baccari et al., 2000, 1998; Chiu and Lagunoff, 1971; Vitiello et al., 1997).

MC granules store and secrete a wide variety of compounds, including proteases, serotonin and, found more recently, histamine (Dobson et al., 2008; Mulero et al., 2007a). As in mammals, MCs in fish express the stem cell factor receptor kit-like and the high-affinity receptor (FcεRI) for IgE (Galli et al., 2005a), although IgE is not produced in teleost fish (Bengten et al., 2006). Moreover, it has been described that zebrafish (*Danio rerio*) MCs also express the evolutionary conserved gene encoding for carboxypeptidase A5 (*cpa5*) (Dobson et al., 2008) and the myeloid differentiation primary response gene 88 (*myd88*), a TLR adaptor, suggesting the conservation of innate immune responses is mediated through TLRs (Da'as et al., 2011).

Piscine MCs have been described to induce neutrophil chemotaxis and activation when they undergo degranulation (Matsuyama and Iida, 1999, 2001). In fish, antimicrobial peptides released by MCs may partially induce the accumulation of neutrophils at sites of MC degranulation (Crivellato et al., 2015; Prykhozhij and Berman, 2014). Moreover, due to their ability in fish to be motile (Reite and Evensen, 2006), there is evidence of an increased number of MCs at the sites of infection (Dezfuli and Giari, 2008), especially during parasitosis (Dezfuli et al., 2013). Indeed, it is thought that there are two populations of MCs, one resident and the other circulating, the latter moving to the infected site in the case of infection. In this way, MCs are associated with blood vessels and a few are interspersed in connective tissues, increasing around and within capillaries during parasite infections (Dezfuli et al., 2012a, 2012b). Although MCs are classically included with innate immune leukocytes,

there is evidence concerning their role not only in innate immunity, but also in the adaptive immune response (Da'as et al., 2011).

2.1.2. Regulatory molecules

There are many humoral components in fish innate immunity. The most important regulatory molecules involved in this process include cytokines, complement, lysozyme, lectins, antimicrobial polypeptides, eicosanoids, ROS/RNS, natural antibodies, and protease inhibitors.

Cytokines are a vast group of regulatory molecules in the innate immune response which include interlukins (IL), tumor necrosis factor α (TNFα), interferons (IFN), chemokines:

- Interleukin (IL) are classified into six families (1, 2, 6, 10, 12 and 17) (Savan and Sakai, 2006). Those of the IL-1 family participate in pro-inflammatory processes (Dinarello, 1997, 1998a, b) and include IL-1β and IL-18 (Savan and Sakai, 2006). The IL-1β, which are constitutively expressed in macrophages and neutrophils in some teleost species (Engelsma et al., 2001; Fast et al., 2006, 2007), elicit the humoral response (Savan and Sakai, 2006), while IL-18 is stored intracellularly in a vast range of cells, such as macrophages, dendritic cells, and B and T cells and induce interferon (IFN)-γ secretion (Whyte, 2007). In the IL-2 family, IL-2 has been found to modulate the production of IFN-γ (Díaz-Rosales et al., 2009) and drive lymphocyte differentiation (Corripio-Miyar et al., 2012). IL-12 is also an important inflammatory mediator as it induces IFN-γ secretion (Whyte, 2007). IL-6, a member of the IL-6 family (Savan and Sakai, 2006), is involved in inflammatory processes (Zante et al., 2015) and IL-10, from the IL-10 family (Savan and Sakai, 2006), acts as an anti-inflammatory cytokine, inhibiting the production of pro-inflammatory innate immune mediators, such as IL-1 (Karan et al., 2016). In the IL-17 family, IL-17 has been found to regulate the production of IL-6 and IL-8 (Monte et al., 2013) and to be regulated by, the still not fully characterised, regulatory T cells (Secombes et al., 2011).

- TNF-α is an important macrophage-activation factor produced by leukocytes that lead to increased respiratory activity, phagocytosis and RNS (Uribe et al., 2011; Whyte, 2007). TNF-α also induce the expression of various immune genes including IL-1β, IL-8 and cyclooxygenase 2 (Zou et al., 2003).

- IFN-α and IFN-β play and essential role in the defence against viral infection by inhibiting the nuclear acid replication of viruses within infected cells (Robertsen,

2006; Uribe et al., 2011) or protecting other cells from viral infection by modulating the expression of a variety of genes, some of them encoding for antiviral proteins (De Veer et al., 2001; Samuel, 2001). IFN-γ is produced by non-specific cytotoxic cells and T cells (Rubio-Godoy, 2010) in response to IL-12, IL-18, mitogens or antigens (Whyte, 2007), and has the same function as its mammalian homologue (Zou and Secombes, 2011).

- Chemokines are a superfamily of cytokines produced by different cell types. They possess chemoattractant properties that induce the recruitment, activation and adhesion of cells to sites of infection or tissue damage, and are crucial in chaging the innate immune response into the adaptive response (Whyte, 2007). This family is divided into CXC, CC, C and CX_3C classes. IL-8, the first fish chemokine known, induces the recruitment of neutrophils, T cells and basophils, increases cytosolic calcium levels and respiratory burst, and produces changes in neutrophil shape (Whyte, 2007).

The complement is responsible for three immune functions: i) opsonization, ii) inflammation by stimulating the contraction of smooth muscle, vasodilation and the chemoattraction of leukocytes, and iii) the lysis of pathogens through perforation of their membranes. The third component (C3), which is mainly produced in liver (Boshra et al., 2006), is essential in the activation of the complement system (Rubio-Godoy, 2010). After activation of C3, it is divided into C3a and C3b, the latter being the main phagocytosis-promoting factor. Macrophages and neutrophils have been found to possess receptors specific for the complement (Yano, 1996). Like that of higher vertebrates, it can be activated by three pathways (the classic, alternative and lectin pathways) (Holland and Lambris, 2002; Sakai, 1992; Sunyer and Lambris, 1998).

Lysozyme is a bacteriolytic enzyme widely distributed throughout the body (Uribe et al., 2011). It acts as a lytic enzyme of the peptidoglycan component of the bacterial cell wall and can also have an opsonic function (Manning, 1998). The sources of lysozyme are monocytes/macrophages, neutrophils and MCs of the intestine (Uribe et al., 2011).

Lectins are proteins or glycoproteins of non-immune origin that bind to particular carbohydrates, leading to their recognition. They participate in the recruitment of microorganisms and the precipitation of different substances (Arason, 1996; Russell and Lumsden, 2005), can activate the complement system (Rubio-Godoy, 2010). They are classified into at least six families: legume lectins, cereal lectins, P-, S-, C-type lectins and

pentraxins (Whyte, 2007). Pentraxins and C-reactive protein (CRP) are evolutionary conserved proteins that are generally associated with the acute phase response, and play an important role in fish innate immunity (Cartwright et al., 2004; Lund and Olafsen, 1999; Richards et al., 2003; Whyte, 2007). The pentraxins, CRP and serum amyloid protein (SAA), particularly, are associated with early response to infection or tissue damage (Rubio-Godoy, 2010; Uribe et al., 2011). CRP is an opsonin which, like SAA, activates the complement system after binding to the bacterial surface (De Haas et al., 2000; Nakanishi et al., 1991).

Antimicrobial polypeptides, such as histone H2B and three 60S ribosomal proteins, L40, L36A and L35, appear in integumental secretions of fish and in the skin mucus (Bergsson et al., 2005; Cole et al., 2000; Noga et al., 2002; Patrzykat et al., 2001; Robinette and Noga, 2001; Robinette et al., 1998), and they participate in fish immunity against bacteria or viruses (Chinchar et al., 2004; Ellis, 2001; Ingram, 1980). Piscidins are antimicrobial polypeptides of 22 aminoacids forming an alpha helix with potent, broad-spectrum antibacterial activity against fish pathogens (Silphaduang and Noga, 2001; Silphaduang et al., 2006). They are found in mucous tissues and immune cells (Crivellato et al., 2015; Rubio-Godoy, 2010). Piscidins are inhibitors of the cell wall, nucleic acids, and protein synthesis or even enzymatic activity of bacteria (Campagna et al., 2007; Mulero et al., 2008a).

The family of *eicosanoids* includes prostaglandins, thromboxanes and leukotrienes, which take part in several processes such as immune regulation and inflammation by increasing phagocytosis and acting as chemoattractants for neutrophils (Gómez-Abellán and Sepulcre, 2016; Manning, 1998; Secombes, 1994, 1996).

ROS and *RNS*, such as superoxide, hydrogen peroxide and nitric oxide, are produced by neutrophils and macrophages, and have physiological, toxicological and immunoregulatory effects (Lincoln et al., 1997; Moncada et al., 1991). Superoxide and hydrogen peroxide are generated by nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase (Swindle et al., 2004) and nitric oxide formation needs nitric oxide synthase (type II NOS) (Koranteng et al., 2000). In immune responses, when neutrophils and macrophages are activated, ROS/RNS are produced at relatively high levels (Koranteng et al., 2000).

Natural antibodies are produced at low regulated levels in the absence of antigenic stimulation of cells in healthy individuals (Uribe et al., 2011; Whyte, 2007). They are found at high levels in the serum (Boes, 2000; Lange et al., 2001; Magnadottir et al., 1999a, b; Ochsenbein and Zinkernagel, 2000; Sinyakov et al., 2002), providing protection against

bacterial and viral pathogens. In mammals, they are generated by B-1 cells (CD5+), a subset of long-lived, self-replenishing B cells active during embryonic and early developmental stages (Baumgarth et al., 2005). The intensity of production of natural antibodies depends on the species and the environmental conditions (Whyte, 2007). Natural antibodies are key factors not only in innate immunity but also in adaptive immunity.

Protease inhibitors are found in the serum and other body fluids of fish (Bowden et al., 1997). Their function is to maintain fluid homeostasis and they are involved in acute phase reactions and against proteolytic secreting-pathogens (Magnadottir, 2010).

2.1.3. Histamine and histamine receptors

Histamine is one of the first inflammatory mediators released after infection or tissue damage. For decades, histamine was believed to belong only to higher vertebrates. Nevertheless, in 2007 this hypothesis changed when histamine was found in the granular content of gilthead seabream MCs (Mulero et al., 2007a), although our knowledge of the role of histamine in teleost fish remains poor.

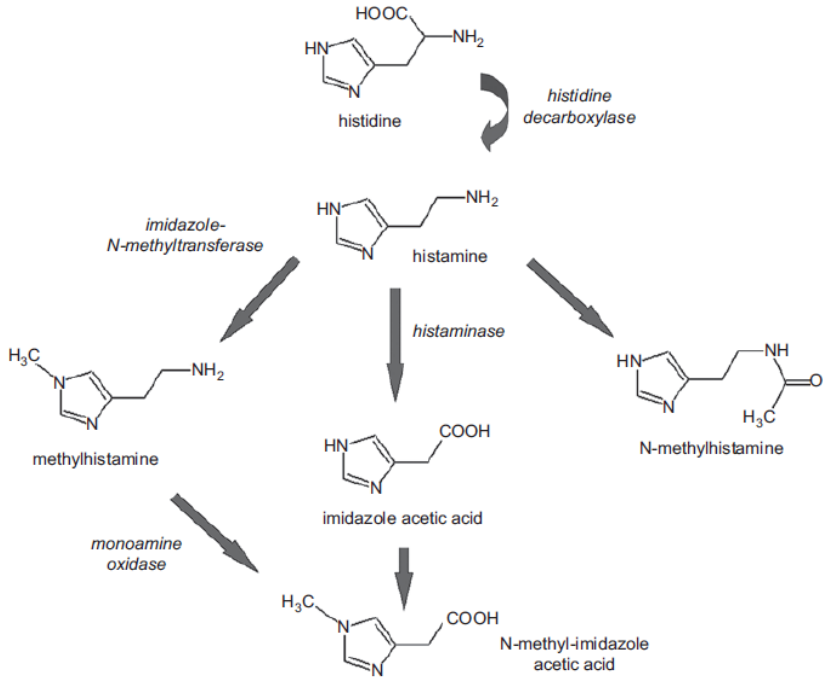


Figure 1. Histamine molecular structure and metabolic pathways of histamine synthesis and its derivates (Giusi et al., 2010).

Histamine (β-emidazolylethylamine) is a very important amine in mammals, in which it is involved in a wide variety of biological processes such as neurotransmission and numerous brain functions, the secretion of pituitary hormones, and the regulation of gastrointestinal and circulatory functions, and inflammatory reactions (Leurs et al., 1995). In mammals, histamine

is synthesized by the enzyme histidine decarboxylase (HDC) (Ohtsu et al., 2001) and its levels of histamine are controlled by the diamine oxidase and histamine N-methyltransferase enzymes (Abe et al., 1993; Yamauchi et al., 1994) (Fig. 1). Although histamine is mainly produced by, stored in, and released by MCs, it has also been found to be produced by platelets, dendritic cells and T cells (Kubo and Nakano, 1999; Radvany et al., 2000; Saxena et al., 1989).

Histamine has a role in the progression of allergic-inflammatory responses by increasing the synthesis of IL-1α and β, IL-6, IL-8 and decreasing the production of TNFα (Bayram et al., 1999; Meretey et al., 1991; Vannier and Dinarello, 1994; Vannier et al., 1991). Histamine also affects monocytes (IL-1, IL-2, IL-18, IL-19, TNF-α production), dendritic cells (changes in T cell-polarising capacity, intracellular Ca^{2+} transients, actin polymerization, chemotaxis, intracellular cAMP, IL-10 and IL-12 production), and B and T cells (affection Th1 and 2 cells balance and Ig synthesis, IFN-γ, IL-4, IL-13 production) (Fig. 2) (Ferstl et al., 2012; Jutel et al., 2002).

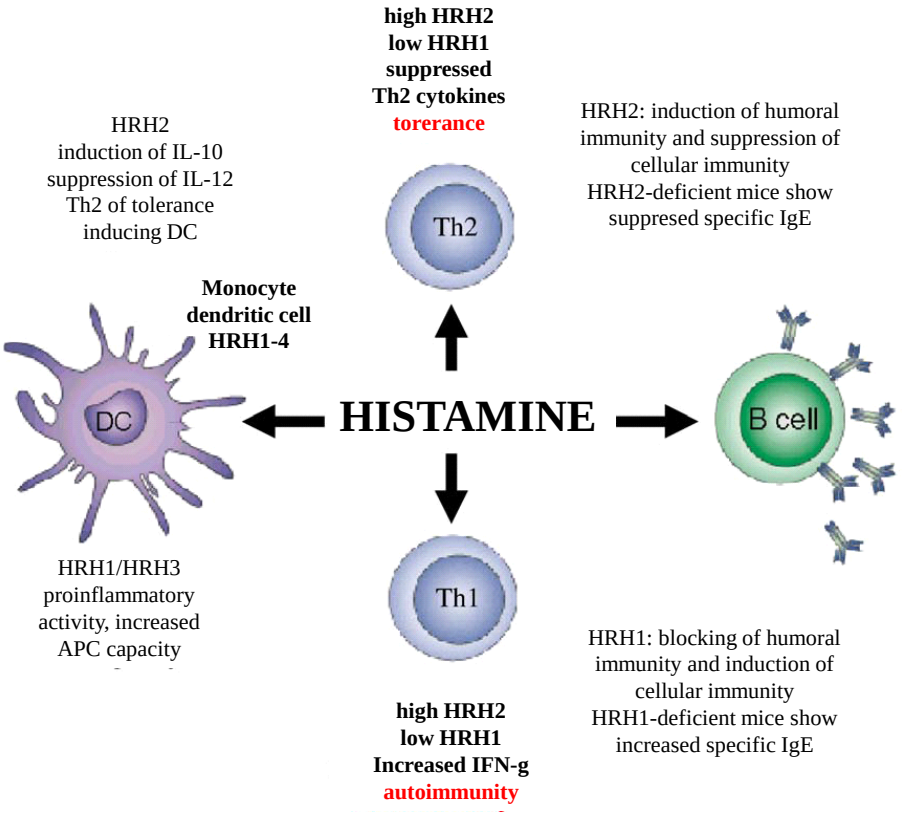


Figure 2. Histamine regulates monocytes, dendritic cells, and B and T cells in lymphatic organs and subepithelial tissues (modified from Akdis and Simons, 2006).

In mammals, four membrane histamine receptors (HRs) have been identified and named as HR H1, H2, H3 and H4 (HRH1, HRH2, HRH3 and HRH4) (Ash and Schild, 1966;

Lovenberg et al., 1999; Oda et al., 2000). All of them are heptahelical G-protein-coupled receptors, with seven putative transmembrane domains, amino-terminal glycosylation sites and phosphorylation sites for protein kinase A (PKA) and protein kinase C (PKC). HRH1 is coupled to Gαq, while the HRH2, HRH3 and HRH4 signalling mechanisms depend on adenylate cyclase (AC), whereby HRH2 is coupled to Gαs and its activation induces AC and PKA activation, and HRH3 and HRH4 are coupled to Gαi/0 receptors and their activation leads to the inhibition of AC (Fig. 3) (Leurs et al., 1995; Oda et al., 2000; Shahid et al., 2009).

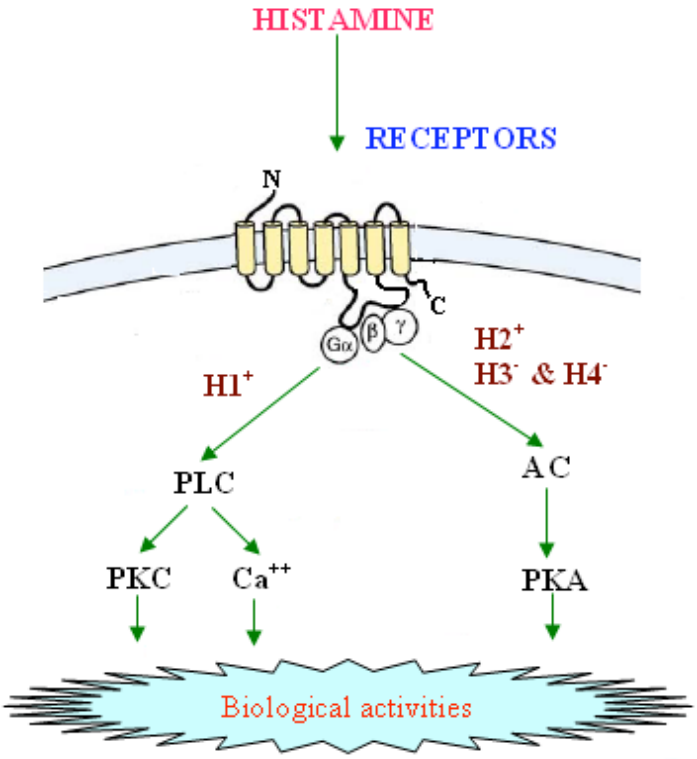


Figure 3. The classical binding sites of histamine and their main signaling pathways such as AC (adenylate cyclase), PKC (protein kinase C), PKA (protein kinase A), PLC (phospholipase C) (Shahid et al., 2009).

HRH1 and HRH2 are expressed in many cell types, including nerve cells, airway and vascular smooth muscle, hepatocytes, chondrocytes, endothelial cells, neutrophils, eosinophils, monocytes, dendritic cells, and B and T cells (Leurs et al., 1995). Meanwhile, HRH3 has been described on histaminergic neurons in the brain and on some peripheral tissues (Jutel et al., 2002). Moreover, HRH4 is found on haematopoietic cells, including neutrophils, eosinophils and Th cells, basophils and MCs (Liu et al., 2001; Zhu et al., 2001). Constitutive activity has been described in all four types of HR (Leurs et al., 2002). Stage of cell differentiation and micro-environment regulate the expression pattern of HR (Ferstl et al., 2012).

The role of histamine in the immune system of mammals is very controversial. Even though histamine was thought to be a pro-inflammatory mediator of immunity, it also plays an anti-inflammatory role, depending on the predominance of a certain type of HR (Jutel et al., 2002). It was described that the lack of the histamine producing enzyme HDC and inhibition of HRH1 or HRH4 downregulate the production of IL-6 (Desai and Thurmond, 2011; Horvath et al., 2002). Moreover, histamine stimulation induces IL-10 secretion through HRH2 in both dendritic and T cells (Jutel et al., 2002; Osna et al., 2001). HRH4 activation induces the accumulation of inflammatory cells (particularly eosinophils and mast cells) at sites of allergic inflammation (Akdis and Simons, 2006). Furthermore, histamine is involved in the adaptive immunity affecting B cell proliferation mediated by HRH1 and antibody production through HRH1 and HRH2 (Banu and Watanabe, 1999; Jutel et al., 2001). In summary, despite some opposing studies, HRH1 is generally considered to have a pro-inflammatory role in immune cells, increasing their migration to the inflammation area and modulating their effectors functions, while HRH2 is thought to be a potent anti-inflammatory suppressor of inflammation; and the pro- or anti-inflammatory roles of HRH3 and HRH4 remain to be elucidated (Akdis and Simons, 2006).

Histamine is biologically active and regulates the inflammatory response in gilthead seabream since histamine can regulate "professional phagocyte" function through Hrh1 and Hrh2 (Mulero et al., 2007a). Furthermore, Hrh2 agonist, but not Hrh1 or Hrh3 agonists, induce cell death or smooth muscle contraction in this teleost fish (Mulero et al., 2007a). Moreover, three homologues of mammalian HR (Hrh1, Hrh2 and Hrh3) have been described in zebrafish (*Danio rerio*) (Peitsaro et al., 2007). The same study revealed that the genes encoding for Hrh1 and Hrh2 are expressed throughout development and that *hrh3* is hardly expressed 3 hours post fertilization but is clearly visible later (Peitsaro et al., 2007). Moreover, these receptors showed different expression patterns in numerous tissues of adult zebrafish: *h1hr* was found in intestine, liver and spleen, *h2hr* expression was visible in brain, gills, heart and spleen, and *h3hr* was strongly expressed in brain, heart and spleen but weakly expressed in liver, eyes and gills (Peitsaro et al., 2007). Moreover, the three HRs are expressed in the gonads of gilthead seabream (García-García et al., 2016).

Before the presence of histamine in perciformes was confirmed, several studies tried to determine the capacity of MC degranulation in response to certain compounds. For instance, injection of exotocins from *Aeromonas salmonicida* in rainbow trout (*Oncorhynchus mykiss*) resulted in a decrease in the histamine content in the gut, EGCs degranulation in the intestinal

wall and an increase of histamine in the blood, suggesting a histaminogenic role for EGCs in rainbow trout (Ellis, 1985). Moreover, *A. salmonicida*, Co 48/80 (a classic MC degranulation inducer) and concanavalin A induced EGCs degranulation in the lower intestine and rectum of rainbow trout, which was inhibited by the Hrh1 and Hrh2 antagonists promethazine and cimetidine, respectively (Vallejo and Ellis, 1989). Additionally, the intraperitoneal injection of substance P and capsaicin into rainbow trout induced extensive degranulation of EGCs of the intestine (Powell et al., 1991). Furthermore, substance P and compound 48/80 increased resistance in branchial vessels of rainbow trout (Reite, 1997). Also, the injection of formalin-killed *Escherichia coli*, proteose peptone, Co 48/80 or Hank's balanced salt solution into the swim bladder of tilapia (*Oreochromis niloticus*) caused degranulation of EGC at the injection site and the rapid migration of neutrophils from the peritoneal cavity (Matsuyama and Iida, 1999).

Compared with the numerous studies regarding to the role of histamine in the immune system in mammals, the regulatory function of histamine in the fish immune system has received little attention. Although Co 48/80, substance P and capsaicin (classic chemical substances able to activate MCs or induce histamine release in mammals) have been demonstrated to induce fish MC degranulation (Manera et al., 2011; Mulero et al., 2007a; Powell et al., 1991), new studies using these MC-activating agents are necessary to fully understand the role of histamine in the immune system of fish.

2.2. Adaptive immune system

The appearance of functional lymphocytes let teleost fish develop a specific capacity to recognize particular antigenic insults, become activated in their presence, and show memory immune responses (Rubio-Godoy, 2010). Together, lymphocytes analogous to the mammalian B and T cells, and the humoral components, Ig and cytokines, of the adaptive immunity permit a rapid and an efficient pathogen elimination immune response upon secondary exposure to the same antigen (Rubio-Godoy, 2010).

2.2.1. Cell types

The immunological memory of B and T cells allows teleost fish to present a more competitive response against successive exposures against pathogens. It is due to this adaptive immune response that fish can be protected from pathogens through vaccines (Uribe et al., 2011).

B cells are mainly produced in the head kidney, followed by the thymus and spleen of teleost fish (Crowhurst et al., 2002; Dos Santos et al., 2000; Koumans-van Diepen and Steiner, 1995; Salinas et al., 2011; Trede et al., 2001) and they populate peripheral organs such as the intestine (Rombout et al., 2005) or the peritoneal cavity (Parra et al., 2016). There is some evidence showing that B cells are also developed in the pancreas (Danilova and Steiner, 2002). Teleost B cells produce Ig to label specifically altered-host or foreign cells in order to agglutinate or precipitate soluble antigens, promoting phagocytosis (Rubio-Godoy, 2010). Based on the discovery of fish IgM, IgD and IgT/Z (see next section), three B cell subsets have been identified in catfish (IgM^+/IgD^-, IgM^+/IgD^+ and IgM^-/IgD^-) (Edholm et al., 2010) and two in rainbow trout ($IgM^+/IgD^+/IgT^-$ and $IgM^-/IgD^-/IgT^+$) (Zhang et al., 2010), while four B cell subsets have been described in teleost fish: B cells expressing only IgM, IgD or IgT and a B cell subset coexpressing IgM and IgD (Salinas et al., 2011).

T cells are mainly produced in the thymus, followed by the head kidney and the spleen of teleost fish (Dos Santos et al., 2000; Nakanishi et al., 2015; Rombout et al., 2005; Salinas et al., 2011) but they may also be found in skin, gills, gut or blood (Rombout et al., 2005). In mammals, T cells are categorized into two general populations: Tc and Th cells. The existence of Tc cells in fish, where they would be involved in specific cell-mediated cytotoxicity, similar to that which occurs in higher vertebrates has been suggested (Fisher et al., 2006). Recently, fish Tc and Th cells have been identified as $CD8^+$ and $CD4^+$ cells, respectively, in ginbuna crucian carp (*Carassius auratus langsdorfii*) (Toda et al., 2009, 2011). Unlike mammalian species, the existence of teleost fish Th cells and their polarization into Th1, Th2, Th17 and regulatory T cells has not been demonstrated yet but thereis evidence that Th cells do exist teleost fish, similar to their mammalian counterparts (Secombes, 2008; Yamaguchi et al., 2015). Whatever the case, teleost T cells would be involved in allograft rejection (ability of T cells to recognize genetically different major histocompatibility complex molecules of transplants and induce an inflammatory reaction), stimulation of phagocytosis and antibody production by B cells (Powell, 2000; Zapata and Amemiya, 2000). As in mammals, the adaptive immune response of teleost T cells is thought to be mediated by the production of cytokines (Rubio-Godoy, 2010; Uribe et al., 2011).

2.2.2. Regulatory molecules

The way in which B cells defend the host body against pathogens involves the production of specific antibodies. Ig production by B cells results in protective responses against antigens through the neutralization of small molecular weight molecules by agglutination and/or

precipitation of soluble antigens by the antibodies, working as opsonins, by coating antigens and promoting phagocytosis. Moreover, antibodies can activate the complement system when they bind to the antigens, producing conformational changes in the Fc region (Uribe et al., 2011). Furthermore, as mentioned in the innate immunity section, although fish have not been previously exposed to an exogenous specific antigen, B cells can produce low levels of Ig against this specific antigen, working as natural antibodies and helping the innate humoral immune system.

Teleost antibodies are found systemically in plasma (Uribe et al., 2011), skin (Cain et al., 2000; Hatten et al., 2001), intestine (Jones et al., 1999; Rombout et al., 1986), gill (Lumsden et al., 1993), and bile (Jenkins et al., 1994).

Three different Ig have been identified in fish: IgM (Acton et al., 1971), IgD (Wilson et al., 1997) and IgT/Z (Danilova et al., 2005; Hansen et al., 2005). A tetramer of the IgM class containing eight antigenic combining sites is the most predominant Ig in teleost fish (Acton et al., 1971). Also, a monomer of IgM has been found in the serum of some teleost fish (Wilson and Warr, 1992). The IgM concentration in serum varies among teleost species (Scapigliati et al., 1997; Uchida et al., 2000; Vilain et al., 1984) and depends on animal size (Magnadottir et al., 1999b; Sánchez et al., 1993), water temperature (Sánchez et al., 1993), water quality (Olesen and Jogersen, 1986) and season of the year (Magnadottir et al., 2001; Olesen and Jogersen, 1986). IgD was the second isotype identified in channel catfish (*Ictalurus punctatus*). As in mammals, fish IgD gene sequence is located immediately under the IgM gene and is expressed in B cells (Wilson et al., 1997). IgT was described in teleost fish (Danilova et al., 2005; Hansen et al., 2005), and is also named IgZ in cyprinids (Parra et al., 2016). B cells exclusively expressing surface IgT or IgM have been described in rainbow trout (Zhang et al., 2010) and B cells expressing IgZ or IgM in zebrafish (Page et al., 2013; Schorpp et al., 2006).

There is no evidence concerning the presence of IgE, IgA or IgG in teleost fish, although a functional FceRI receptor has been described (Da'as et al., 2011), which, in mammals, acts as an IgE receptor. Moreover, although there is some evidence showing that antibodies found in mucus are slightly different structurally from serum Ig (Lobb and Clem, 1981), a distinct class of secretory Ig analogous to mammalian IgA has not been found in fish.

T cells produce a variety of soluble factors, cytokines, to mediate cell interaction in order to defend the organism against pathogens. Although the existence of Th1 and Th2 cells in

teleost fish has not yet demonstrated, some evidence suggests that they could exist and have a similar role to their mammalian counterparts (Secombes, 2008; Yamaguchi et al., 2015). Indeed, the cytokines, IFN-γ and IL-4, known to be produced by mammals Th1 and Th2 cells, respectively, seems to mediate similar Th1 and Th2 cells responses in teleost fish (Li et al., 2007; Rubio-Godoy, 2010; Zou et al., 2005)

3. Estrogens and immunity

3.1. Estrogen and estrogen receptors

The synthesis of sex steroids, steroidogenesis, mainly takes place in the gonads (testis and ovaries) as well as in the adrenal glands (Young et al., 2004) that produce end-products such as testosterone (T), 11-ketotestosterone (11KT), dihydrotestosterone (DHT), 17β-estradiol (E_2) and estrone (Fig. 4). The regulation of this process depends largely on the follicle stimulating hormone (FSH) and luteinizing hormone (LH), synthesized and secreted by the pituitary (Schulz et al., 2001). E_2 has been considered to be the main hormone of female fish; however, recent studies have suggested that estrogens are ‘‘essential” for normal male reproduction (Amer et al., 2001; Hess, 2003; Miura et al., 1999).

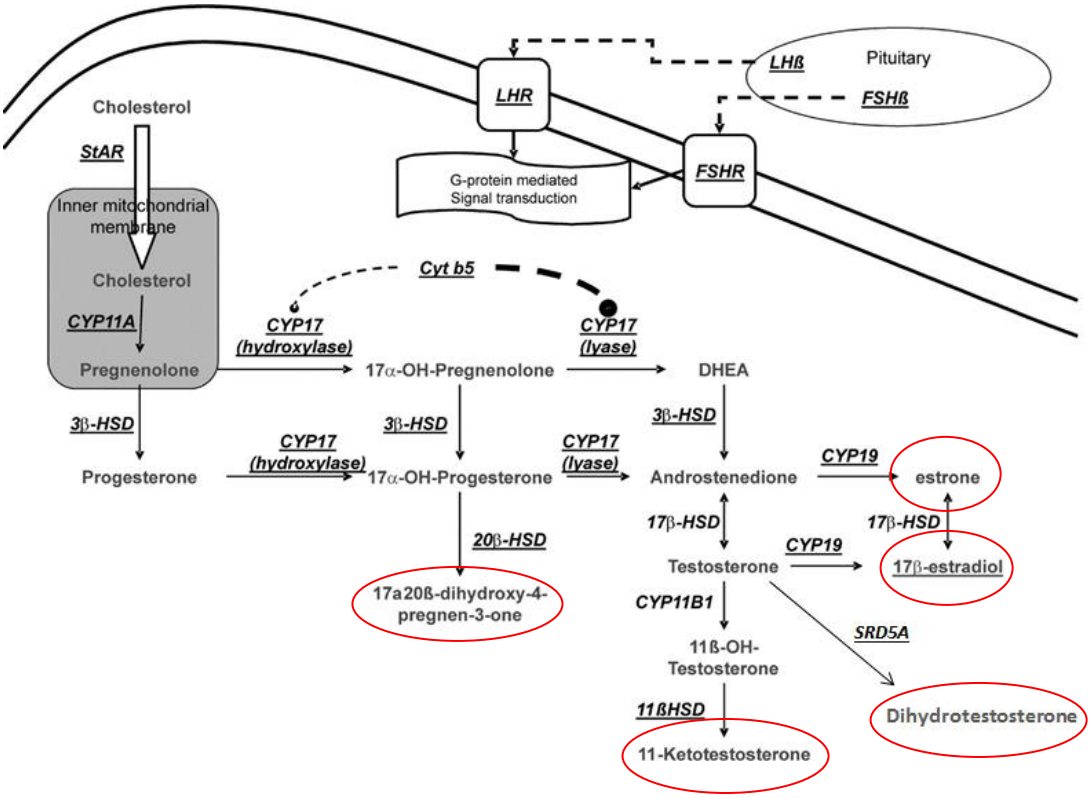


Figure 4. Schematic representation of the key steps involved in steroidogenesis in teleost (modified from (modified from Villeneuve et al., 2007).

Gilthead seabream, a protandrous hermaphrodite fish, develops functionally as male, at least, during the two first years of life, although their gonads possess a non-developed ovarian area (Chaves-Pozo et al., 2005b; Liarte et al., 2007), depending on the natural environment of the populations studied. The reproductive cycle of males consists of four stages: spermatogenesis, spawning, post-spawning and resting (Fig. 5), in which the levels of E_2 as well as those of the androgens, T and 11KT, the main androgens in this species, vary (Chaves-Pozo et al., 2008a). Resting is succeeded by a testicular involution stage when the fish are ready to undergo sex change (Chaves-Pozo et al., 2005b; Liarte et al., 2007). During post-spawning and resting, a marked increase in serum levels of E_2 and decreases in T and 11KT have been observed (Chaves-Pozo et al., 2008a).

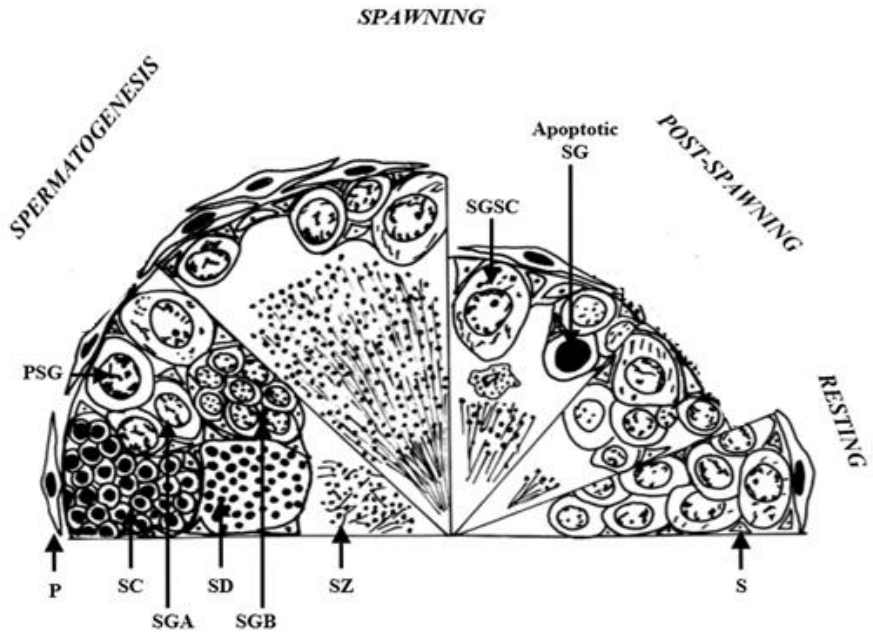


Figure 5. Schematic illustration of the changes in the testis of gilthead seabream. Germinal compartment. SGSC: spermatogonia stem cell; PSG: primary spermatogonia; SGA: spermatogonia A; SGB: spermatogonia B; SC: spermatocyte; SD: spermatid (Chaves-Pozo et al., 2005b).

Estrogens act via classical nuclear estrogen receptors (ERs), ERα and ERβ. Moreover, it is accepted that the species of the class Actinopterygii have two ERβ subtypes, ERβ-I and ERβ-II (Nelson and Habibi, 2013). Classically, it has been described that ERα and ERβ mediate genomic actions (Thomas et al., 2010). Nevertheless, there are physiological responses to estrogens that cannot be explained by the activation of these classical nuclear ERs. Although some research has indicated the possibility of estrogen binding sites at the outer cell membrane (Pietras and Szego, 1977), the scientific community required 23 years more to identify an estrogen cell surface receptor, the G protein-coupled estrogen receptor,

GPR30 or GPER1, with non-genomic activity (Filardo et al., 2000). Endogenous estrogens such as E_2 are non-selective activators of the three ERs known to date, ERα, ERβ and GPER1 (Prossnitz and Barton, 2011).

The presence of genomic and non-genomic ERs has been demonstrated in different tissues including ovary, testes, liver, heart, muscle, pituitary, skin, stomach, duodenum, midgut, rectum, kidney and brain (Casanova-Nakayama et al., 2011; Smith and Thomas, 1990), including immune cells (Lynn et al., 2008; Shved et al., 2009).

3.1.1. Nuclear estrogen receptors: ERα and ERβ

When estrogen ligands are lacking, ERs are mainly found in the cytoplasm. Estrogens induce the formation of homodimers and heterodimers of ERα and ERβ in the nucleus, where they interact with specific regions of the DNA, or some transcriptional factors already associated with DNA, to induce transcriptional activity of the complex (Fig. 6) (Nelson and Habibi, 2013). Alternatively, activated ERs can modulate the function of other classes of transcriptional factors (TF) through protein–protein interactions. Thus, subpopulations of E_2-activated ER at the plasma membrane interact with adaptor proteins and signalling molecules, including c-Src, which mediate rapid signalling via PI3K/Akt and MAPK pathways (Fig. 6) (Prossnitz and Barton, 2011). This classic genomic mechanism is relatively slow, typically taking place over a time scale of hours (Thomas, 2012). Unlike mammals, teleost ERβ forms are able to bind E_2 with higher affinity than ERα (Menuet et al., 2002; Xia et al., 1999, 2000). Moreover, nuclear ERs are primarily responsible for the induction of vitellogenin (precursor protein of egg yolk) (Nagler et al., 2010).

In gilthead seabream, expression of the nuclear *er* has been identified in different cells and tissues: i) *era* in endothelial cells (Liarte et al., 2011a), head kidney macrophages and lymphocytes (Liarte et al., 2011b), kidney (Pinto et al., 2006; Rodenas et al., 2015), white muscle, liver, heart, stomach, duodenum, midgut, rectum, pituitary, ovary, skin and testis (Pinto et al., 2006); ii) *erb1* in endothelial cells (Liarte et al., 2011a), head kidney macrophages stimulated with genomic DNA from *Vibrio anguillarum* (VaDNA) (Liarte et al., 2011b), cell fractions of head kidney after GA removal (Liarte et al., 2011b), white muscle, liver, heart, stomach, duodenum, midgut, rectum, pituitary, ovary, skin, testis and kidney (Pinto et al., 2006); and iii) *erb2* in VaDNA-stimulated head kidney macrophages (Liarte et al., 2011b), white muscle, liver, heart, stomach, duodenum, midgut, rectum, pituitary, ovary, skin, testis and kidney (Pinto et al., 2006).

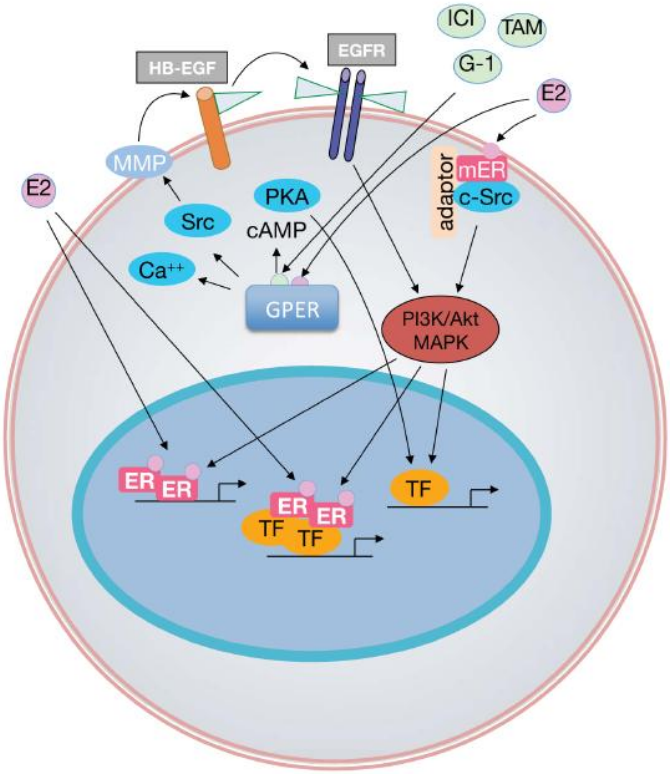


Figure 6. Non-genomic and genomic estrogen signaling pathways. E2: 17β-estradiol; ER: Estrogen receptor; GPER: G protein-coupled estrogen receptor; TF: transcriptional factor; TAM: tamoxifen; ICI: fulvestrant; mER: ER at the plasma membrane; adaptor: adaptor protein; MMP: matrix metalloproteinases; HB-EGF: pro-heparin-binding-epidermal growth factor; EGFR: EGF receptors; cAMP: cyclic adenosine monophosphate; PI3K: phosphoinositide 3-kinase; Akt: protein kinase B; MAPK: mitogen-activated protein kinase (Prossnitz and Barton, 2011).

3.1.2. Cell membrane estrogen receptor: GPER1

GPER1 is as an estrogen-binding intracellular membrane G protein-coupled receptor predominantly located intracellularly. E_2, selective agonists (*e.g.*, G1), selective estrogen receptor downregulators (*e.g.*, fulvestrant), or selective estrogen receptor modulators (*e.g.*, tamoxifen) can activate GPER1. Such GPER1 activation stimulates cAMP production, calcium mobilization and c-Src, followed by the activation of matrix metalloproteinases, which cleave pro-heparin-binding-epidermal growth factor (HB-EGF), releasing free HB-EGF that transactivates EGF receptors (EGFR). Then, EGFR activates the MAPK and PI3K/Akt pathway, inducing rapid (non-genomic) effects, or genomic effects regulating gene transcription (Fig. 6) (Prossnitz and Barton, 2011). This non-genomic signalling involves rapid activation of intracellular signal transduction pathways within a few minutes (Thomas, 2012).

GPER1, expressed on the cell surface of fish oocytes, mediates estrogen inhibition of oocyte maturation (Thomas, 2012). In fact, the decrease of E_2 stops meiosis during oocyte maturation (Yaron and Levavi-Sivan, 2011).

The expression of the GPER1 in gilthead seabream immune cells and tissues has been identified in head kidney (Cabas et al., 2013b; Rodenas et al., 2015), spleen, liver, testes and head kidney AGs (Cabas et al., 2013b).

3.2. Estrogen regulation of immunity

The existence of ERs in immune cells has increased the interest of researchers in the possible effects of estrogens in fish immune modulation. Indeed, during gonadal maturation, leukocyte recruitment varies in brown trout (*Salmo trutta* L.) (Pickering, 1986) and when salmonids migrate to freshwater and suffer sexual maturation, the plasma levels of gonadal steroids increase, accompanied by the higher production of serum lysozyme activity and antibody-producing cell numbers (Maule et al., 1996). Estrogens modulate the expression of immune genes, including lectins, components of the complement or cytokines in hepatic cells of the European flounder (*Platichthys flesus*) and rainbow trout (Casanova-Nakayama et al., 2011; Tilton et al., 2006; Williams et al., 2007). Also, sexually mature salmonids have been shown to suffer a high number of ectoparasitic infestations (Pickering and Christie, 1980). Furthermore, during spawning, rainbow trout serum shows reduced bactericidal activity (Iida et al., 1989). Interestingly, estrogens enhance susceptibility to pathogens in goldfish (*Carassius auratus*) and rainbow trout (Casanova-Nakayama et al., 2011; Wang and Belosevic, 1994). Moreover, E_2 reduces phagocytosis (Watanuki et al., 2002; Yamaguchi et al., 2001), as well as respiratory burst activity in common carp (*Cyprinus carpio*) (Watanuki et al., 2002), but increases respiratory burst activity in seabass, where it increases the number of leukocyte, and plasma Ig levels, but decreases serum lysozyme and bacterial activity (Thilagam et al., 2009). Others studies also confirm that fish plasma Ig levels are sensitive to endogenous and exogenous estrogens (Cuesta et al., 2007a; Hou et al., 1999; Saha et al., 2002; Suzuki et al., 1997).

Previous studies in our laboratory demonstrated that E_2 regulates gilthead seabream AGs and macrophages (Liarte et al., 2011b, 2011c) and profoundly alter the gene expression profile of macrophages, especially gene ontology category immune-related processes and pathways (Liarte et al., 2011c). Moreover, *in vitro* the long term treatment of head kidney leukocytes with E_2 revealed an activating effect on the production of ROS and the accumulation of IL-1β (Chaves-Pozo et al., 2003). However, short term treatment with higher

concentrations of E_2 inhibited the phagocytic capability of head kidney leukocytes, while the percentage of phagocytic cells and the VaDNA-stimulated production of ROS and cell migration activity remained steady (Liarte et al., 2011b). Moreover, a correlation between E_2 and leukocyte functions and the expression profile of immune-relevant genes in the gonad have been observed (Chaves-Pozo et al., 2008b). For example, the AGs infiltration that occurs in the testis has been related to: (i) post-spawning and testicular involution stages, (ii) E_2 and T hormone peaks, (iii) increased expression of gonadal aromatase, the enzyme that transforms to T to E_2, and (iv) increase of E_2 serum levels (Chaves-Pozo et al., 2003, 2005b, 2005c, 2007, 2008b).

3.2.1. Estrogen regulation of mammalian mast cells

The role of estrogens in the modulation of MCs is unclear. Although some studies in mammals have paid their attention to this field, no studies on the role of estrogens in fish MCs have been conducted. It has been described that mammalian MCs possess ERs (Nicovani and Rudolph, 2002; Vliagoftis et al., 1992), which suggests that estrogens could modulate MC functions. Variations in plasma E_2 levels induce, in a dose-dependent manner, variations in MC number, histamine concentrations and ER expression in mammary gland (Jing et al., 2012). In addition, E_2 stimulate MC degranulation (Theoharides et al., 1993) and histamine and serotonin secretion (Vliagoftis et al., 1992), while physiological concentrations of E_2 increase the MC histamine release mediated by IgE molecules present on the MC membrane (Cocchiara et al., 1990, 1992). However, high concentrations of E_2 induce MC degranulation (Spanos et al., 1996; Vliagoftis et al., 1992) or inhibit the same (Harnish et al., 2004). Also, E_2 acts through ERα to IgE-induce the secretion of β-hexosaminidase by HMC-1 human MC (Zaitsu et al., 2007). Moreover, E_2 inhibits the secretion of IL-6 and TNFα by stimulated HMC-1 human MC (Kim et al., 2001).

Some evidence suggests that estrogens could be responsible for some human diseases mediated by MCs, such as asthma or migraine (Levy et al., 2007; Loewendorf et al., 2016). Asthma is more frequent in women than in men (De Marco et al., 2002; Mannino et al., 2002; Schatz and Camargo, 2003), when taking hormone replacement therapy (Barr et al., 2004) or showing ER polymorphisms (Dijkstra et al., 2006). In addition, serum concentrations of MC-released leukotriene C_4 are higher during the perimenstrual phase in asthmatic women, compared with healthy women (Nakasato et al., 1999). Migraine, too, shows a higher prevalence in women than in males, peaking at age 30-40 years (Loewendorf et al., 2016). Moreover, one-third to one-half of female patients suffering migraine report worse migraine

during ovulation but a decrease during pregnancy, lactation and menopause (Loewendorf et al., 2016).

4. Endrocine disruption

The State of the Science of Endocrine Disrupting Chemicals (EDCs)-2012 is an update of scientific knowledge, including main conclusions and key concerns concerning EDCs as part of the ongoing collaboration between the World Health Organization (WHO) and the United Nations Environment Programme (UNEP) to address concerns about the potential adverse health effects of chemicals on humans and wildlife (reviewed by (UNEP/WHO, 2013). This document defines an endocrine disruptor as *an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations* and a potential endocrine disruptor as *an exogenous substance or mixture that possesses properties that might be expressed to lead to endocrine disruption in an intact organism, or its progeny, or (sub) populations*.

We live in a world in which man-made chemicals have become a part of everyday life. Close to 800 chemicals are known or suspected to be capable of interfering with hormone receptors, hormone synthesis or hormone conversion. However, only a small fraction of these chemicals have been investigated in tests capable of identifying overt endocrine effects in intact organisms. Moreover, over the last 10 years, it has also been established that endocrine disruptors can work together to produce additive effects, even at low doses that individually do not produce observable effects (UNEP/WHO, 2013).

EDCs represent a challenge, as their effects depend on both the level and timing of exposure, being especially critical when exposure occurs during growth. The principal sources of EDCs in the environment and, especially, in the aquatic systems are humans and livestock, wastewater from industrial activities and agricultural runoff (Aris et al., 2014; Miyagawa et al., 2014). Depending on their different origins and nature, EDCs can be classified as alkylphenols, plasticizers, bisphenols [*e.g.*, bisphenol-A (BPA), 4-nonylphenol (NP)], pesticides (*e.g.*, endosulfan), herbicides (*e.g.*, atrazide), metals (*e.g.*, lead, cadmium, mercury), dichlorodiphenyltrichloroethane (DDT) and its metabolites (*e.g.*, o,p'-DDT), or natural and pharmaceutical estrogens [*e.g.*, E_2, 17α-ethynylestradiol (EE_2)] (Prait and Nelson, 2002; Tohyama et al., 2016).

Some EDCs can act as agonist (binding and activating natural hormone receptor) or antagonist (blocking the receptor's action). Others can act directly on any number of proteins

that control the delivery of a hormone to its normal target cell or tissue. EDCs interfere in some way with hormone action and, in doing so, can produce adverse effects on human and wildlife health. The physiological systems affected by this disruption very likely include all hormonal systems ranging from the development and function of reproductive organs to adult onset diabetes or cardiovascular disease. Although there are many hormones and hormone systems, most studies of EDCs have focused on chemicals that interact with estrogen, androgen and thyroid hormone systems acting through specific estrogen, androgen and thyroid receptors, respectively (Milla et al., 2011). A growing number of studies, however, indicate that environmental chemicals can interfere with other endocrine systems (Casals-Casas and Desvergne, 2011) such as metabolism, fat storage, bone development and the immune system (Bergman et al., 2013).

The type and intensity of EDC effects depend on their concentration and duration of exposure as well as the fish species (Devlin and Nagahama, 2002; Milla et al., 2011; Miyagawa et al., 2014). In general, sensitivity to endocrine disruption is highest during tissue development, when developmental effects will arise at lower doses than are required for effects in adults (Bergman et al., 2013). Some biomarkers of endocrine disruption have been described: for example, the induction of vitellogenin in cyprinids or salmonids for estrogenic disruption (Christiansen et al., 1998; Fenske et al., 2001; Heppell et al., 1995; Tyler et al., 1996) or the glue protein spiggin in the three-spined stickleback (*Gasterosteus aculeatus*) for androgenic disruption (Katsiadaki et al., 2002, 2006; Sánchez et al., 2008).

4.1. Estronic EDC and its effects in the fish immune system

Estrogenic EDCs, natural or synthetic, mimic endogenous estrogen activity or induce estrogen-like response in an organism. These EDCs can be found in wastewater, surface water, sediments, groundwater, drinking water and food at low concentrations, but become more concentrated higher up the food-chain, retaining their bioactivity for long periods (Campbell et al., 2006; Narita et al., 2007). They include pesticides (*e.g.*, atrazine), surfactants (*e.g.*, alkyphenol-ethoxalates), natural and pharmaceutical estrogens (*e.g.*, E_2, EE_2 and tamoxifen), phytoestrogens (*e.g.*, isoflavonoides and coumestrol) and other industrial compound (*e.g.*, BPA) (Campbell et al., 2006). EE_2, BPA and NP are probably the most studied estrogenic EDCs. As endogenous estrogens, BPA induces the expression of the gene encoding for vitellogenin in zebrafish (Kausch et al., 2008) and tamoxifen in zebrafish and gilthead seabream (Kausch et al., 2008; Rodenas et al., 2015, 2016).

There is increasing evidence that estrogenic EDCs increase the susceptibility to disease in fish due to their interference with steroid activity and immune homeostasis (Milla et al., 2011). Estrogenic EDCs have different effects on fish immunity, including the modulation/alteration of:

Leukocyte cell numbers. The exposure of roach (*Rutilus rutilus*) to estrogen-active effluents from wastewater treatment plants was associated with a differential cell count in the total number of thrombocytes (Liney et al., 2006). Moreover, BPA induced lymphocyte proliferation in goldfish (Yin et al., 2007), while the ER antagonist 3,3',4,4',5-pentachlorobiphenyl (PCB 126) increased the number of specific antibody secreting cells against *Edwardsiella ictaluri* in channel catfish (Rice and Schlenk, 1995). In addition, NP decreased the number of erythrocytes and blood leukocytes and the percentage of red and white blood cells in common carp (Schwaiger et al., 2000) and tamoxifen increased the percentage of IgM+ B cells in head kidney and spleen of gilthead seabream (Rodenas et al., 2016).

ROS/RNS production. The respiratory burst activity of head kidney and spleen leukocytes from redbreast sunfish (*Lepomis auritus*) collected from water polluted by estrogenic EDCs was altered in comparison with individuals of this species living in uncontaminated places (Rice et al., 1996). BPA suppressed macrophage respiratory burst activity in goldfish (Yin et al., 2007). In addition, BPA, NP and different phthalates increased the production of superoxide anions in phagocytic cells from carp (Gushiken et al., 2002; Watanuki et al., 2003). The production of RNS and genes relating to ROS was altered after treatment with permethrin, atrazine or NP in zebrafish (Jin et al., 2010).

Phagocytosis. BPA and NP decreased the phagocytic activity of carp phagocytic cells (Gushiken et al., 2002).

mRNA expression of immune related genes. PCB 126 affects the expression of stress-immune genes in head kidney of rainbow trout (Quabius et al., 2005). Furthermore, the mRNA levels of different cytokines (TNF-α, IFN, IL-1β, IL-8, CXCL-Clc and CC-Chemokine) were altered after permethrin, atrazine or NP treatment in zebrafish (Jin et al., 2010). Moreover, tamoxifen inhibits the production of IL-1β after vaccination with hemocyanin plus aluminium adjuvant in gilthead seabream (Rodenas et al., 2015, 2016).

Humoral innate and adaptive immunity. NP induces the expression of different complement components in winter flounder (*Pseudopleuronectes americanus*) and zebrafish

(Baldwin et al., 2005; Ruggeri et al., 2008). PCB 126 decreases the plasma antibody number against *V. anguillarum* in Chinook salmon (*Oncorhynchus tshawytscha*) (Regala et al., 2001). Moreover, tamoxifen alters the antibody levels after vaccination in gilthead seabream (Rodenas et al., 2016).

4.1.1. 17α-ethynylestradiol

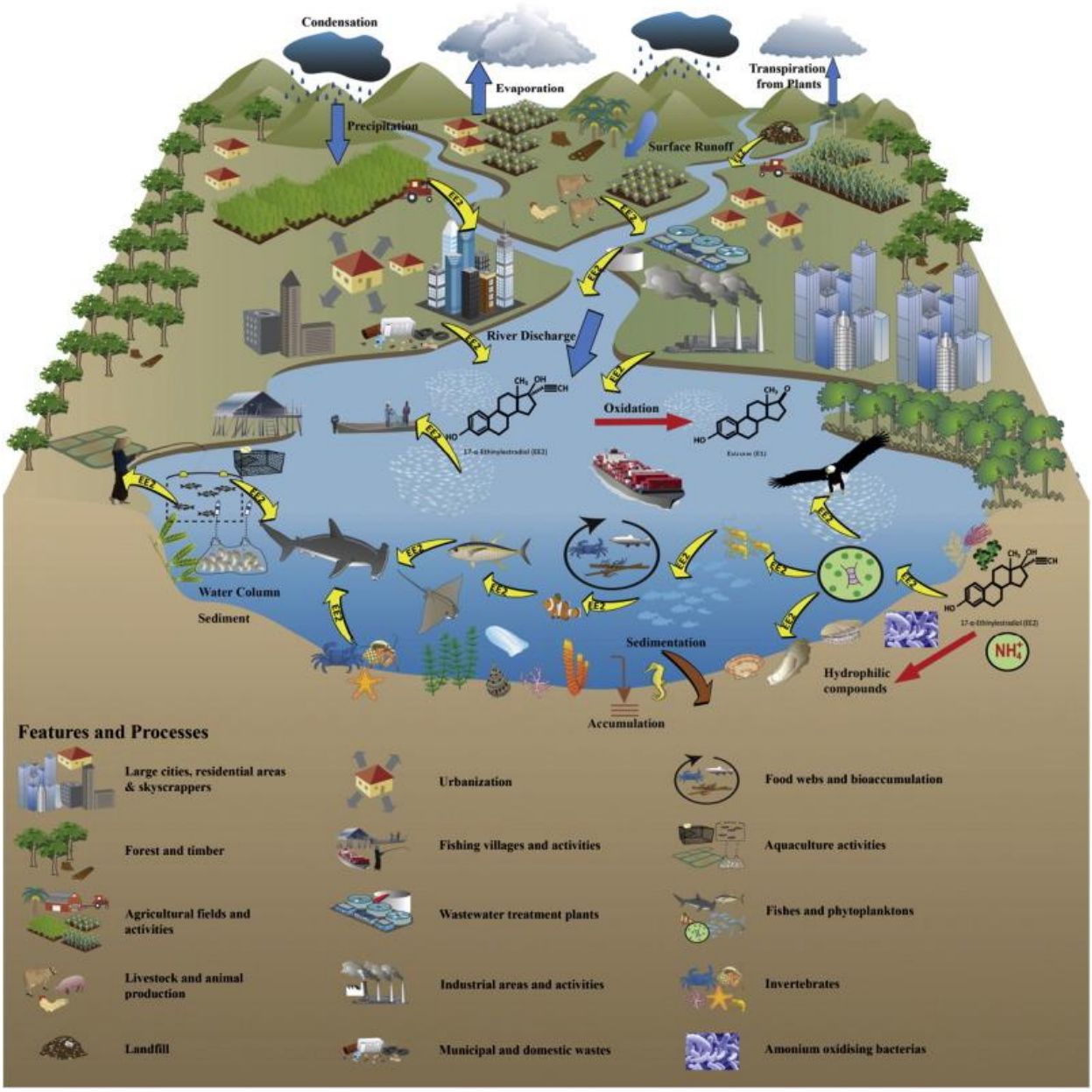
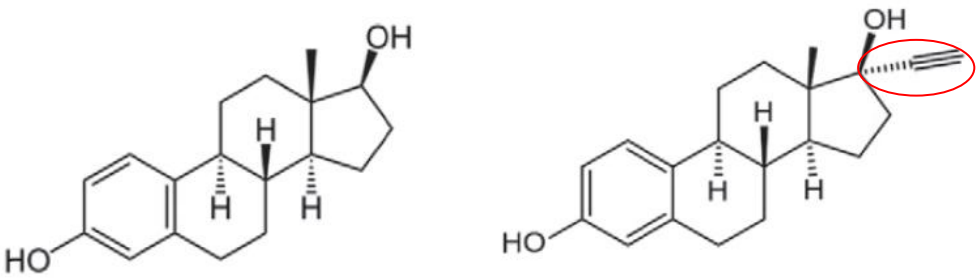


Figure 7. Sources and transport of EE_2 in the aquatic environment (Aris et al., 2014).

EE_2 is an estrogenic EDC released into the environment by human drug consumption (mainly oral contraceptive pill and replacement hormone therapies), livestock (to promote

growth, and prevent and treat reproductive disorders) and agricultural activities, especially in aquaculture (to develop single-sex populations of fish to optimize growth) (Aris et al., 2014). Although human urine is the main source of EE_2 (Pauwels et al., 2008; Vethaak et al., 2005), prior to it excretion, EE_2 is biologically inactivate before becoming a water-soluble sulphate or a glucuronide conjugate (Andrew et al., 2008; Colman et al., 2009; de Mes et al., 2005; Froehner et al., 2012; Nieto et al., 2008). However, EE_2 can be activated into its free form in waste water treatment plants through bacterial modification (Al-Ansari et al., 2010; Atkinson et al., 2012) and be released into the water ecosystems (Aris et al., 2014). EE_2 has become a widespread problem in the environment due to its tendency to absorb organic matter, accumulate in sediment and concentrate in biota (Aris et al., 2014).

EE_2 differs from E_2 because of its ethynyl-group in the 17-position (Fig. 8), making this molecule more resistant to degradation than E_2 (Aris et al., 2014). Moreover, EE_2 is one of the most potent estrogen EDCs, being even 100 times more potent that E_2 in transgenic zebrafish (Legler et al., 2002). EE_2 can also induces the expression of the gene encoding for vitellogenin in zebrafish (Kausch et al., 2008).



Estradiol **Ethynylestradiol**

Figure 8. Structure of 17β-estradiol (E_2) and 17α-ethynylestradiol (EE_2) (modified from Aris et al., 2014).

There is some evidence that EE_2 affects the fish immune system. In carp, EE_2 alters the transcription of lytic enzymes important for protection against bacteria (Moens et al., 2006). EE_2 is also associated with morphological changes of immune organs, reducing, for example, the number and size of the splenic melanomacrophage centres in tilapia (Shved et al., 2009) or the proliferation of reticuloendothelial cells in the spleen of common carp (Schwaiger et al.,

2000). Moreover, chronic exposure to NP combined with EE_2 injection resulted in a lower number of leukocyte in common carp (Schwaiger et al., 2000).

In our laboratory, some studies have determined the effect of EE_2 on the immune response of gilthead seabream in both *in vivo* and *in vitro* studies that focused on the leukocytes of the head kidney. EE_2 was considered a biomarker of endocrine disruption in gilthead seabream (Cabas et al., 2012; Rodenas et al., 2015, 2016) as in other fish (Milla et al., 2011). Interestingly, when the EE_2 treatments ceased, this increase disappeared (Rodenas et al., 2016) and most of the effects on the innate and adaptive immune responses also returned to control values (Rodenas et al., 2015, 2016). In short, and taking into consideration all the results obtained, EE_2 seems to alter the capacity of fish to appropriately respond to infection although it does not behave as an immunosuppressor (Cabas et al., 2012, 2013a; Rodenas et al., 2015, 2016). Moreover, EE_2 induces the infiltration of AGs and B cells into the gonad and increases the expression of E-selectin, an adhesion molecule correlated with a leukocyte infiltration (Cabas et al., 2011).

4.2. Effects of estrogenic EDC on mast cells

Although recent years have seen an increase in the number of scientific publications focusing on the modulatory effect of EDCs on immunity, very little is known about how these water pollutants alters MC immune responses.

In rodent models and cell lines, estrogenic EDCs induce MC degranulation and activation (Mizota and Ueda, 2006; Narita et al., 2007; O'Brien et al., 2014; Rajkovic et al., 2014; Uchida et al., 2003). In human MC lines, estrogenic EDCs promote MC degranulation and an additive effect has been described in the presence of various estrogenic EDCs (Kennedy et al., 2012; Narita et al., 2007). Furthermore, much of these effects are mediated by ERα (Narita et al., 2007). In addition, BPA changes the behaviour of rats suffering the MC-mediated disease migraine, which it exacerbates (Vermeer et al., 2014).

Nowadays, the possible effects of estrogenic EDCs on fish MC immune responses are widely unknown. There is evidence that PCB 216 induces agradual increase of MCs in the gills and the intestine of gilthead seabream (Lauriano et al., 2012). Moreover, endosulfan and atrazine can modulate neurobiological features, such as appetite and motor behaviour through the HRs present in brain cells (Giusi et al., 2010). However, to the best of our knowledge, information regarding estrogenic EDC modulation of fish MCs is not available.

OBJECTIVES

This work has the following specific objectives:

1. To develop a protocol to isolate gilthead seabream peritoneal mast cells.

2. To analyze the suitability of histamine and compound 48/80, a histamine release inducer, as efficient adjuvants for gilthead seabream intraperitoneal vaccination.

3. To obtain a monoclonal antibody specific to gilthead seabream mast cells.

4. To determine the effects of the dietary intake of EE_2 on the peritoneal exudate of gilthead seabream and to analyze if these possible effects disappear when the treatment ceases.

CHAPTER I: Isolation of mast cells from the peritoneal exudate of the teleost fish gilthead seabream (*Sparus aurata* L.)

Abstract

Inflammation is the first response of animals to infection or tissue damage. *Sparus aurata* (Perciformes) was the first fish species shown to possess histamine-containing mast cells at mucosal tissues. We report a separation protocol for obtaining highly enriched (over 95% purity) preparations of fish mast cells in high numbers (5-20 million mast cells per fish). The peritoneal exudate of *S. aurata* is composed of lymphocytes, acidophilic granulocytes, macrophages and mast cells. We separated the lymphocyte fraction through discontinuous density gradient centrifugation. The remaining cells were cultivated overnight in RPMI-1640 culture medium containing 5% fetal calf serum, which allowed macrophages to adhere to the cell culture flasks. Finally, acidophilic granulocytes were separated from the mast cells though a Magnetic-Activated Cell Separation (MACS) protocol, using a monoclonal antibody against these cells. The purity of mast cells-enriched fractions was analyzed by flow cytometry and by transmission electron microscopy. The functionality of purified mast cells was confirmed by the detection of histamine release by ELISA after stimulation with compound 48/80 and the induction of the pro-inflammatory cytokines IL-1b and IL-8 following stimulation with bacterial DNA. This fish mast cells separation protocol is a stepping stone for further studies addressing the evolution of vertebrate inflammatory mechanisms.

1. Introduction

Inflammation is the first response of animals to infection or tissue damage. Inflammation involves changes in vascular tissues, and the recruitment of immune cells to the inflammatory site. Histamine plays an essential role in the initiation of inflammation (Beer and Rocklin, 1987; Crivellato and Ribatti, 2010), but this role has only been demonstrated in specific groups of vertebrates (perciform fish, reptiles, birds, and mammals) (Crivellato and Ribatti, 2010; Mulero et al., 2007a; Reite, 1972). In these organisms histamine is primarily stored in and secreted by mast cells (Crivellato and Ribatti, 2010; Reite, 1972). Mast cells are pro-inflammatory granular leukocytes located at host-environment interface, thus being exposed firsthand to invading microorganisms and environmental stressors (Crivellato and Ribatti, 2010; Theoharides and Cochrane, 2004). Mast cells derive from mononuclear precursors undergoing final differentiation in the tissues. These cells possess unique sets of proteases and exhibit functional diversity depending on the tissue in which they differentiate, thus resulting in phenotypic heterogeneity (Crivellato and Ribatti, 2010).

Mast cells are activated by their interaction with specific microbial components, or host molecules released by tissue damage. Upon activation, mast cells release a variety of pro-inflammatory mediators, which are essential for the recruitment and activation of other leukocytes (Crivellato and Ribatti, 2010; Marshall, 2004). Among these molecules are preformed mediators (e.g. histamine, tryptase, chymase and TNF-α); and newly synthesized mediators (e.g. prostaglandins, leukotrienes, cytokines, chemokines, and growth factors). In mammals, many of these pro-inflammatory molecules are released upon mast cell activation via the Fcε receptor (Gilfillan and Tkaczyk, 2006), which remains the best studied model of mast cell activation (Beaven and Baumgartner, 1996; Tkaczyk and Gilfillan, 2001). Little information exists on the activation mechanisms of mast cells in lower vertebrates lacking membrane-bound receptors for antibodies, for example teleost fish (Hamuro et al., 2007; Stafford et al., 2006; Xu et al., 2010).

Thanks to the development of various methods for the isolation of mast cells from various tissues and their *in vitro* development (Cooper and Stanworth, 1976; Ishizaka et al., 1977; Lappalainen et al., 2007; Lawrence et al., 1987; Massey et al., 1991; Morgan et al., 2008), the molecular mechanisms involved in mammalian mast cell activation have been well characterized. Many mammalian receptors capable of activating mast cells are known (Gilfillan and Tkaczyk, 2006), as are many of the intracellular signaling mechanisms that regulate this activation (Gilfillan and Tkaczyk, 2006). In comparison, very little is known about the mechanisms regulating non-mammalian mast cells.

The lack of information on many aspects of non-mammalian mast cells biology is partly due to the lack of available protocols for the isolation of mast cells from lower vertebrates. Here we describe a protocol for obtaining mast cells from the teleost fish *Sparus aurata* peritoneal exudate with high yield, purity and viability.

2. Material and methods

2.1. Animals

Healthy specimens of the hermaphroditic protandrous marine fish gilthead sea bream (*S. aurata* L, Perciformes, Sparidae) were maintained at the Centro Oceanográfico of Murcia, Instituto Español de Oceanografía (Mazarrón, Murcia). Fish (approximately 500 g mean weight) were kept in running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/h) at 23 °C under natural photoperiod, and fed twice a day with a commercial pellet diet (Skretting, Spain) at a feeding rate of 1.5% of fish biomass. Specimens were anesthetized with 40 ppm of clove oil and injected intraperitoneally with 10 ml PBS, adjusted with 0.35% NaCl to sea bream serum osmolarity (353.33 mOs), containing heparin (1000 i.u./mL). The abdomens of the fish were massaged for 10 min to dislodge tissue-attached cells into the PBS solution. Incisions were made below of the lateral fin to access the peritoneum, and the peritoneal exudates then aspirated, collected into 15 mL Falcon tubes, and kept on ice for their transport to the University of Murcia. All experiments comply with the Guidelines of the European Union Council (86/609/EU), the Spanish RD 53/2013, and the Bioethical Committees of the University of Murcia and the IEO for the use of laboratory animals.

2.2. Ficoll gradient

The peritoneal exudates (approximately 9 mL) were passed through 70 µm cell strainers to remove large clumps of cells. After centrifugation at 400 x g for 5 min cells were resuspended in 10 mL PBS + 0.35% NaCl, and overlaid over 5 mL Ficoll-Histopaque-1077 (Sigma-Aldrich). Cells suspensions were then centrifuged at 600 x g for 10 min at 4 °C. Mononuclear cells (mostly lymphocytes) remained at the interface, and were discarded. The cell pellet was resuspended in 10 mL PBS + 0.35% NaCl and centrifuged at 400 x g for 5 min to remove all traces of Ficoll.

2.3. Cell culture

The cells recovered from the pellet, after the Ficoll gradient, were resuspended at 1 x 10^7 cells/mL in sbRPMI (RPMI-1640 culture medium + 0.35% NaCl). The medium was supplemented with 5% fetal bovine serum (FBS) and containing 100 i.u./ml penicillin and 100 µg/ml streptomycin and 1% glutamine. Cells were incubated in cell culture flasks at 25 °C, under a 5% CO_2 atmosphere, for 18 h. On the next day, non-adherent cells were recovered, washed once with PBS + 0.35% NaCl, and resuspended at 1 x 10^7 in PBS + 0.35% NaCl. When necessary, adherent cells were recovered from the culture flasks using 0.25% trypsin, washed, and resuspended in PBS + 0.35% NaCl.

2.4. Magnetic-activated cell sorting (MACS)

Acidophilic granulocytes, and few contaminating lymphocytes, were separated from the remaining cells by MACS as described before (Roca et al., 2006). Non-adherent cells obtained from the previous step were resuspended in MACS buffer (PBS + 0.35% NaCl + 5% FBS + 2mM EDTA) at 1 x 10^8 cells/mL. Twenty to fifty million cells were used for the separation of acidophilic granulocytes from each fish. Cells were incubated on ice for 20 min with a 1:10 dilution of a monoclonal antibody specific against gilthead sea bream acidophilic granulocytes (G7 mAb) (Sepulcre et al., 2002), plus a 1:100 dilution of a commercial mAb against gilthead sea bream IgM (9H8, Aquatic Diagnostics). Cells were washed with 10 mL MACS buffer, resuspended at 1 x 10^8 cells/mL in MACS buffer, and incubated with a 1/20 dilution of micromagnetic bead-conjugated anti-mouse IgG antibody (Miltenyi Biotec) for 30 min at 4 °C. Cells were washed with 10 mL MACS buffer, resuspended in 500 µL MACS buffer, and mast cells-enriched fractions were obtained by negative selection using Miltenyi Biotec LD magnetic columns, according to the manufacturer's specifications.

2.5. Flow cytometry

To analyze the purity of mast cells preparations, cells (0.5 x 10^6 cells in 100 µL MACS buffer) from MACS-positive ($G7^+$ and $9H8^+$) or MACS-negative fractions were re-stained with the G7 antibody, followed by 10 µg/mL Alexa 488-conjugated goat anti-mouse IgG. After a 20 min incubation on ice, cells were washed and fluorescence analyzed by flow cytometry. Cell preparations were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). Cell viability was also analyzed by flow cytometry, by incubating the cells (0.5 x 10^6 cells in 100 µL) with 1 µg/mL propidium iodide in PBS +

0.35% NaCl for 2 min, before flow cytometry analysis. Cells from a minimum of fifteen fish were analyzed by flow cytometry.

2.6. Transmission electron microscopy

In order to analyze the purity of the negative and positive MACS fractions, cells (1 x 10^6 per sample) were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and processed for electron microscopy analysis as described (Mulero et al., 2007a). Cells were examined using a JOEL JEM-1011 transmission electron microscope. Cells from a minimum of four fish were analyzed by electron microscopy.

2.7. Analysis of mast cells histamine content by flow cytometry

Mast cell's histamine content was analyzed by flow cytometry, by performing an intracellular anti-histamine staining. Cells (1 x 10^6 cells in 100 µL) were fixed overnight with 2% 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) in PBS + 0.35% NaCl. Cells were permeabilized by adding 100 µL of 0.2% saponin in PBS. After 30 min on ice, cells were centrifuged at 1500 x g for 3 min. After completely removing the supernatant by aspiration, cells were resuspended in 500 µL PBS + 4% FBS and incubated on ice for 1 h. After this blocking step, cells were centrifuged at 1500 x g for 3 min, and resuspended in 100 µL PBS + 4% FBS containing the anti-histamine antibody (Rabbit polyclonal; Sigma-Aldrich) at a 1:200 dilution. After a 1 h incubation on ice, cells were washed with 1 mL PBS + 4% FBS, centrifuged at 1500 x g for 3 min, and resuspended in 100 µL of PBS + 4% FBS containing 10 µg/mL Alexa Fluor 488 Goat Anti-Rabbit IgG (Life Technologies). Cells were kept on ice for 1 h, washed as above, and finally resuspended in 100 µL 1% paraformaldehyde in PBS. Cells were analyzed by flow cytometry, as described above. Cells from a minimum of nine fish were analyzed though this method.

2.8. Analysis of mast cells histamine release

Histamine release by mast cells was analyzed using a commercial competitive ELISA kit, according to the manufacturer's specifications (Cayman Chemical). Histamine was detected in the supernatant of cells (2 x 10^6 per sample in 100 µL PBS + 0.35% NaCl) stimulated with the indicated concentrations of compound 48/80 for the indicated times. After stimulation, cells were centrifuged at 2000 x g for 5 min, and the supernatant recovered to perform the ELISA assay. Experimental replicates were performed for all samples. Because histamine

release was measured using a competitive assay, data are expressed as the reciprocal of light absorbance (1/A405) ± SEM for clarity.

2.9. Analysis of gene expression

For the analysis of gene expression cells (2 x 10^6) were stimulated with 50 µg/mL *Vibrio anguillarum* DNA for 6 h, and the total RNA then isolated using TRIzol Reagent (ThermoFisher Scientific). The expression of sea bream IL-1β and IL-8, TNF-α, and CCL4 genes was analyzed by real-time PCR as described previously (Angosto et al., 2013; Cabas et al., 2012).

2.10. Statistical analysis

Depending on the experimental design, data were analyzed by independent-samples *T*-Tests, or one-way ANOVA and Tukey *post hoc* tests, using the software IBM SPSS statistics v 20.0.

3. Results

3.1. Separation of small mononuclear cells from the peritoneal exudate of *S. aurata*

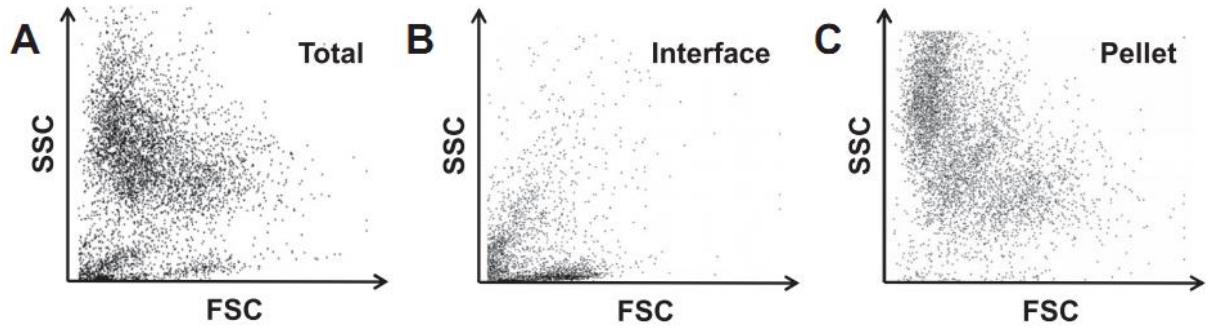


Figure 1. Separation of small mononuclear cells from *S. aurata* peritoneal exudate by density gradient centrifugation. Cells from the peritoneal exudate of *S. aurata* were analyzed by flow cytometry, plotting cell size (Forward light Scatter, FSC) *vs.* internal complexity (Side light Scatter, SSC). A) Total cell population; B) small mononuclear cells recovered from the interface of the Ficoll-Histopaque gradient, after centrifugation; C) cells recovered from the bottom of the Ficoll-Histopaque gradient, after centrifugation.

Preliminary results from our laboratory indicated that the peritoneal exudate of *S. aurata* was composed of at least three different cell types, observed by transmission electron microscopy: mast cells, granulocytes, and lymphocytes (Meseguer et al., 1993). Ficoll

gradients have been used for a various decades for the separation of lymphocytes, and small mononuclear cells from larger more granular cell types (Alexander et al., 1978; Berthold, 1981; Goldrosen et al., 1977; Hokland and Heron, 1980; Rola-Pleszczynski and Churchill, 1978). Flow cytometry analysis of the cells of the peritoneal exudate reveals a heterogeneous distribution in dot-plots (Forward light Scatter, FSC; *vs*. Side light Scatter, SSC) (Fig. 1A). The peritoneal exudate was layered onto Ficoll-Histopaque and centrifuged, which resulted in a clear separation of small non-granular cells, recovered from the interface of the gradient (Fig. 1B). The cells recovered at the bottom of the gradient appeared as a mixed population of more granular (SSC^{high}) cells of variable size (FCS), distributed in more discrete cell populations (Fig. 1C).

3.2. Separation of macrophages from the peritoneal exudate

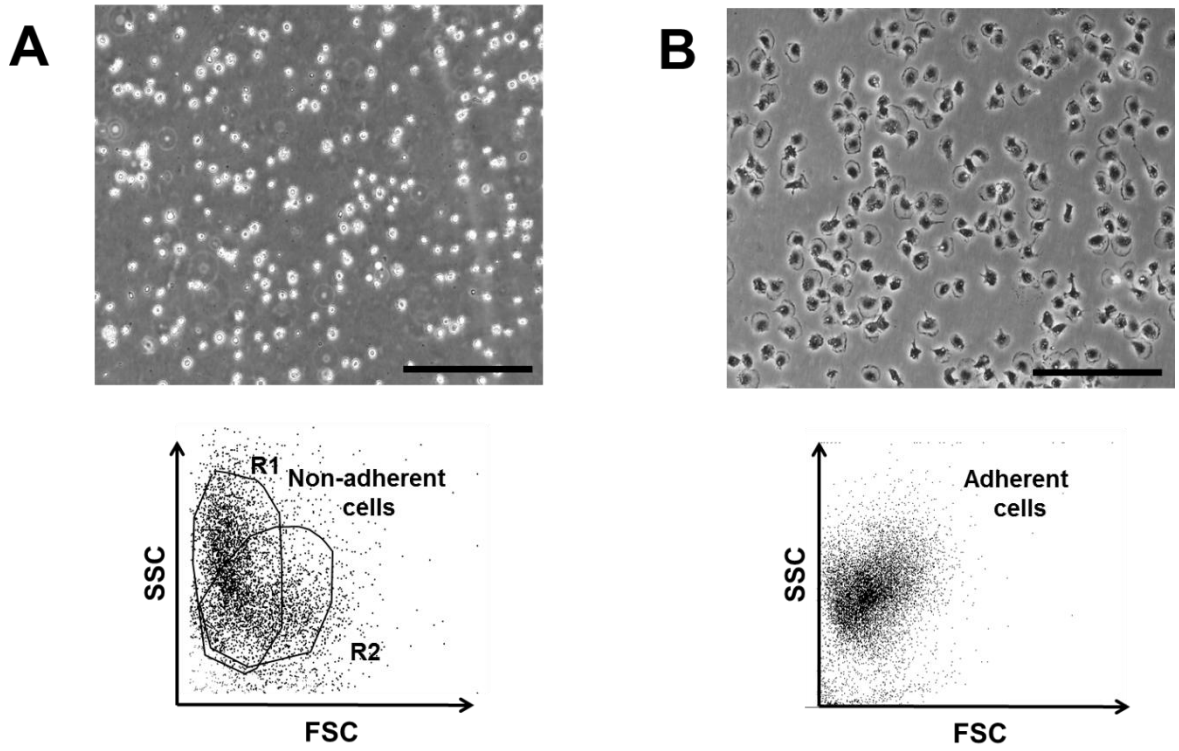


Figure 2. Macrophage separation from *S. aurata* peritoneal exudate through cell culture. Cells recovered from the bottom of the Ficoll-Histopaque gradient after centrifugation, were resuspended in RPMI-1640 culture medium containing 5% FBS. After overnight incubation, granular cells in suspension were separated from adherent cells. Top panels are phase-contrast microscopy photographs of cells in suspension (A) or adherent cells (B). Bottom panels represent the flow cytometry analysis of the same cells. Scale bar 50 µm.

A distinctive feature of macrophages is that they are highly adhesive (Verschoor et al., 2012). To separate macrophages from the remaining cells, once lymphocytes were eliminated

by density gradient centrifugation, cells were cultivated in tissue culture flasks for 18 h. This cultivation step was sufficient to allow macrophages to fully adhere to the bottom of the flask (Fig. 2), and the easy recovery of cells in suspension. Flow cytometry analysis of the cells in suspension showed the presence of two overlapping cell populations, differing in size and internal complexity (Fig. 2). Recovery of the adherent cells by trypsin treatment revealed that their distribution in SSC *vs.* FCS dot-plots was clearly different from the distribution of non-adherent cells (Fig. 2B), and also different from the distribution of freshly isolated cells (Fig. 1).

3.3. Enrichment of mast cells by negative selection

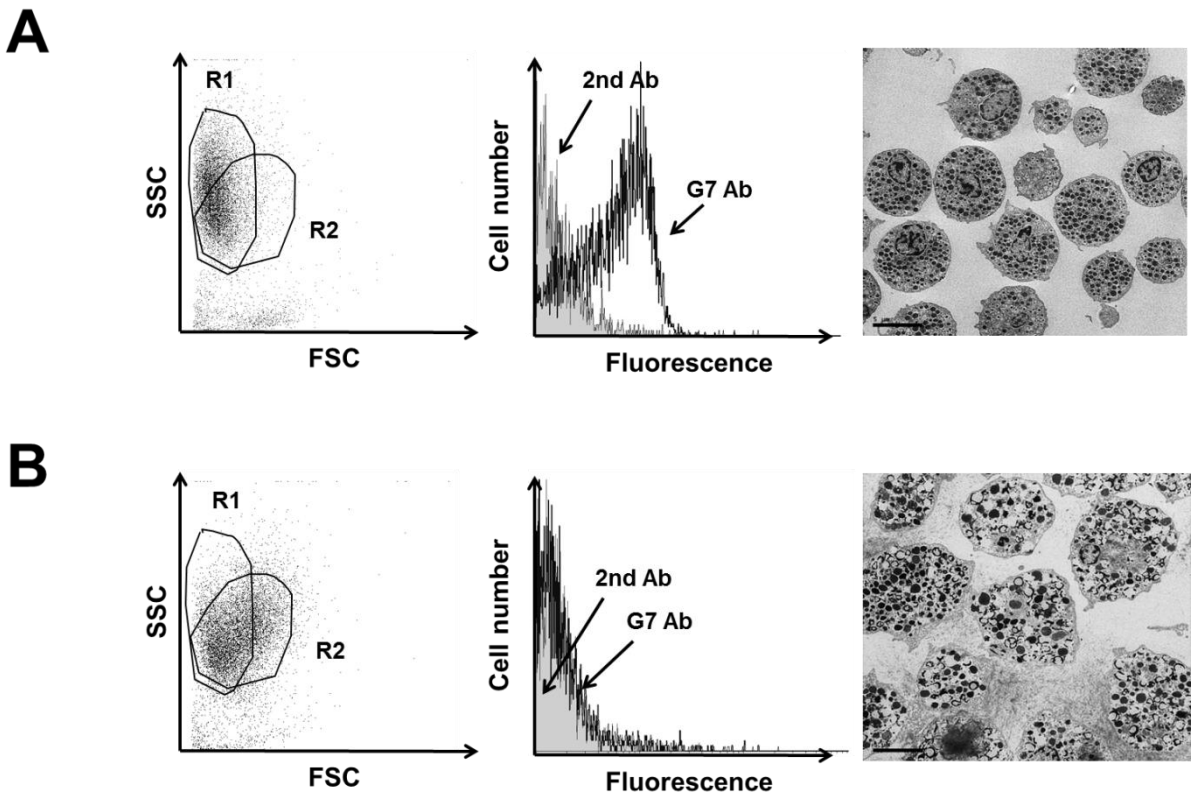


Figure 3. Isolation of *S. aurata* mast cells by negative selection using a Magnetic-activation Cell Separation protocol. Acidophilic granulocytes from *S. aurata* peritoneal exudate were labeled with the monoclonal antibody G7 (A) and magnetically separated from mast cells, which were recovered as a negative fraction (B). Purity of the negative fraction was evaluated by fluorescent re-staining using the G7 antibody (middle panels). Basal fluorescence was estimated by labeling cells with the secondary antibody alone (2nd Ab). R1 gate represents acidophilic granulocytes, while the R2 gate represents mast cells. The purity of the fractions was also evaluated by transmission electron microscopy analysis (right panels). Scale bar 5 µm.

Magnetic-activated cell sorting (MACS) technology is state-of-the-art technology, allowing the quick and simple isolation of numerous cell types with high purity, yield, and viability (Grutzkau and Radbruch, 2010). Our laboratory has developed a specific mAb against *S. aurata* acidophilic granulocytes (G7) (Sepulcre et al., 2002). These cells constitute a large fraction of the peritoneal exudate of this fish. We used this antibody to deplete acidophilic granulocytes from the cell suspensions by negative selection. In addition, we used an antibody against *S. aurata* IgM (9H8, mAb) to eliminate as much as possible the few remaining contaminating lymphocytes (B cells). Flow cytometry analysis of the positive fraction of the MACS protocol showed a homogeneous cell population, which was positive for G7 as indicated by fluorescence staining (Fig. 3A). The homogeneity of this cell preparation was also evident by examination of cell morphology by transmission electron microscopy (Fig. 3A). The negative fraction of the MACS protocol constituted a homogeneous cell population as indicated by flow cytometry analysis, which overlapped partially with G7-positive acidophilic granulocytes according to FSC and SSC parameters, but was negative for G7 (Fig. 3B). The homogeneity of this mast cells-enriched fraction was evidenced by transmission electron microscopy (Fig. 3B). Importantly, the cells present in the mast cells-enriched preparations had the morphological features reported for *S. aurata* intestinal mast cells (Mulero et al., 2007a). Cell viability was analyzed in the mast cells-enriched fraction by propidium iodide staining and flow cytometry analysis. Over 90% (91.97 ± 0.9 SEM, n=6) of the mast cell preparations were propidium iodide-negative.

3.4. Examination of mast cells histamine content

One of the key features of mammalian mast cells is their ability to regulate the earliest phase of the inflammatory process by the release of histamine (Crivellato and Ribatti, 2010). *S. aurata* is among the few fish species possessing histamine-containing mast cells (Mulero et al., 2007a). Using immuno-histochemistry, and transmission electron microscopy plus immuno-gold, we previously reported the presence of histamine in intestinal mast cells of *S. aurata* (Mulero et al., 2007a). We confirmed the presence of histamine in mast cells of the peritoneal exudate of this species, by intracellular staining using an anti-histamine antibody, followed by flow cytometry analysis (Fig. 4). Mast cells preparation from different fish showed variable levels of histamine content, but in every case histamine-positive cells represented over 90 % (91.62 ± 0.85 SEM, n=8) of cell population (Fig. 4).

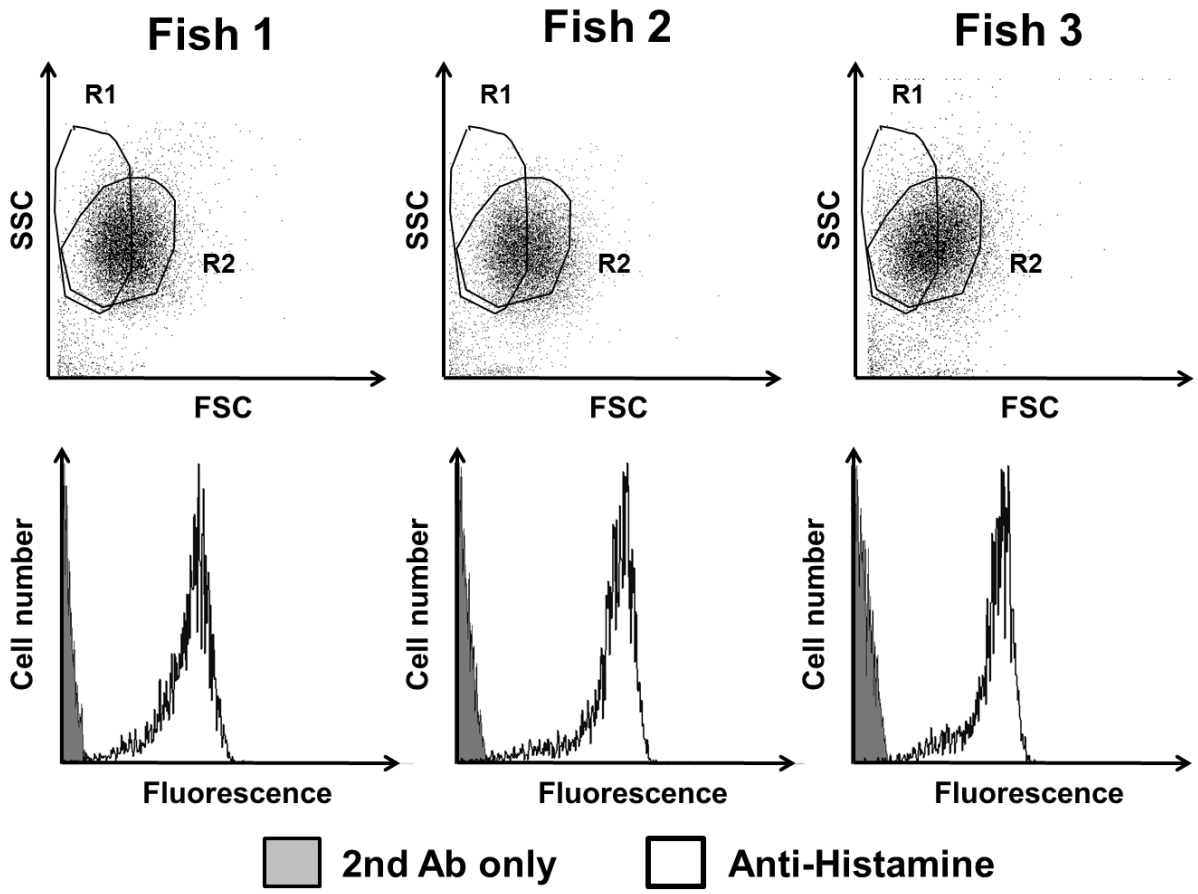


Figure 4. *S. aurata* mast cells contain histamine. *S. aurata* mast cells were stained intracellularly using an anti-Histamine antibody. Basal fluorescence was estimated by labeling cells with the secondary antibody alone (2nd Ab). R1 gate represents acidophilic granulocytes, while the R2 gate represents mast cells.

3.5. Histamine is released upon mast cells stimulation by c48/80

Compound 48/80 (c48/80) is a synthetic secretagogue, classically used to promote mast cells activation and histamine release in mammals (Conti et al., 1992; McGowen et al., 2009; Meng et al., 2013) and fish (Mulero et al., 2007a)). To determine whether mast cells-enriched fractions were functionally competent, we stimulated them with increasing concentrations of c48/80, and measured histamine release by ELISA. Fig. 5A shows that c48/80 (at 10 µg/mL) induced a significant increase in the concentration of histamine in cells supernatants. Maximal histamine release was observed 15 min after cell stimulation (Fig. 5B).

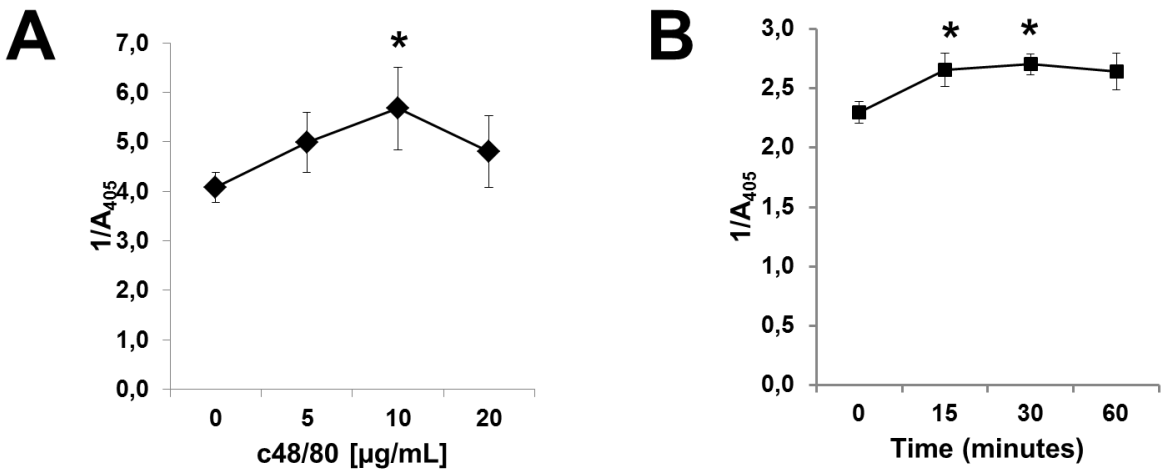


Figure 5. Compound 48/80 promotes histamine release from *S. aurata* mast cells. A) *S. aurata* mast cells were stimulated with the indicated concentrations of compound 48/80 for 15 min; B) Mast cells were stimulated with 10 µg/mL c48/80 for the indicated times. After stimulation the supernatants were collected and histamine detected using a competitive ELISA assay. Data are the mean ± SEM of two independent experiments, using 3 animals each time. Data are expressed as the reciprocal of light absorbance ($1/A_{405}$). Asterisks indicate statistically-significant differences at $p < 0.05$ according to a one-way ANOVA test.

3.6. Mast cell stimulation with V. anguillarum DNA induces the expression of genes encoding pro-inflammatory cytokines

In addition to the rapid release of pro-inflammatory molecules (e.g. histamine), mast cells regulate the inflammatory process through the production of various cytokines, whose genes are regulated at the transcriptional level (Abraham and St John, 2010; Gilfillan and Tkaczyk, 2006). In the past we have used *V. anguillarum* DNA (VaDNA) to stimulate cytokine gene expression in S. aurata acidophilic granulocytes and macrophages (Sepulcre et al., 2007). Fig. 6 shows that simulation of mast cells with VaDNA significantly up-regulated the transcript levels of S. aurata IL-1β and IL-8. There was also a small, but not significant, increase in the mRNA levels of the gene encoding of the chemokine CCL4, whereas VaDNA had no effect on the transcript levels of TNFα.

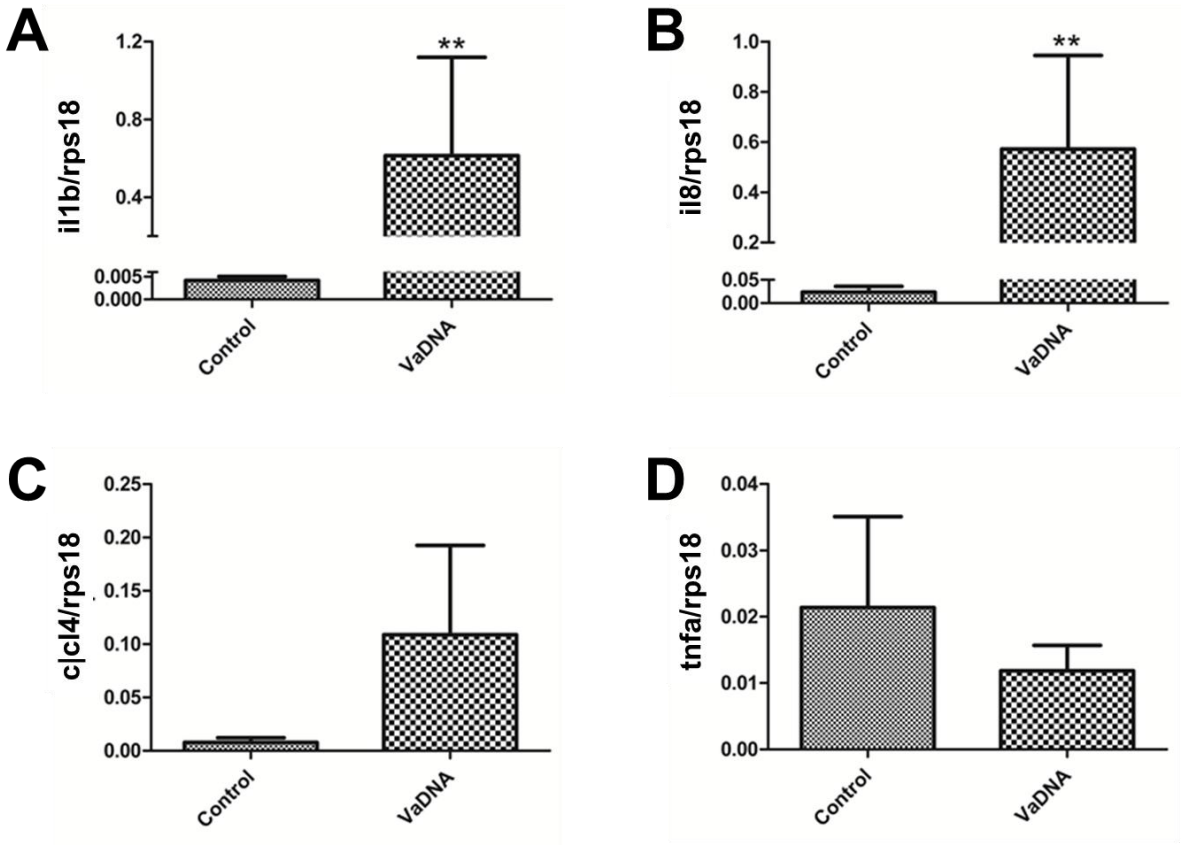


Figure 6. *S. aurata* mast cells up-regulate pro-inflammatory cytokine genes upon stimulation with bacterial DNA. *S. aurata* mast cells were stimulated with *V. anguillarum* DNA (*Va*DNA) at 50 µg/mL. Six h after stimulation cells were recovered, lysed with Trizol, and the RNA extracted for quantitative PCR analysis, using gene encoding the ribosomal protein S18 as an endogenous control. Data represent gene expression *versus* the endogenous control. A) IL-1β; B) IL-8; C) CCL4; D) TNFα. Data are mean ± SEM of four individuals. Asterisks indicate statistically-significant differences at $p < 0.05$ according to a Student *t* test.

4. Discussion

Mast cells are pro-inflammatory granular leukocytes located at host-environment interface, exposed firsthand to invading microorganisms and environmental stressors (Crivellato and Ribatti, 2010; Theoharides and Cochrane, 2004). They have an essential role in the initiation of the inflammatory process, and the subsequent modulation of the immune response (Crivellato and Ribatti, 2010; Theoharides and Cochrane, 2004). The extraordinary importance of mast cells for vertebrate immunity is underlined by the existence of multiple pathologies related to altered mast cell functions (Bischoff, 2009; Carvalho et al., 2005; Theoharides and Cochrane, 2004; Wang et al., 2003). In mammals, environmental stressors are known to produce alterations of mast cell functions, often resulting in harmful acute or chronic inflammatory processes (Bischoff, 2009; Holgate et al., 1995; Parronchi et al., 2000;

Theoharides and Cochrane, 2004). Little is known about the consequences of altered mast cell function in other vertebrates.

Here we describe a separation protocol for obtaining highly enriched (over 95% purity) preparations of fish mast cells. From a 200-400 g fish we typically obtained 5-20 million mast cells per fish. Purity of our mast cells-enriched preparations was analyzed by flow cytometry, and by transmission electron microscopy. In addition to high yield and purity, the mast cells recovered through this protocol were fully functional: the entire population of mast cells was positive for histamine; histamine was released in response to stimulation with c48/80; and bacterial DNA stimulated the expression of important pro-inflammatory cytokine genes.

Histamine has a key role in the regulation of the inflammatory process in vertebrates. Histamine modulates blood flow and vascular permeability (Eto et al., 2001; Jin et al., 2006; Schuschke and Saari, 1989; Shepherd and Duling, 1996), and also a number of cellular mechanisms of host defense (Jutel et al., 2006; O'Mahony et al., 2011). The correlation between histamine storage inside mast cells, and its ability to regulate inflammation and immunity has been firmly established for various decades (Crivellato and Ribatti, 2010; Mulero et al., 2007a; Reite, 1965, 1972; Reite and Evensen, 2006). In fish, histamine-containing mast cells have only been observed in perciform fish (the largest and most evolutionarily advanced order of teleosts), including *S. aurata* (Mulero et al., 2007a). The methodology described in this paper represents the only available protocol for the isolation of high numbers of mast cells from a non-mammalian species. We previously reported the role of exogenous histamine in the regulation of vascular permeability and leukocyte functions in *S. aurata* (Mulero et al., 2007a). This fact, together with the current observation that *S. aurata* mast cells release histamine and are capable of cytokine production, makes *S. aurata* an ideal experimental model for the study of histamine-regulated inflammatory mechanisms in lower vertebrates.

Compound 48/80 is often used to promote mast cells activation and histamine release (Conti et al., 1992; McGowen et al., 2009; Meng et al., 2013). This compound was indeed able to promote histamine release from *S. aurata* mast cells *in vivo* (Mulero et al., 2007a) and *in vitro* (this study). In mammals, along with histamine mast cells secrete proteases, eicosinoids (prostaglandins and leukotrienes), and glycosaminoglycans, among other molecules. It will be interesting to determine what other immuno-regulatory molecules are released from *S. aurata* mast cells upon stimulation with c48/80, or other pro-inflammatory agents.

We have used bacterial (*V. anguillarum*) DNA to stimulate various cell functions of *S. aurata* granulocytes and macrophages, including cytokine gene expression (Sepulcre et al., 2007; Sepulcre et al., 2011; Sepulcre et al., 2002) . Mammalian mast cells are an important source of IL-1, which is the prototypic pro-inflammatory cytokine (Ashraf et al., 1996; Kandere-Grzybowska et al., 2003; Lorentz et al., 2000; Nigrovic et al., 2007). Stimulation of *S. aurata* mast cells with *Va*DNA resulted in a significant up-regulation of this gene. Similarly, mammalian mast cells release pre-formed and newly-synthetized IL-8, which is one of the major mediators of the inflammatory response, functioning as a chemoattractant and a potent angiogenic factor (Buckley et al., 1995; Gibbs et al., 2001; Moller et al., 1993). *Va*DNA stimulation of *S. aurata* mast cells also resulted in a significant up-regulation of the IL-8 gene. Although there is abundant evidence that TNFα is produced by mammalian mast cells (Bissonnette et al., 1995; Bradding et al., 1995; Eklund et al., 1997; Gordon and Galli, 1990; Rasheed et al.; Tashiro et al., 1997; Williams and Coleman, 1995), we observed no up-regulation of this gene by *Va*DNA stimulation. Similarly, although mammalian mast cells have been reported to produce CCL4 (Macrophage inflammatory protein-1) (Sun et al., 2005; Yano et al., 1997), we observed no up-regulation of this gene upon *S. aurata* mast cell stimulation with *Va*DNA. It will be interesting to determine the full array of cytokine genes *S. aurata* mast cells are capable of inducing upon stimulation with other pro-inflammatory agents.

Many mammalian receptors capable of activating mast cells are known (Gilfillan and Tkaczyk, 2006), as are many of the signaling pathways that regulate this activation (Gilfillan and Tkaczyk, 2006). In contrast, the study of the molecular mechanisms that regulate mast cell activation in non-mammalian organisms has barely received attention. The high number of cells that can be obtained from a single fish, through the isolation described here, will help to conduct experiments to explore these issues in teleost fish. The information obtained from this analysis will shed light onto the evolution of vertebrate mast cells. The use of *S. aurata* as an experimental model to address this evolutionary question represents an important asset, due to the striking similarities between *S. aurata* and mammalian mast cells.

The protocol described here allows for the separation of *S. aurata* mast cells in high numbers and high viability. Fish mast cells isolated through this protocol are fully functional, capable of releasing histamine and increasing mRNA transcripts for pro-inflammatory cytokines upon stimulation. This fish mast cells separation protocol is a stepping stone for further studies addressing the evolution of vertebrate inflammatory mechanisms.

CHAPTER II: Histamine and mast cell activator compound 48/80 are safe but inefficient systemic adjuvants for gilthead seabream

Abstract

Histamine has a key role in the regulation of inflammatory and innate immune responses in vertebrates. Gilthead seabream (*Sparus aurata* L.), a marine hermaphrodite teleost of great commercial value, was the first fish species shown to possess histamine-containing mast cells (MCs) at mucosal tissues. MCs are highly abundant in the peritoneal exudate of gilthead seabream and compound 48/80 (Co 48/80), often used to promote MC activation and histamine release, is able to promote histamine release from gilthead seabream MCs *in vitro* and *in vivo*. The aim of the present study was to analyze the effect of histamine and Co 48/80 on the immune responses of gilthead seabream. For this purpose, histamine and Co 48/80 were intraperitoneally injected alone or combined with 10^9 heat-killed *Vibrio anguillarum* cells and their effects on head kidney and peritoneal exudate were analyzed. The results indicated that although histamine and Co 48/80 were both able to alter the percentage of peritoneal exudate and head kidney immune cell types, only Co 48/80 increased reactive oxygen species production by peritoneal leukocytes. In addition, histamine, but not Co 48/80, was able to slightly impair the humoral adaptive immune response, i.e. production of specific IgM to *V. anguillarum*. Notably, both histamine and Co 48/80 reduced the expression of the gene encoding histamine receptor H2 in peritoneal exudate leukocytes. These results show for the first time in fish that although systemic administration of histamine and Co 48/80 is safe, neither compound can be regarded as an efficient adjuvant for gilthead seabream vaccination.

1. Introduction

Inflammation, a biological process that consists of the production and release of mediators, changes in the vascular tissues and the recruitment of leukocytes to the inflammatory site, is associated with both tissue damage and infection. Histamine is one of the first biomolecules to reach the inflammation site. Histamine (2-[4-imidazole]-ethylamine) is mainly stored and released by mast cells (MCs) in mammals, reptiles, birds and perciform fish (Crivellato et al., 2015; Mulero et al., 2007a). There are two theories on the origin of histamine synthesis in MCs: (i) histamine in MCs is of ancient origin, (ii) histamine production in MCs has had multiple independent origins in different phylogenetic species.

In mammals, MCs are relatively well-known. It is thought that MC precursors leave the bone marrow and, via blood circulation, reach mucosal sites where they mature (Reite, 1996, 1998b). Several studies have described not only the role of MCs in inflammation, including the mechanisms through which the release of immune mediators, such as histamine, is induced, but also the MC role in cellular growth modulation and leukocytes differentiation (Dawicki and Marshall, 2007; Marshall, 2004). Although bone marrow and lymph nodes are lacking in fish, this vertebrate group possesses head-kidney (HK), spleen, thymus and mucosa-associated lymphoid tissue (Press and Evensen, 1999). HK is a very important hematopoietic organ (Fange, 1986) that shows morphological similarities with the bone marrow of higher vertebrates (Meseguer et al., 1995). MC precursors leave this organ and reach the intestinal mucosa, dermis and gills (also eye, hypothalamus, pancreas and corpuscles of Stannius) (Sfacteria et al., 2015) where they differentiate into mature MC, presenting a range of phenotypic heterogeneity. Moreover, preliminary studies by our group have shown an enrichment of MCs in the peritoneal exudate (PE) (Gómez-González et al., 2014), as occurs in rat (Diamant, 1990). However, knowledge of the mechanism of MC activation and the release of pro- and anti-inflammatory mediators in fish is still lacking.

The effects of histamine are mediated by four histamine receptors (HRs) in mammals (HR H1, H2, H3 and H4) and three in fish (HR H1, H2 and H3), all of which belong to the G-protein-coupled receptor family (Cofiel and Mattioli, 2006; Holstein, 1986; Peitsaro et al., 2000; Peitsaro et al., 2007; Temma et al., 1989). In mammals, the expression of these receptors have been amply studied (Akdis and Simons, 2006) but little information is available about fish HR activation.

The gilthead seabream (*Sparus aurata* L.) is a marine, protandrous hermaphrodite teleost, which has been described as the first fish species known to possess histamine in the granules of its MCs (Mulero et al., 2007a). In addition, histamine regulates the respiratory burst of gilthead seabream professional phagocytes and compound 48/80 (Co 48/80), a potent synthetic degranulation agent classically used to induce MC activation in mammals (Lagunoff et al., 1983; Paton, 1951), is able to induce the release of histamine in gills and the contraction of the intestinal smooth muscle *ex vivo*. Moreover, stimulation of seabream isolated MCs with Co 48/80 induces the release of histamine and increases the transcript levels of the genes encoding IL-1ß and IL-8 following stimulation with bacterial DNA (Gómez-González et al., 2014).

The present study aims analyzing the effect of non lethal doses of histamine and Co 48/80 on the innate and adaptive immune response of gilthead seabream. For this purpose, fish were intraperitoneally (i.p.) injected with heat-killed *Vibrio anguillarum* (hkVa) in combination with histamine or Co 48/80. In addition the presence of the three known HR was checked and the impact of the above treatments on their expression was analyzed.

2. Materials and methods

2.1. Animals and experimental design

Healthy specimens of gilthead seabream *Sparus aurata* L. (Actinopterygii, Perciformes, Sparidae) were maintained at the Centro Oceanográfico de Murcia (Instituto Español de Oceanografía, Mazarrón, Murcia, Spain), where they were kept in 2 m^3 tanks with running seawater (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/h), a suitable aeration and filtration system and natural photoperiod and temperature. Fish were fed twice a day with a commercial pellet diet (44% protein, 22% lipids; Skretting) at feeding rate of 1.5% of fish biomass.

In vivo treatments were carried out in December (2014) with mature gilthead seabream males (n = 150) with a body weight of 205 ± 31 g (Fig. 1). Briefly, specimens were injected i.p. with PBS (control fish), histamine (10 mg/kg bw; Sigma-Aldrich) or Co 48/80 (0.25 mg/kg bw; Sigma-Aldrich) alone or combination with 10^9 hkVa (strain R82, serotype 01)/specimen at day 0 (priming) and 49 days post-priming (booster). The concentrations of histamine and Co 48/80 used were not lethal for gilthead seabream according to a previous study (Mulero et al., 2007).

Fish were fasted for 24 h before sampling at 1 dpp (day post-priming) and 1 mpb (month post-booster). Specimens (n=5 fish/treatment/sampling time) were tranquilized by 8 µL/L of clove oil and, immediately, anesthetized using 40 µL/L of clove oil and weighed. Serum samples from trunk blood were obtained by centrifugation and immediately frozen and stored at -80ºC until use. After that, the fish were i.p. injected with 5 mL of sRPMI culture medium [RPMI culture medium (Sigma-Aldrich) supplemented with 2 mM glutamine (Sigma-Aldrich), 100 I.U./mL penicillin and 100 µg/ml streptomycin (Sigma-Aldrich) and adjusted with 0.35% NaCl to sea bream serum osmolarity (353.33 mOs)] and tissue-attached cells were dislodged into the sRPMI by massaging the abdomen for 10 minutes. Incisions were made below of the lateral fin to access the peritoneum and the peritoneal exudate was aspirated and collected into 15 mL Falcon tubes. Then, the specimens were decapitated and the HK were removed and kept on sRPMI or RNAlater (Sigma-Aldrich) for reactive oxygen species (ROS) production assays and gene expression analysis, respectively, as described below. The experiments described were approved by the Consejería de Agua, Agricultura y Medio Ambiente of the Región de Murcia (approval number A13160507).

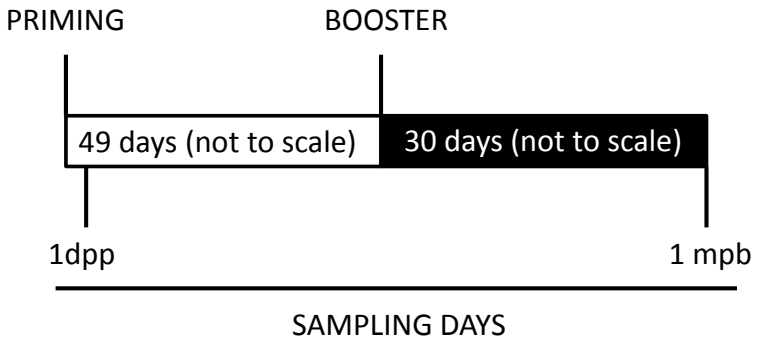


Figure 1. Experimental design and sampling schedule. Fish were injected with PBS, histamine (H) or compound 48/80 (Co) alone or in the presence of heat-killed *V. anguillarum* (hkVa) at day 0 (priming) and 49 (booster). Samples were collected 1 day post-priming (dpp) and 1 month post-booster (mpb). The six experimental groups were: C: PBS; H: histamine; Co: Co 48/80; Va: hkVa; H+Va: histamine plus hkVa; Co+Va: Co 48/80 plus hkVa.

2.2. Peritoneal exudate and head kidney cell suspensions

The PE (approximately 5 mL) was passed through 70 µL cell strainers to remove large clumps of cells. After centrifugation at 600 x *g* for 5 min, cells were resuspended in 5 mL of sPBS (PBS, Sigma-Aldrich + 0.35% NaCl). The HK cells were disaggregated using a 100 µL cell strainer and resuspended in 5 mL sPBS. After centrifugation at 600 x *g* for 5 min, cells were washed twice and resuspended in 5 mL of sPBS. The total cell number in PE was

counted using a Neubauer chamber while the number of total HK cells was analyzed by an automatic cell counter (Biorad).

2.3. Viability assay

Aliquots (0.5 x 10^6) of PE and HK cell suspensions were diluted in 200 µL of PBS containing 40 µg/mL propidium iodide (PI), which stains dead cells. The mean fluorescence intensity was analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed with the FlowJo v10.0.4 software.

2.4. Determination of acidophilic granulocytes and IgM positive cells

To analyze the percentage of acidophilic granulocytes (AGs) and IgM^+ B lymphocytes, 10^5 PE and HK cells were incubated with a monoclonal antibody (mAb) specific to gilthead seabream AGs (G7, 1:10) (Sepulcre et al., 2002) or a commercial mAb against gilthead seabream IgM (Aquatic Diagnostics, 9H8, 1:100) in 100 µL of FACS buffer [PBS supplemented with 2% fetal bovine serum (FBS) (Sigma-Aldrich) and 0.05% sodium azide (Sigma-Aldrich, S8032)], respectively. After 30 min at 4°C, cells were centrifuged at 600 x *g* for 5 min, and the supernatant was removed by aspiration before washing the cells twice with FACS buffer. Cells were stained for 30 min at 4°C with Alexa Fluor 488 F(ab')2 fragment of goat anti-mouse IgG (H+L) (1:500, ThermoFisher Scientific), washed twice and the fluorescence intensity was measured using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed with FlowJo v10.0.4. Appropriate isotype control antibodies were used, as previously described (Sepulcre et al., 2002).

2.5. Determination of mast cells

The percentage of MCs in the PE was analyzed by transmission electron microscopy, according to their ultrastructural features described by Mulero et al. (2007). For this, PE cells (5 x 10^6) were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and processed for transmission electron microscopy as described (Mulero et al., 2007a). Cells from 5 fish were examined using a JOEL JEM-1011 transmission electron microscope. At least, 20 pictures in random locations of the samples were taken and the percentage of MCs was determined.

2.6. ROS production assay

ROS production was measured as the dihydrorhodamine 1,2,3 (DHR 123)-dependent fluorescence produced by 0.5 x 10^6 PE or HK cell suspensions. The ROS activity was determined by incubating the cells with 10 µM DHR 123 (Sigma-Aldrich, D1054) in Hanks' Balanced Salt Solution (HBSS) (Sigma-Aldrich) for 5 min and 1 µg/mL phorbol myristate acetate (PMA) (Sigma-Aldrich) for 30 min at 21°C. Two technical replicates were used for all biological samples. Cells were analyzed using a FACSCalibur flow cytometer and data were analyzed with the FlowJo v10.0.4 software.

2.7. Analysis of gene expression

Gene	Accession number	Name	Sequence (5'-3')	Used
actb	X89920	F3	ATCGTGGGGCGCCCCAGGCACC	RT-PCR
		R3	CTCCTTAATGTCACGCACGATTTC	
rps18	AM490061	F1	AGGGTGTTGGCAGACGTTAC	RT-qPCR
		R1	CTTCTGCCTGTTGAGGAACC	
il1b	AJ277166	F2	GGGTCTGAACAACAGCACTCTC	RT-qPCR
		R3	TTAACACTCTCCACCCTCCA	
il10	JX976621	F1	TGCTTCGTAGAAGTCTCGGATGT	RT-qPCR
		R1	TGGAGGGCTTTCCTGTCAGA	
il4/il13	AM970029	F1	AAGAGGACTCCGGAGGTTGA	RT-qPCR
		R1	TGAGGCTCGGTGGAAGAGTA	
hrh1	LN875558	F3	CATGCCTCTGAACCTGGTGT	RT-qPCR
		R3	AAATTGAGGCTGTGCTTGCC	
hrh2	KP728255	F1	CCTAACACGCTTCACTCCGT	RT-qPCR
		R1	AGCTGCAGTTTTCTGTGGGA	
hrh3	KP728256	F1	CTGTTTCAGCACACGGCTTC	RT-qPCR
		R1	GGCACACACGTACCACTACA	

Table 1. Gene accession numbers and primer sequences used for gene expression analysis. The gene symbols followed the Zebra Fish Nomenclature Guidelines (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines) (F and R are forward and reverse, respectively).

HK samples were defrosted and RNAlater was removed. Total RNA was extracted from PE cell suspensions and HK tissues with TRIzol Reagent (ThermoFisher Scientific) following

the manufacturer's instructions and quantified with a spectrophotometer (NanoDrop, ND-1000). The RNA was treated with DNase I (amplification grade, 1 unit/µg RNA, ThermoFisher Scientific) to remove genomic DNA traces that might interfere with the PCR reactions, and SuperScript III RNase H- Reverse Transcriptase (ThermoFisher Scientific) was used to synthesize first strand cDNA with oligo $(dT)_{18}$ from 1 µg of total RNA, at 50°C for 50 min. The ß-actin (*actb*) gene was analyzed by PCR with and an Eppendorf Mastercycle Gradient Instrument (Eppendorf). The reaction mixture was incubated for 3 min at 95°C, followed by 35 cycles of 45 s at 95°C, 45 s at the specific annealing temperature and 1 min at 72°C, and finally at 72°C for 10 min. Real-time PCR was performed with an ABI PRISM 7500 (ThermoFisher Scientific) using SYBR Green PCR Core Reagents (ThermoFisher Scientific). Reaction mixtures were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and finally 15 s at 95°C. For each mRNA, gene expression was corrected by the ribosomal protein S18 gene (*rps18*) content in each sample. The gilthead seabream specific primers used are shown in Table 1. In all cases, each PCR was performed with three technical replicates. Less than 3% variation in the *rps18* gene expression was observed among samples.

2.8. Determination of IgM specific titer

The *V. anguillarum* specific IgM titer was determined 1 mpb by ELISA in serum of hkVa-vaccinated groups. Ninety-six-well ELISA plates were pre-coated with carbonate/bicarbonate solution and left for 45 min and then coated again and left for 16 h with 10^5 hkVa/well. The following day, the plates were washed three times with low salt buffer (2.42 g TRIS, 22.22 g NaCl and 2.5 ml Tween 20 dissolved in 1 l of distilled water, pH 7.3). Unspecific bindings were blocked by adding 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS to the plate and shaking for 2 h. The plates were washed once with low salt buffer before adding doubling-dilutions of serum and shaking for 2 h. The plates were then washed three times with high salt buffer (2.42 g TRIS, 29.22 g NaCl and 5 ml Tween 20 dissolved in 1 l of distilled water, pH 7.7) followed by incubating with an mAb specific to gilthead seabream IgM (F03, Aquatic Diagnostics Ltd). The plates were rinsed three times with high salt buffer, incubated for 1 h with an anti-mouse IgG (whole molecule)-peroxidase antibody produced in goat (Sigma-Aldrich) and washed again three times with high salt buffer. Finally, the chromogen tetramethylbenzidine (TMB) (Sigma-Aldrich) was added, the reaction was stopped with 2M H_2SO_4 and the absorbance was read at 450 nm using a FLUOstart luminometer (BGM, Lab Technologies).

2.9. Statistical analysis

Data were analyzed by independent-samples T-Tests (control group compared with hkVa group) or by one-way ANOVA and Tukey *post hoc* tests (comparisons among control, histamine and Co 48/80 groups and among the hkVa-treated groups), using the software GraphPad Prism 5.

3. Results

3.1. Cell viability is not modified by histamine or compound 48/80 used alone or combined with hkVa

None of the treatments used in this study affected PE or HK cell viability compared with the control specimens (Fig. 2).

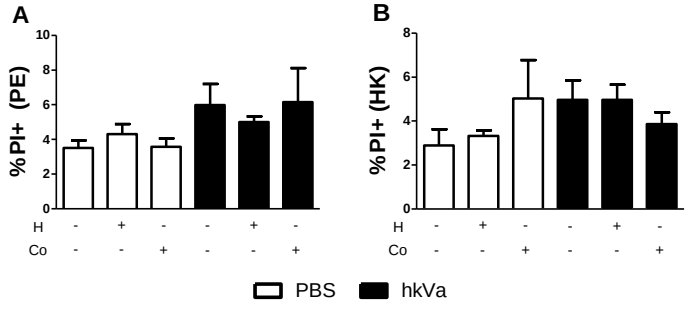


Figure 2. Histamine and Co 48/80 did not affect cell viability. Specimens were injected i.p. with PBS (control), histamine (H) or Co 48/80 (Co) alone (open bars) or combined with hkVa (solid bars). PE (A) and HK (B) non-viable cells were assayed at 1 dpp by flow cytometry as the percentage of PI^+ cells. Data are shown as the mean ± SEM of 5 individuals. Statistical analysis was performed among groups according to one-way ANOVA and Tukey post-hoc test.

3.2. Histamine and Co 48/80 alter the percentage of acidophilic granulocytes and mast cells and the *ight* transcript levels of PE and HK

As expected (Chaves-Pozo et al., 2005), hkVa injection resulted in increased total PE cells at 1 dpp (Fig. 3A). However, the presence of histamine decreased the percentage of PE AGs compared with the control group, while the combination of histamine plus hkVa increased this percentage compared with the hkVa group (Fig. 3B). As regards the HK, hkVa-treated fish had a lower percentage of AGs compared with the control group, while the combination of Co 48/80 and hkVa decreased the percentage compared with the hkVa group (Fig. 3C). Of

particular note was the fact that the percentage of MCs in PE was significantly lower in hkVa-immunized groups than in the PBS-injected groups (Fig. 3D). However, no changes were observed in the percentage of IgM^+ B lymphocytes in the PE (Fig. 3E) and HK (Fig. 3F) for any of the treatments used in this study. Finally, *ight* transcript levels decreased in the PE of immunized fish (Fig. 3G), while Co 48/80 slightly decreased them in the HK of immunized fish (Fig. 3H).

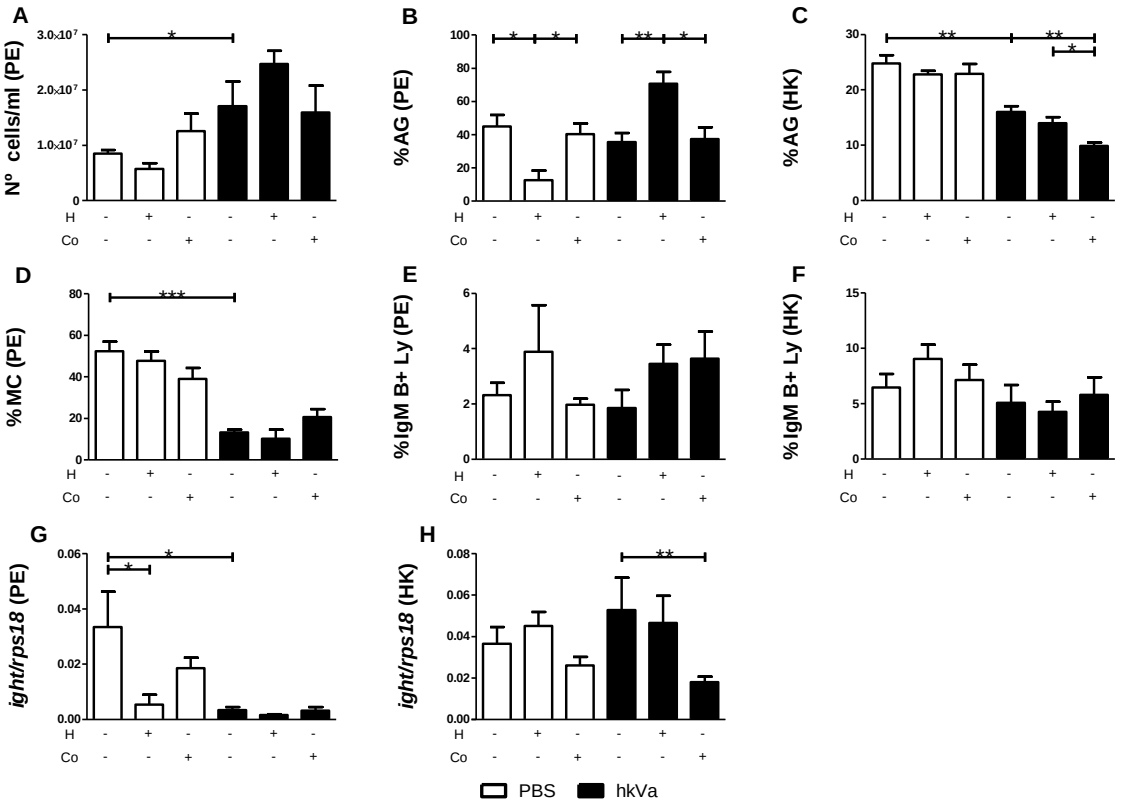


Figure 3. Histamine and Co 48/80 modulate the percentages of AGs and MCs and the transcript levels of *ight* in PE and HK. (A) Total PE cells in fish i.p. injected with PBS (control), histamine (H) or Co 48/80 (Co) alone (open bars) or combined with hkVa (solid bars) 1 dpp. (B- H) The percentages of AGs [$G7^+$ cells (B) and FSC^{high}/SSC^{high} (C)], MCs (D) and IgM^+ B lymphocytes ($9H8^+$) (E and F), and the mRNA expression of *ight* (G and H) were analyzed in PE (B, D, E and G) and HK (C, F, H) cell suspensions at 1 dpp. Data are mean ± SEM of 5 individuals. The asterisks denote statistically significant differences among groups according to a Student *t*-test (control vs. hkVa) or one-way ANOVA and Tukey post-hoc test (control vs. H and Co 48/80; hkVa vs. H plus hkVa and Co 48/80 plus hkVa). *$p < 0.05$, **$p<0.01$ and ***$p<0.001$.

3.3. Co 48/80 and exogenous histamine alters the innate immune system

As expected from previous studies (Chaves-Pozo et al., 2005a; Chaves-Pozo et al., 2004), hkVa injection significantly increased ROS production (Fig. 4A and C), the percentage of

ROS-producing cells (Fig. 4B, D), and the transcript levels of *il1b* (Fig. 5A and C) and *il10* (Fig. 5B and D) expression in PE and HK, respectively, at 1 dpp.

Moreover, compound 48/80 but not histamine increased ROS production (Fig. 4) and the percentage of ROS positive cells (Fig. 4B) in PE from 1 dpi specimens compared with the control group, while neither histamine nor compound 48/80 had any effect on the same parameters at this time in HK (Fig 4A-D).

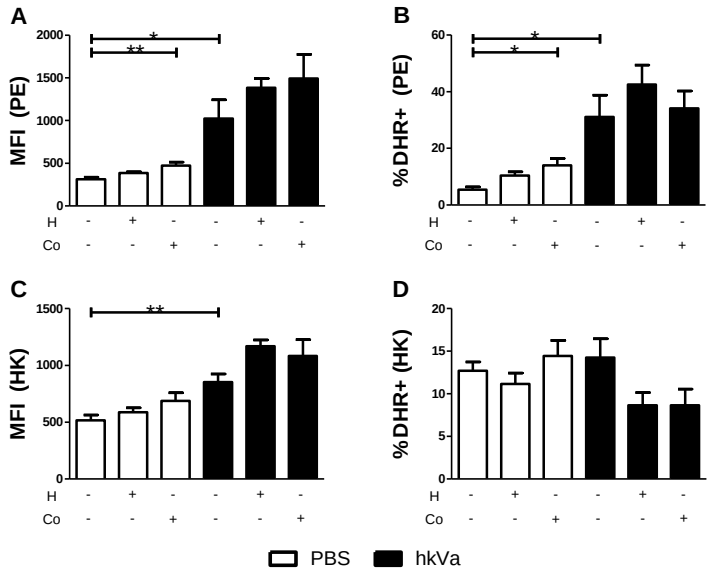


Figure 4. Compound 48/80 increases the ROS production and ROS-producing cells in PE. PE (A and B) and HK (C and D) cells collected at 1 dpp from fish i.p. injected with PBS (control), histamine (H) or Co 48/80 (Co) alone (open bars) or in combination with hkVa (solid bars) were labeled with DHR 123 and stimulated for 30 min. The mean fluorescent intensity (MFI) (A and C) and the percentage of DHR+ cells (B and D) were then analyzed by flow cytometry. Data are mean ± SEM of 5 individuals. The asterisks denote statistically significant differences among groups according to a Student *t*-test (control vs. hkVa) or one-way ANOVA and Tukey post-hoc test (control vs. H and Co 48/80; hkVa vs. H plus hkVa and Co 48/80 plus hkVa). *$p < 0.05$ and **$p<0.01$. (DHR+: positive DHR 1, 2, 3 cells).

hkVa injection significantly increased the transcript levels of *il1b* (Fig. 5A and D) and *il10* (Fig. 5B and E) expression in PE and HK, respectively, at 1 dpp. Although neither histamine nor Co 48/80 promoted any alteration in the mRNA levels of *il1b*, *il10* and *il4/il13* in the PE and HK of hkVa-injected fish, both compounds decreased the transcript levels of *il4/il13* in the PE of control animals (Fig. 5C).

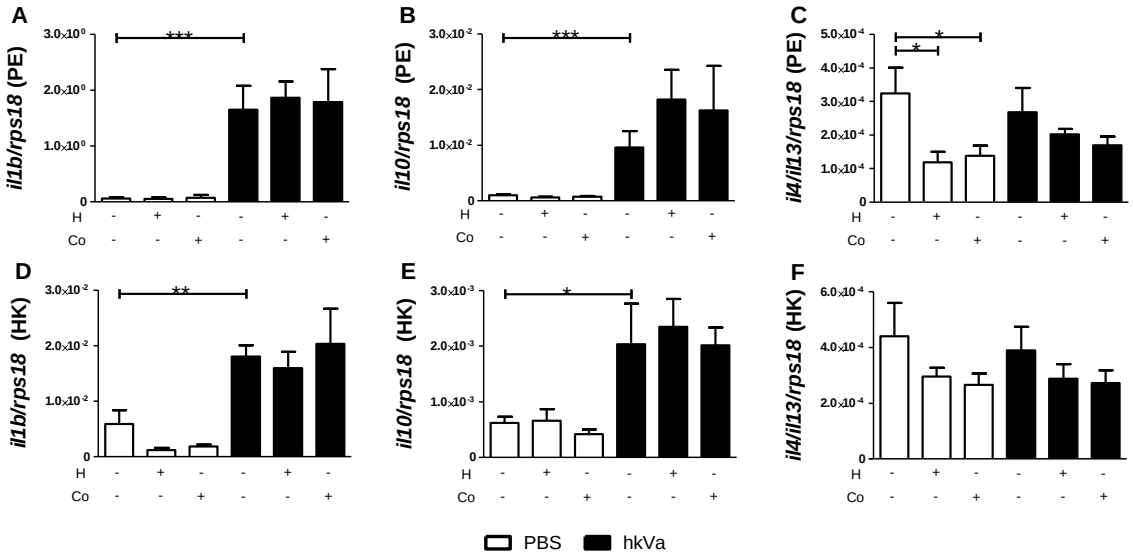


Figure 5. hkVa injection increases the transcript levels of *il1b*, *il10* in PE and HK while histamine and compound 48/80 diminish the mRNA expression of *il4/il13* in PE. The transcript levels of *il1b* (A and D), *il10* (B and E) and *il4/il13* (C and F) were determined by RT-qPCR in PE (A-C) and HK (D-F) cells collected at 1dpp from fish i.p. injected with PBS (control), histamine (H) or Co 48/80 (Co) alone (open bars) or in combination with hkVa (solid bars). The *rps18* was used as an endogenous control. Data represent gene expression *vs.* the endogenous control shown as the mean ± SEM of 5 individuals. The asterisks denote statistically significant differences among groups according to Student *t*-test (control vs. hkVa) or one-way ANOVA and Tukey post-hoc test (control vs. H and Co 48/80; hkVa vs. H plus hkVa and Co 48/80 plus hkVa). *p < 0.05, **p<0.01 and ***p <0.001.

3.4. Histamine, but not Co 48/80, alters the humoral adaptive immune response

All groups of fish immunized with hkVa showed increased specific IgM titers at 1 mpb (Fig. 6). Although Co 48/80 was found to have no effect on this response, histamine slightly, but significantly, impaired the production of specific IgM (Fig. 6A). However, neither histamine nor Co 48/80 altered the specific IgM titers at 3 mpb (data not shown).

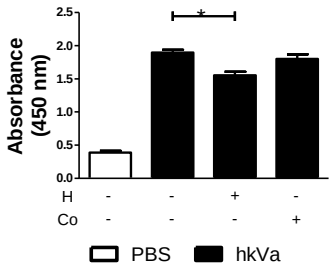


Figure 6. Histamine modulates adaptive immunity. The titers of specific IgM to Va were determined by ELISA in serum collected at 1 mpb from fish i.p. injected with PBS (control), histamine (H) or Co 48/80 (Co) alone (open bar) or in combination with hkVa (solid bars). Data are mean ± SEM of 5 individuals. The asterisks denote statistically significant differences among groups according to one-way ANOVA and Tukey post-hoc test (control vs. H and Co 48/80; hkVa vs. H plus hkVa and Co 48/80 plus hkVa). *p < 0.05.

3.5. Histamine, Co 48/80 and hkVa all inhibit hrh2 expression in PE cells

PE and HK leukocytes constitutively expressed *hrh1*, *hrh2* and *hrh3* (Fig. 7). Histamine, Co 48/80 and hkVa treatments all decreased the transcript levels of *hrh2* in PE cells at 1 dpp (Fig. 7B) but not those of *hrh1* or *hrh3* (Fig. 7A and C). However, no changes in the mRNA levels of *hrh1*, *hrh2* and *hrh3* were found in HK leukocytes (Fig. 7D, E and F).

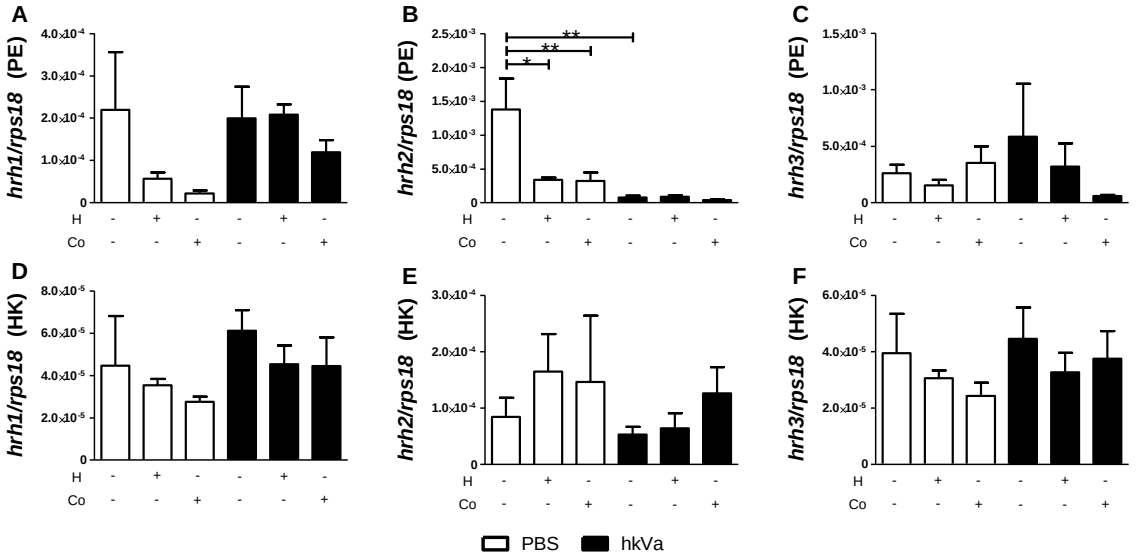


Figure 7. Histamine, Co 48/80 and hkVa inhibit *hrh2* expression in PE leukocytes. The mRNA levels of *hrh1* (A and D), *hrh2* (B and E) and *hrh3* (C and F) were determined by RT-qPCR in PE (A-C) and HK (D-F) leukocytes obtained at 1 dpp from fish i.p. injected with PBS (control), histamine (H) or Co 48/80 (Co) alone (open bars) or in combination with hkVa (solid bars). The *rps18* was used as an endogenous control. Data represent gene expression *vs.* the endogenous control and are shown as the mean ± SEM of 5 individuals. The asterisks denote statistically significant differences among groups according to Student t-test (control vs. hkVa) or one-way ANOVA and Tukey post-hoc test (control vs. H and Co 38/80; hkVa vs. h plus hkVa and Co48/80 plus hkVa). *p < 0.05 and **p<0.01.

4. Discussion

The role of histamine in the immune system of fish and the mechanisms responsible for histamine release from MCs are largely unknown. Previous studies demonstrated that the most evolutionarily advanced fish, the Order Perciformes, were able to produce and store

histamine in the granules of MCs (Gómez-González et al., 2014; Mulero et al., 2007a). In addition, histamine is used as inflammation mediator in gilthead seabream, since it induces fish death when injected i.p., promotes contraction of the intestinal smooth muscle *ex vivo* and regulates the respiratory burst of AGs (Mulero et al., 2007a). Notably, compound 48/80, which has been successfully used to promote MC degranulation in phylogenetically distant fish species (Manera et al., 2014; Manera et al., 2011; Vallejo and Ellis, 1989), induces histamine release from intestinal, gill and peritoneal MCs of gilthead seabream both *in vivo* and *in vitro* (Gómez-González et al., 2014; Mulero et al., 2007a).

In the current study, we used hkVa as priming/immunization model in gilthead seabream to further investigate the role of exogenous and endogenous histamine in the immune response of perciform fish. To achieve this, non lethal doses of histamine and Co 48/80 alone or combined with hkVa were used. As expected, hkVa immunization induced the recruitment of leukocytes to the peritoneal cavity. Surprisingly, however, hkVa injection resulted in a pronounced decrease of MCs in the peritoneal cavity, whereas the percentages of AGs, which are functionally equivalent to mammalian neutrophils (Sepulcre et al., 2002) and of IgM^+ B lymphocytes were unaltered. The increased number of PE cells coincided with the depletion of AGs in the HK, suggesting that these cells are mobilized from the HK (the hematopoietic compartment) to the inflammatory site, as previously reported in fish injected i.p. with live Va (Chaves-Pozo et al., 2005a). Furthermore, hkVa injection increased ROS production by PE leukocytes, induced IL-1β and IL-10 expression in both PE and HK leukocytes, and led to the high production of serum IgM antibodies specific to *V. anguillarum*, indicating that hkVa promotes both local and systemic immune responses.

The injection of histamine modulated the innate and adaptive immune responses to hkVa. Furthermore, the injection of histamine alone decreased the number of AGs in the PE, but, when combined with hkVa, it increased the number. These results suggest that exogenous histamine increases the migration of AGs from the HK to the peritoneal cavity in response to hkVa. However, exogenous histamine failed to further enhance the innate immune response to hkVa, i.e. ROS production and cytokine gene expression. As AGs are the main source of ROS and IL-1β in gilthead seabream (Mulero et al., 2008a; Sepulcre et al., 2007) and these cells increased in the PE of the fish treated with histamine and hkVa, histamine seems to inhibit the production of ROS and IL-1β in response to hkVa.. These results may also explain the slight impairment of adaptive immune responses, i.e. IgM production to hkVa, observed in fish injected with histamine and hkVa. This effect of histamine agrees with previous studies in

rabbits, in which histamine reduced IgM titers specific to sheep red blood cells (Tripathi et al., 2010). In sharp contrast, however, it was found that histamine enhances the *in vitro* proliferative responses of anti-IgM-stimulated B cells via HRH1 signaling (Banu and Watanabe, 1999), which induces IgM secretion in the human B lymphoma cell line BMNH but not in the lymphoblastoid B cell line CESS or human peripheral monocytes (Falus, 1993). Histamine was also found to selectively enhance IgE and IgG4, but not IgG1, IgG2, IgG3, IgM, IgA1 or IgA2, production induced by anti-CD58 monoclonal antibody in primary human B cells (Kimata et al., 1996).

Since we previously demonstrated that Co 48/80 is also able to induce MC activation and histamine release in gilthead seabream (Gómez-González et al., 2014; Mulero et al., 2007a), while the same compound has been shown to act as a powerful vaccine adjuvant in mammals (McLachlan et al., 2008), we evaluated the impact of Co 48/80 in hkVa-immunized fish. Although Co 48/80 reduced the number of AGs in the HK, a concomitant increase of the same was not observed in the peritoneal cavity. This result suggest that endogenous histamine release promotes AG mobilization from the HK to other organs, as has been demonstrated in mice, in which the subcutaneous or nasal administration of small-molecule MC activators, including Co 48/80, with vaccine antigens increased dendritic cell and lymphocyte recruitment to draining lymph nodes (McLachlan et al., 2008). This result deserves further investigation.

In contrast to exogenous histamine, Co 48/80 treatment increased ROS production by PE leukocytes, while the percentage of AGs remained unaffected, indicating that the release of endogenous histamine from MCs increases ROS production of AGs, as has been shown *in vitro* with the Hrh2 agonist dimaprit (Mulero et al., 2007a). Unexpectedly, however, Co 48/80 failed to enhance the expression of genes encoding major cytokines and the humoral adaptive immune response to hkVa, unlike exogenous histamine did.

Another interesting finding of this study is the identification of histamine receptors in leukocytes and their corresponding expression profile. We previously demonstrated that gilthead seabream HK leukocytes express *hrh1* (García-García et al., 2016). In this study, we extend these observations by demonstrating that gilthead seabream PE and HK leukocytes express *hrh1*, *hrh2* and *hrh3*. More interestingly, the injection of histamine, Co 48/80 or hkVa resulted in the drastically reduced expression of *hrh2* in PE leukocytes, while that of *hrh1* and *hrh3* was unaffected. These results suggest that immune challenge by both exogenous and endogenous MC-derived histamine would potentiate the inhibitory effects of histamine on PE

leukocytes by impairing Hrh2 signaling (Mulero et al., 2007a). Further studies are required to better understand the role of the three histamine receptors in the immune response of fish.

To summarize, this is the first report showing that exogenous and endogenous MC-derived histamine is able to modulate *in vivo* the immune response of fish. Unfortunately, although systemic administration of histamine and Co 48/80 is safe, neither compound can be regarded as an efficient adjuvant for seabream vaccination.

CHAPTER III: Generation of a mAb, GB10, against a cell surface receptor of fish mast cell that induces oncosis

Abstract

Mast cells (MCs) are pro-inflammatory granular leukocytes located at the host-environment interface. The lack of information on many aspects of non-mammalian MC biology is partly due to the scarcity of tools available for the labeling and isolation of MCs from lower vertebrates. In this study, we report the production and characterization of a monoclonal antibody (mAb), GB10, which specifically recognizes gilthead seabream MCs, as assayed by immunofluorescence and flow cytometry. GB10 reacted with 20-30% of peritoneal exudate leukocytes. By propidium iodide staining, this antibody was shown to rapidly induce the death of gilthead seabream MCs, independently of temperature. Flow cytometry and transmission electron microscopy analysis indicated that the MC death in the peritoneal exudates of gilthead seabream occurred via oncosis. *In vivo* assays, in which GB10 will be intraperitoneally injected in seabream, will help to understand the evolutionary aspects of both MCs and histamine. Moreover, a second mAb, BG4, wich specifically recognizes MCs has been obtained.

1. Introduction

Mast cells (MCs) are a well-studied type of leukocyte known to participate in innate host defense in mammals, where they take part in inflammation and are one of the first cellular mediators of this biological process. Furthermore, in recent years, studies have demonstrated that MCs also regulate acquired immunity (Da'as et al., 2011; Galli et al., 2005b; Krystel-Whittemore et al., 2015). In general, MCs are found in mucosal tissues, near the surface, in contact with potential enemies from the environment. This strategic localization is needed for their main role as the first fighters against exogenous pathogens. Different types of MCs have been identified depending on the tissue in which they differentiate and the functions they play (Crivellato et al., 2015). MCs are present in species very distant in evolution, including all classes of vertebrate species. Moreover, mast-like cells have been observed in some invertebrate species (Baccari et al., 2011; Crivellato et al., 2015). However, the number of studies that focus on the functions of MCs is much lower.

During years, mammalian MCs were thought to be the only MCs able to produce, store and release histamine. Nevertheless, we now know that some non-mammalian species can also present this function. In fact, vertebrate classes, including reptiles, amphibians, birds, and fishes (Baccari et al., 2000; Baccari et al., 1998; Chiu and Lagunoff, 1971; Mulero et al., 2007a; Vitiello et al., 1997), and invertebrate classes, such as ascidians (de Barros et al., 2007), have been found to possess histamine in MCs. In mammals, histamine is mainly release by MCs as one of the first pro-inflammatory mediators and its role in immunity has been widely studied. Nevertheless, the role of histamine in non-mammalian species is very much unknown.

Nowadays, aquaculture is the main source of fish for human consumption. As in others farming activities, the increasing demand for food involves mass production processes, which, in the case of fish, may be associated with epidemics and pathogenic diseases. For this reason, the increasing demand for healthy and affordable farmed fish has led to new research in fish immunology. The fish species gilthead seabream, *Sparus aurata* L., is a protandric hermaphrodite marine teleost of great commercial value in Mediterranean aquaculture. It was also the first fish species known to possess histamine in its MCs (Mulero et al., 2007a). Gilthead seabream MCs containing histamine have been detected in intestine and peritoneal exudate by using a commercial antibody against histamine (Gómez-González et al., 2014; Mulero et al., 2007a). However, no antibodies able to label piscine MCs exist. Although some findings on the role of MCs and histamine are available for fish (Da'as et al., 2011; Dobson et

al., 2008; Gómez-González et al., 2014; Manera et al., 2011; Matsuyama and Iida, 1999; Mulero et al., 2007a), the lack of tools to study them, such as antibodies against MCs, is delaying our complete understanding the role of these cells in fish immunity.

In this context, we previously developed in our laboratory a protocol to purify MCs from peritoneal exudates of gilthead seabream, which allowed us to obtain MCs in sufficient numbers to develop a protocol for antibodies generation (Gómez-González et al., 2014). In the present study, we describe the production of a monoclonal antibody (mAb) that specifically recognizes the MCs of gilthead seabream. This mAb, GB10, produces MC death by oncosis. Moreover, we are already producing another mAb against MCs of gilthead seabream.

2. Material and Methods

2.1. Fish

Healthy specimens of adult (300-500 g mean weight) gilthead seabream, *Sparus aurata* L., were maintained in tanks with running seawater with a flow rate of 20% aquarium volume/h, 6 ppm dissolved oxygen and suitable aeration and filtration systems in a natural photoperiod and temperature at the Centro Oceanográfico de Murcia (Instituto Español de Oceanografía, IEO, Mazarrón, Murcia, Spain). Specimens were fed twice a day with a commercial pellet diet (44% protein, 22% lipids; Skretting, Spain) with a feeding rate of 1.5% of fish biomass.

All experiments were carried out under the approved Guidelines of the European Union Council (2010/63/EU), the Spanish RD 53/2013, and the Bioethical Committee of the University of Murcia for the use of laboratory animals.

2.2. Isolation of peritoneal mast cells and head kidney cell suspensions

Fish were tranquilized by 8 ml/L of clove oil and then immediately anesthetized using 40 ml/L of clove oil. Then, fish were intraperitoneally injected with 10 mL sRPMI [RPMI (Sigma-Aldrich), containing 100 i.u./mL penicillin, 100 µg/mL streptomycin and 1% glutamine, adjusted with 0.35% NaCl to seabream serum osmolarity (353.33 mOs)]. The fish abdomens were massaged for 10 min to dislodge tissue-attached cells into the sRPMI medium. An incision was made below of the lateral fin to access to the peritoneum, and the

peritoneal exudate was then aspirated, collected into 15 mL Falcon tubes, and kept at 4 °C. Then, the specimens were decapitated and the head-kidney were removed and kept on sRPMI.

Peritoneal gilthead seabream MCs were isolated as described previously (Gómez-González et al., 2014) with slight modifications. MC-enriched fractions were obtained by negative selection.

The head-kidney were disaggregated and passed through 100 µL cell strainers and resuspended in 5 mL sPBS (PBS, Sigma-Aldrich + 0.35% NaCl). After centrifugation at 600 x *g* for 5 min, cells were washed twice and resuspended in 5 mL of sPBS. The cells were counted using a Neubauer chamber.

2.3. Production of GB10 and BG4 mAbs

BALB/c mice, *Mus musculus*, were housed in specific pathogen-free conditions with suitable atmosphere, 12 h day/12 h night photoperiod and natural temperature at the Animalarium of the University of Murcia (Campus de Espinardo, Murcia, Spain).

Mice were immunized three times by intraperitoneal injections of 2-50 x 10^6 isolated gilthead seabream peritoneal MCs every 2 weeks. Three days after the last immunization, mice were sacrificed by cervical dislocation and the spleen was aseptically harvested and transferred to a sterile 100 mm-diameter Petri dish with 10 mL of sterile complete serum-free RPMI medium (sfRPMI). After that, the spleen was washed with 5 mL of sfRPMI and disaggregated through a 100 µm pore diameter sieve to obtain a splenocyte cell suspension. Splenocyte number and viability were counted using a Neubauer chamber and trypan blue (Sigma-Aldrich). Then, the cells were centrifuged at 500 x *g* for 5 min at room temperature and resuspended in 10 mL of sfRPMI medium.

The myeloma cell line SP2/0-Ag14 was grown in a non-adherent flask using RPMI-10 [RPMI medium + 10% fetal bovine serum, (FBS)] at 37 °C and 5% CO_2 atmosphere. Before fusion, myeloma cells were counted and viability was assessed using an automatic cell counter (Bio-Rad) and trypan blue. Then, cells were washed three times in 10 mL sfRPMI medium and centrifuged 5 min at 250 x *g* at room temperature and resuspended in 10 mL of sfRPMI medium.

2.3.1. Fusion of splenocytes cell suspension and myeloma cell line

Splenocytes and the myeloma SP2/0-Ag14 were centrifuged at 500 x *g* for 5 min at room temperature and then fused using 1 mL of polyethylene glycol (PEG, Sigma-Aldrich) in a proportion of 4:1. After that, the cells were centrifuged in 10 mL of RPMI-20 (RPMI medium + 20% FBS) at 500 x *g* for 5 min at room temperature, and then resuspended in RPMI-20 medium (20 x 10^6 cells/mL) and incubated at 37 °C and 5% CO_2 atmosphere allowing the hybridomas to grow. The day after the fusion, RPMI-HAT (hypoxanthine/aminopterin/thymidine (HAT) supplemented RPMI medium with 20% FBS) was added.

Fourteen days after fusion, the hybridomas were screened to identify which of them were able to produce antibodies against gilthead seabream peritoneal MCs. The screening was analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences). Forward and side scatter parameters (FSC and SSC) dot-plots, and fluorescence (FL1) histograms were collected and analyzed with the software FlowJo v10.0.4.

The selected positive hybridomas were incubated in serial 1/2 dilutions in 200 mL of RPMI-20 at 37 °C and 5% CO_2 atmosphere for 10-15 days to allow monoclonal hybridoma growth. Then, a second screening of monoclonal hybridomas was performed, as previously described. A second cloning and a third screening were carried. When the GB10 hybridoma was obtained, 5 x 10^4 hybridoma cells were grown in RPMI-10 at 37 °C and 5% CO_2 in a non-adherent flask for 5-6 days. Supernatants containing antibodies were collected, filtered using a 45 µm pore diameter to eliminate cellular debris, and frozen at -20 °C until use.

2.3.2. mAb cell staining and viability analysis

Gilthead seabream peritoneal leukocytes (1 x 10^5) were incubated with 100 µL of the hybridoma culture supernatant, GB10 (1/1) or FACS buffer (control medium) (PBS + 2% FBS + 0.05% sodium azide) for 10, 20 or 30 min at 4 °C or 22 °C. After these incubations, cells were centrifuged at 400 x *g* for 5 min at 4 °C, and then washed twice with FACS buffer. After that, cells were labelled with the mAb Alexa Fluor 488 F(ab')2 fragment (1:500) of goat anti-mouse IgG (H+L) (Life Technologies) for 30 min at 4 °C. Then, the cells were centrifuged and washed twice, as previously described. After mAb staining, cell viability was measured with propidium iodide (PI) (Sigma-Aldrich). Forward and side scatter (FSC and SSC) dot-plots, and fluorescence (FL1 and FL3) histograms of MCs staining and viability were analyzed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences).

Data were analyzed with the software FlowJo v10.0.4 as mentioned previously. A similar protocol was developed using G7 (1:100) or BG4 (1/1), which is specific for gilthead seabream acidophilic granulocytes (Sepulcre et al., 2002).

2.4. Apoptosis

Apoptosis is a death cell pathway that requires the activation of caspase signaling. The possible apoptosis cell death produced by GB10 was studied by treating cells with a caspase inhibitor, qVD-opH (P.CAS, SM Biochemicals). Once, total peritoneal leukocytes had been obtained, 1 x 10^5 cells were incubated with RPMI-10 or RPMI-10-P.CAS [RPMI-10 + 10 mM P.CAS] for 30 min at 22 °C and 5% CO_2 atmosphere, and then centrifuged at 400 x *g* for 5 min. After that, cells were resuspended in 100 µL GB10 (1/1) or in 100 µL GB10-P.CAS [GB10 (1/1) + 10 mM P.CAS], respectively, and then incubated during 30 min at 22 °C and 5% CO_2 atmosphere. As controls, 1 x 10^5 cells were incubated with RPMI-10 [C(-)], RPMI-10- Dimethyl sulfoxide (DMSO) [RPMI-10 + 0,2% DMSO, C(DMSO) and C(GB10+DMSO)] or RPMI-10-P.CAS [C(P.CAS)] for 30 min at 22 °C and 5% CO_2 atmosphere and then centrifuged at 400 x *g* for 5 min. Then, the cells were resuspended in RPMI-10 [C(-)], RPMI-10-DMSO [C(DMSO)], GB10 + 0.2% DMSO [C(GB10+DMSO)] or RPMI-10-P.CAS [C(P.CAS)], respectively, and then incubated for 30 min at 22 °C in a 5% CO_2 atmosphere. Viability and cell morphology was analyzed by flow cytometry, as previously mentioned.

2.5. Oncosis

Peritoneal cells were resuspended in sbRPMI with 10% FBS (control medium) or GB10 hybridoma supernatant for 30 min or 2 h at 22 °C and 5% CO_2 atmosphere. Viability and cell structure and complexity were analyzed by flow cytometry.

2.5.1. Transmission electron microscopy

After the oncosis assay, peritoneal cells (control and GB10 treated) were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and processed for electron microscopy analysis, as previously described (Mulero et al., 2007a). Cells were examined using a JOEL JEM-1011 transmission electron microscope.

3. Results

3.1. GB10 mAb staining of gilthead seabream peritoneal mast cells

The presence of acidophilic granulocytes, MCs, macrophages, lymphocytes and other immune cells has previously been described by electron microcopy in gilthead seabream peritoneal exudate (Meseguer et al., 1993), where they are distributed in three main regions, as observed by flow cytometry (Gómez-González et al., 2014). Following these previous results and using flow cytometry (FSC-SSC), we observed that acidophilic granulocytes represent the most frequent population, followed by MCs and, in a smaller proportion, lymphocytes and other immune cells (Fig. 1A).

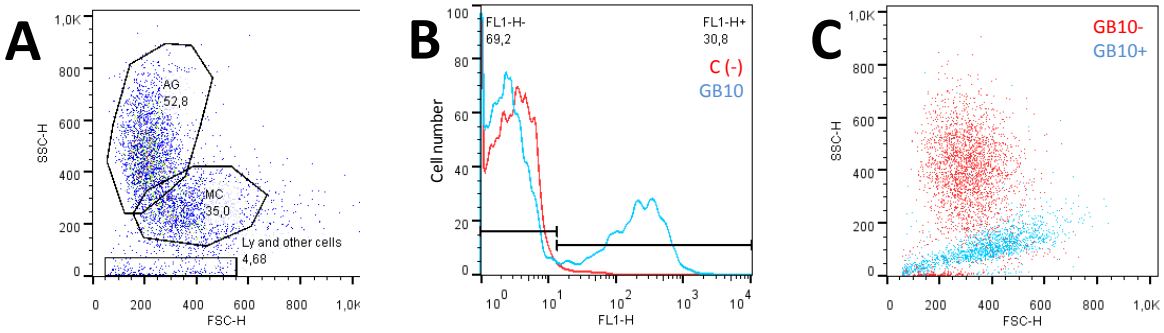


Figure 1. Flow cytometric analysis of gilthead seabream peritoneal leukocytes (A-C) labeled with the GB10 mAb (B-C). A) Representative FSC-SSC dot-plot of peritoneal leukocytes; B) Representative fluorescence histogram (FL1, GB10) showing the fluorescence intensity and the percentage of GB10 labeled peritoneal cells (blue) compared with the negative control (background) (red); C) Representative FSC-SSC dot-plot of positive (blue) and negative (red) GB10 staining cells. Positive GB10 cells were found in the MC region. FSC-H: forward scatter cell parameters, SSC-H: side scatter cell parameters; FL1-H; fluorescence 1 cell parameters. The data are representative of 6 independent experiments.

The obtained mAb GB10 was used as primary antibody for the extracellular immunofluorescence staining of total peritoneal leukocytes at 4º C (Fig. 1B, C). GB10 stained 30.8% of peritoneal cells, as shown from the increase in fluorescence intensity (FL1) in the flow cytometry histogram (Fig. 1B). Particularly, the size and complexity of the GB10 positive cells seen in this histogram confirm that the GB10 mAb specifically labels gilthead seabream peritoneal MCs, since GB10 positive cells were found in the MC region (Fig. 1C).

GB10 immunofluorescence staining of head-kidney total leukocytes was also performed to confirm the specificity of the antibody, since it is known that head-kidney does not contain MCs. No staining was found (data not shown).

3.2. GB10 produces rapid mast cell death

Alteration of the morphology and the complexity of the peritoneal MCs was observed by flow cytometry after incubation of peritoneal leukocytes with GB10 for 30 min at 4 °C (Fig. 2). However, no changes were found in the rest of the peritoneal cell types.

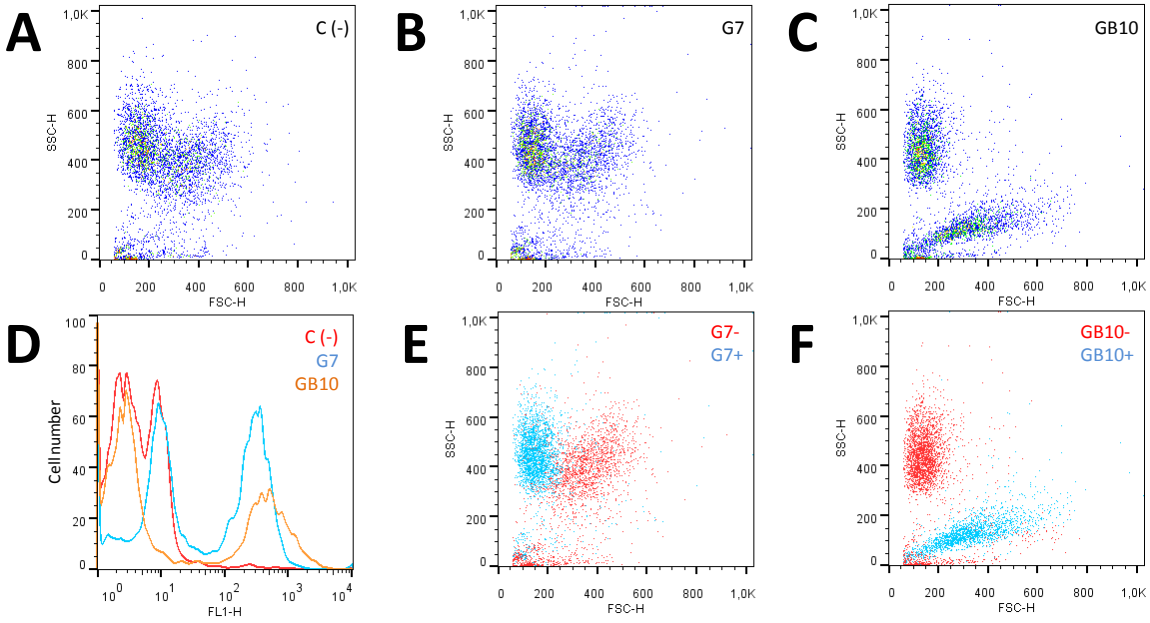


Figure 2. GB10 alters seabream peritoneal MC morphology. A-C) Flow cytometry FSC-SSC dot-plot of total peritoneal leukocytes after 30 min incubation with medium (C-)(A) or the mAb G7 (B) and GB10 (C); D) Percentage of peritoneal cells labeled with G7 (blue), GB10 (orange) compared with the background (red); E) Positive (blue) and negative (red) G7 labeled peritoneal cells; F) Positive (blue) and negative (red) GB10 labeled peritoneal cells. C(-): control medium; G7: mAb specific to acidophilic granulocytes; FSC-H: forward scatter cell parameters; SSC-H: side scatter cell parameters; FL1-H: fluorescence 1 cell parameters. The data are representative of 6 independent experiments.

The comparative analysis of the flow cytometry dot-plots of untreated total peritoneal leukocytes (Fig. 2A), total peritoneal leukocytes incubated with G7, a gilthead seabream specific mAb against acidophilic granulocytes (Fig. 2B), and total peritoneal leukocytes incubated with GB10 (Fig. 2C) demonstrated that GB10 altered the MC size and complexity compared with untreated peritoneal cells or those incubated with G7. This observation was confirmed when the fluorescence intensity of the untreated (C-) peritoneal cells and those incubated with G7 or GB10 was analyzed (Fig. 2D, E, F). G7 positive cells were located in the acidophilic granulocytes region (Fig. 2D, E), while GB10 positive cells, MCs (Fig. 2D, F) showed an altered cell size and complexity (Fig. 2F).

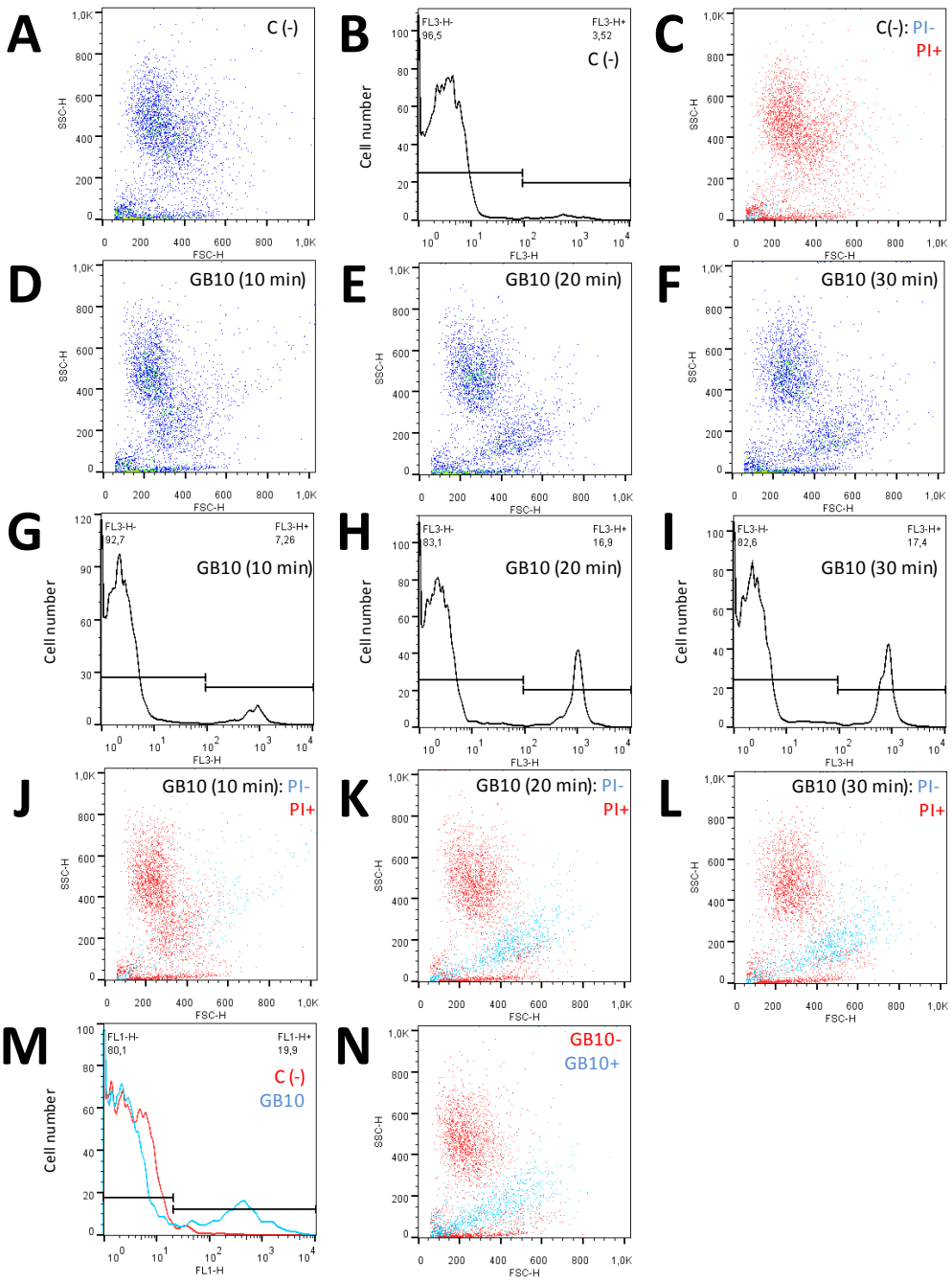


Figure 3. GB10 induces MC death. A) Flow cytometry FSC-SSC dot-plot of peritoneal exudates after 30 min incubation with control medium C(-); B) Percentage of peritoneal cells incubated with control medium (C-) labeled with PI; C) FSC-SSC dot-plot of PI positive cells of peritoneal exudate incubated with control medium (C-); D-F) Flow cytometry FSC-SSC dot-plots of total peritoneal leukocytes incubated with GB10 for 10, 20 and 30 min; G-I) Percentage and fluorescence intensity of GB10 positive peritoneal leukocytes incubated for 10, 20 and 30 min compared with the their GB10 negative peritoneal leukocytes, respectively (red); J-L) Flow cytometry FSC-SSC dot-plot of PI positive peritoneal leukocytes compared with PI negative peritoneal leukocytes (red); M) Percentage and fluorescence intensity of GB10 stained peritoneal leukocytes (blue) compared with the control (C-) (red); N) Flow cytometry FSC-SSC dot-plots of GB10 positive peritoneal leukocytes (blue) compared with the negative control (red). C(-): control medium; PI: propidium iodide; FSC-H: forward scatter cell parameters; SSC-H: side scatter cell parameters; FL3-H: fluorescence 3 cell parameters. The data are representative of 4 fish.

As GB10 induced alterations in the morphology of MCs after 30 min of incubation at 4 °C, the next step was to analyze whether a lower incubation time of 10 or 20 min, with GB10 also altered the MC size and complexity, as well as the viability of MCs at the mentioned time points (Fig. 3). The relative cell size and complexity (Fig. 3A), intensity fluorescence (Fig. 3B) and viability (Fig. 3C) of total peritoneal cells incubated with medium alone at 4 °C were seen to be similar after 10, 20 (data not shown) and 30 min (Fig. 3A-C) of incubation. As expected, more than 95% of viability was reached in the negative control (medium alone). However, when peritoneal cells were incubated with GB10, modifications in cell size and complexity (Fig. 3D-L) and a decrease in viability (Fig. 3J-L) were observed at all the studied time points, with a time-response that peaked at 20 min (Fig. 3E, H and K). Moreover, the percentage of PI positive cells after 20 and 30 min of GB10 incubation (Fig. 3H and I) and the percentage of GB10 positive cells (Fig. 3M), together with the fact that PI positive cells (Fig. 3J-L) and GB10 positive cells (Fig. 3N) were located in the same regions of the cytometric FSC-SSC dot-plots, indicated that GB10 labeled and induced the death of gilthead seabream MCs. Furthermore, no changes in cell size, complexity or viability were detected in other peritoneal leukocytes. Interestingly, when peritoneal leukocytes were incubated with GB10 at 22 °C for 30 min, the FSC-SSC parameters and the viability data obtained were similar (data not shown) to those described above at 4°C, indicating that the MC death induced by GB10 was temperature independent.

3.3. GB10 does not produce Apoptosis

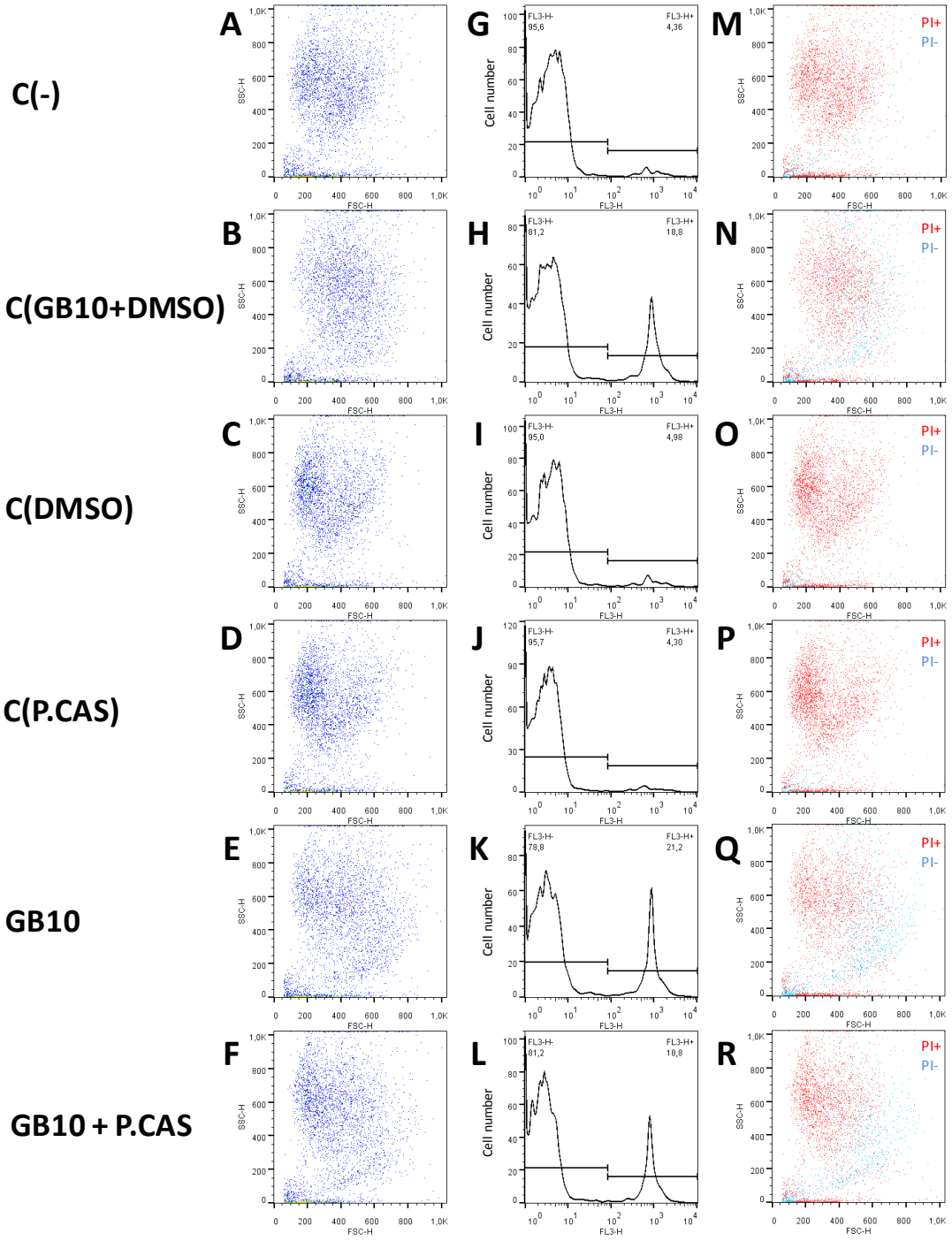


Figure 4. GB10 does not induce MC death by apoptosis. A-F) Flow cytometry FSC-SSC dot-plots of the controls C(-), C(GB10+DMSO), C(DMSO) and C(P.CAS), and GB10 and GB10 + P.CAS, respectively. G-L) Intensity fluorescence of peritoneal total leukocytes stained with PI of the controls C(-), C(GB10+DMSO), C(DMSO) and C(P.CAS), and GB10 and GB10 + P.CAS, respectively. M-R) Flow cytometry FSC-SSC dot-plots of PI- (red, FL3-H-) and PI+ (blue, FL3-H+) of total peritoneal leukocytes of the controls C(-), C(GB10+DMSO), C(DMSO) and C(P.CAS), and GB10 and GB10 + P.CAS, respectively. P.CAS: caspase inhibitor; PI: propidium iodide; FSC-H: forward scatter cell parameters, SSC-H: side scatter cell parameters; FL3-H: fluorescence 3 parameters. The data are representative of 4 fish.

Apoptosis requires the activation of caspase signaling. If GB10 induces death cell by apoptosis, the inhibition of caspases should allow MCs to survive after GB10 incubation. With this in mind, an assay using an inhibitor of caspases, P.CAS, was used, and the cell size, complexity (Fig. 4A-F) and viability (Fig. 4G-L) of total peritoneal cells were analyzed when incubated with GB10 and/or P.CAS. In addition, the cell size and complexity of PI negative and positive cells were studied (Fig. 4M-R). It was observed that both size and complexity in the dot-plots and intensity fluorescence in the histograms were similar for cells incubated in medium alone (C-; Fig, 4A, G, respectively), DMSO-treated cells (Fig. 4C, I, respectively), and P.CAS treated cells (Fig. 4D, J, respectively). However, GB10 altered both the cell size and complexity in the dot plots and intensity fluorescence in the histograms (GB10+DMSO, Fig. 4B, H, respectively; GB10, Fig. 4E, K, respectively; and GB10+P.CAS, Fig. 4F, L, respectively). These observations were confirmed by analysis of the cell size and complexity of PI negative and positive cells (Fig. 4M-R). In conclusion, the above treatment with caspase inhibitor did not modify the capacity of GB10 to induce seabream MC death, indicating that MC death induced by GB10 did not occur through apoptosis.

3.4. GB10 induces oncosis in mast cells

Gilthead seabream peritoneal leukocyte morphology was analyzed by transmission electron microscopy after 30 min and 2 hours of incubation with control medium or GB10 incubation. After both times with the control medium, the peritoneal leukocyte populations showed the characteristic morphology described by Meseguer *et al*. (1993) (Meseguer et al., 1993). Thus, MCs possess an eccentric and euchromatinic nucleus containing some peripheral heterochromatin and two types of granules differentiated by their electron-density and showing a progressive vacuolation process, while acidophilic granulocytes showed an oval and eccentric nucleus with a small rim of heterochromatin and three types of granules: granules with a homogeneous, medium electron-dense content, granules containing dense cores surrounded by low density material, occasionally elliptical and containing a crystalloid, and granules showing an eccentric electron-dense core surrounded by a light ring (Fig. 5A, B). Interestingly, clear morphological changes were observed in MCs after 30 min or 2 hours of GB10 incubation, including the high electron density of the nucleus, a small number of clearly distinguishable granules with different contents and a very thin, sometimes invisible, cellular boundary (Fig. 5C, D). These morphological changes are characteristic of processes of oncosis, suggesting that GB10 induces oncosis of MC. Nevertheless, no changes were observed in the rest of peritoneal cell types after the different GB10 incubations.

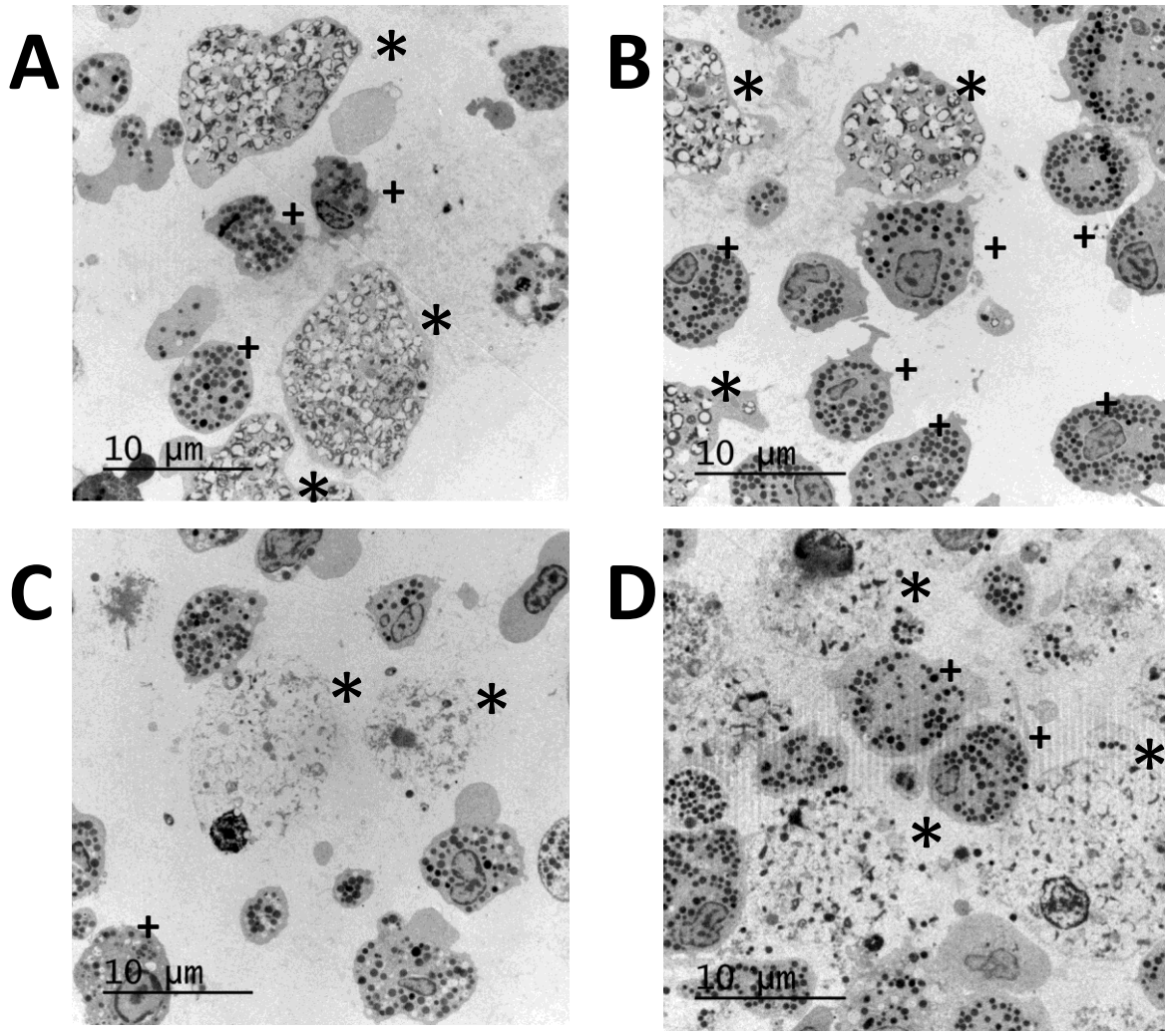


Figure 5. GB10 induces MC death by oncosis. A, C) TEM images of total peritoneal exudate incubated for 30 min and 2 hours with control medium, respectively. B, D) TEM images of total peritoneal exudate incubated 30 min and 2 hours with GB10, respectively. TEM: transmission electron microscopy. *: mast cells; +: acidophilic granulocytes. The data are representative of 4 fish.

3.5. GB10 is an useful tool to produce new mAb against gilthead seabream peritoneal mast cells

Due to the fact that GB10 induces the death of gilthead seabream peritoneal MCs and it cannot be used to isolate biologically functional peritoneal MCs, a new mAb against has been obtained, BG4. Since GB10 labels gilthead seabream peritoneal MCs, it was use as positive control (Fig. 6). Interestingly, BG4 label gilthead seabream peritoneal MCs as the percentage of peritoneal cells stained with BG4 (Fig. 6D) agrees with the percentage of GB10+ cells (Fig. 6C) and with previous observations of the localization of gilthead seabream peritoneal MCs (Gómez-González et al., 2014). New approaches to characterize this new antibody are needed.

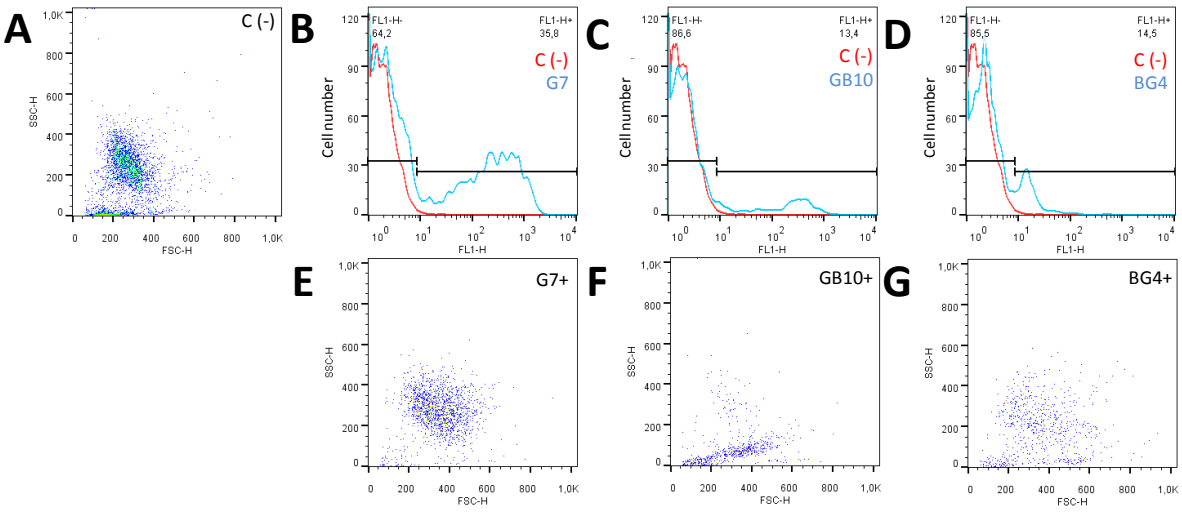


Figure 6. GB10 as a useful tool to produce new mAb against seabream peritoneal mast cells. A) Flow cytometry FSC-SSC dot-plots of total peritoneal leukocytes incubated with medium (C-); B-D) Percentage of peritoneal cells labeled with G7 (B), GB10 (C) and BG4 (D) (red) compared with the background (red); E-G) Positive G7 (E), GB10 (F) and BG4 (G) labeled peritoneal cells. C(-): control medium; G7: mAb specific to acidophilic granulocytes; FSC-H: forward scatter cell parameters; SSC-H: side scatter cell parameters; FL1-H: fluorescence 1 cell parameters.

4. Discussion

MCs are an important cell component of the immunity known to participate in the immune innate response. Their role in mammalian defense has been amply studied due to the availability of antibodies which specifically recognize them. The use of specific mAb against fish leukocytes has allowed these cells to be isolated for *in vitro* and *in vivo* functional studies (Bly et al., 1990; Hamdani et al., 1998; Korytar et al., 2013; Sepulcre et al., 2002), avoiding the need for tedious purification steps that may alter either cell viability or activity. However, fish MCs have been poorly studied mainly due to the lack of specific antibodies against them, and most of our present knowledge has been obtained using histological techniques (Manera et al., 2014; Mulero et al., 2007a; Reite, 1998; Reite and Evensen, 2006).

In this study, we obtained a mAb, GB10, against a cell surface receptor of peritoneal seabream MCs, which specifically recognizes them, as confirmed by immunofluorescence and flow cytometry. A protocol to isolate gilthead seabream peritoneal MCs was previously developed (Gómez-González et al., 2014), improving the ability to obtain a specific antibody against gilthead seabream MCs. GB10 was able to confirm that MCs constitute a notable percentage (20-30%) of cells in the peritoneal cavity of gilthead seabream, as occurs in rats (Diamant, 1990), and in agreement with previous microscopic studies in gilthead seabream

peritoneal exudate (Gómez-González et al., 2014; Meseguer et al., 1993). Although the GB10 obtained in this report may also be directly used in flow cytometry to investigate the physiological activities of these cells, the antibody was found to induce MC death after a very short time of incubation and in a temperature independent way. After confirming that GB10 was able to induce MC death in gilthead seabream, efforts were made to identify the process or processes involved. Classification of the death pathways is a controversial issue. In general, it is thought that cell death can be produced by apoptosis, necrosis or oncosis. The first, apoptosis, is mainly characterized by caspase activity and adenosine triphosphate (ATP) production, DNA fragmentation and irregularity of the nucleus, cell shrinkage and altered orientation of lipids in the plasma membrane. Moreover, the death produced by apoptosis begins 12-24 hours after initiation of the triggering event. The second, necrosis, involves ATP depletion, karyolosis and caspase independent DNA fragmentation, nucleus lysis and cell swelling. In the last mentioned process, oncosis, several morphological features are observed, such as loss of plasma membrane integrity and release of intracellular contents, which results in aggressive and pro-inflammatory damage, ATP depletion, nucleus dilatation and the clumping of chromatine reticular nucleus or cell swelling (Balvan et al., 2015; Tan et al., 2009; Weerasinghe et al., 2013). In contrast, some authors consider that oncosis and apoptosis are followed by necrosis (Majno and Joris, 1995; Trump et al., 1997) or that oncosis is followed by necrosis (Krysko et al., 2004; Zhang et al., 1998), while others do not (Balvan et al., 2015). Considering that oncosis and apoptosis were the possible cause of death produced by GB10, several experiments were performed in order to investigate whether GB10-induced MC death was due to apoptosis or oncosis. Analysis of the obtained results meant that apoptotic MC death cell could be rejected because, on the one hand, a caspase inhibitor was not able to inhibit the GB10 killing and, on the other hand, because the images obtained by transmission electronic microscopy do not show the typical morphology of apoptotic cell death (Balvan et al., 2015). In fact, the MC morphology after GB10 treatment showed a typically feature of oncotic cell death (Balvan et al., 2015; Krysko et al., 2004; Trump et al., 1997). Taking all these data together, i.e., the rapid cell death, the temperature independent process and the MC morphology, it is clear that oncosis is the type of cell death produced by GB10.

GB10 is not the only example of an Ab that induces oncosis cell death. The existence of Abs that induces oncosis has been already published. For example, mAb RE2 has been reported to be cytotoxic toward activated T and B lymphocytes (Matsuoka et al., 1995), the anti-porimin mAb induces oncosis-like death cell in Jurkat cells (Zhang et al., 1998), and the

mAb 216 and A6 (H4C5), derived from VH4-34(VH4.21) gene, binds specifically to human B lymphocyte surface carbohydrate Ag, inducing their death (Bhat et al., 1996). It has also been demonstrated that different VH4-34 (VH4.21) gene-encoded mAb were able to rapidly provoke the death of human B lymphocytes (Bhat et al., 1997), that mAb RAV12 recognizes an N-linked carbohydrate Ag (RAAG12) and is able to kill gastrointestinal adenocarcinoma tumor cells (Loo et al., 2007), that mAb m84 binds to podocalyxin-like protein-1 on human embryonic stem cells, inducing cell death (Tan et al., 2009), and that anti-NGcGM3 14F7 and anti-NeuGcGM3 14F7hT mAb kill L1210 murine tumor cells and human cancer cells, respectively (Dorvignit et al., 2015; Hernández et al., 2011; Roque-Navarro et al., 2008). Nevertheless, to the best of our knowledge this is the first piscine mAb demonstrated as able to induce cell death.

The use of GB10 will be essential for furthering our knowledge of the protective role of MCs in the fish immune response and to improve our understanding of the phylogeny of vertebrate MCs and histamine release. *In vivo* assays, in which GB10 will be intraperitoneally injected in seabream, will help understand the evolutionary aspects of both MCs and histamine. Moreover, GB10 has been used to obtain a new mAb, i.e. BG4, specific to gilthead seabream MCs.

CHAPTER IV: 17α-Ethinylestradiol alters the peritoneal immune response of gilthead seabream

Abstract

17α-ethinylestradiol (EE_2), a synthetic estrogen used in most oral contraceptives pills and hormone replacement therapies, is found in many water bodies, where it can modulate the fish immune response. EE_2 acts as an endocrine disruptor in gilthead seabream, *Sparus aurata* L., a marine teleost fish of great economic value in Mediterranean aquaculture, as it induces hepatic vitellogenin gene (*vtg*) expression. Moreover, EE_2 also alters the capacity of gilthead seabream to appropriately respond to infection. Nevertheless, previous studies have mainly focused on the head kidney leukocytes and no information exists on peritoneal leukocytes, including mast cells. In the present work, juvenile gilthead seabream fish were fed a pellet diet supplemented with EE_2 for 76 days and intraperitoneally injected with hemocyanin plus imject alum adjuvant at the end of EE_2 treatment and 92 days later, and the peritoneal immune response was analyzed. EE_2 supplementation induced *vtg e*xpression but returned to basal levels by 3 months post-treatments. Moreover, EE_2 induced the expression of genes encoding for the nuclear estrogen receptor α and the G protein-coupled estrogen receptor 1 in peritoneal leukocytes. Interestingly, EE_2 induced an inflammatory response in the peritoneal cavity at the end of the treatment in unvaccinated fish, which was largely maintained for several months after the cessation of the treatment. Moreover, EE_2 modulated histamine receptor gene expression, which may modulate histamine signaling in peritoneal cells. Taken together, the study provides fresh information about endocrine immune disruption, focusing on peritoneal leukocytes and histamine signaling.

1. Introduction

It is well known that estrogens are key modulators of the immune system (Straub, 2007), including that of fish, due to the presence of nuclear estrogen and androgen receptors (ER and AR, respectively) in piscine leukocytes (Cabas et al., 2013b; Liu et al., 2009; Lynn et al., 2008; Shved et al., 2009; Slater et al., 1995; Todo et al., 1999). Moreover, the identification of a G protein-coupled estrogen receptor 1 (GPER1), a membrane estrogen receptor that binds estradiol (E_2) (Revankar et al., 2005; Thomas et al., 2005) and other estrogens (Prossnitz and Hathaway, 2015), and its identification in immune cells of both mammals and fish (Blasko et al., 2009; Brunsing et al., 2013; Cabas et al., 2013b), has opened up the possibility of exploring additional estrogen-mediated effects.

Anthropic activity produces the release of a wide variety of chemical compounds into the environment, most of which affect in some way the species living there. Among these compounds, endocrine disruptor chemicals (EDCs) are a group of substances able to mimic and/or antagonize the effects of endogenous endocrine hormones, and disrupt the synthesis and metabolism of endogenous hormones and the synthesis of hormone receptors (Aris et al., 2014). EDCs alter fish reproduction, but also growth, osmoregulation, stress and immunity (Filby et al., 2007).

17α-Ethinylestradiol (EE_2), considered a xeno-estrogen EDC, is a synthetic estrogen and the main active component of contraceptive pills, which are also used in hormonal therapy. EE_2 can be found not only in sewage treatment works (Baronti et al., 2000; Desbrow et al., 1998; Quinn et al., 2004; Vethaak et al., 2005), but also in natural bodies of water (Desbrow et al., 1998; Vethaak et al., 2005). Due to its capacities to be bio-accumulated and to alter endocrine homeostasis, much research effort has focused on its effects on animals living in aquatic environments (Aris et al., 2014). It has been found that EE_2 can modify the expression of ERs and ARs and the expression of the genes classically regulated by ERs and ARs (Milla et al., 2011), such as the vitellogenin gene (Kausch et al., 2008), which is widely accepted to be a biomarker of endocrine disruption by xeno-estrogens (Sumpter and Jobling, 1995).

The gilthead seabream (*Sparus aurata* L.), a marine protandrous teleost fish of great commercial value in the Mediterranean Sea and in aquaculture, has been widely used to study the effect of EE_2 on the reproductive (Cabas et al., 2011) and immune systems at different times of its reproductive cycle (Cabas et al., 2012, 2013a; Rodenas et al., 2015, 2016). Thus, EE_2 provokes an estrogenic response, inducing hepatic vitellogenin gene expression (Cabas et

al., 2012, 2013a; Rodenas et al., 2015, 2016), and alters the expression of the gene encoding for the ERα of head kidney leukocytes (Cabas et al., 2012). Although no evidence of immunesuppression has been observed, EE_2 alters the capacity of the innate and adaptive immune system to appropriately respond to an immune challenge, as assayed *in vivo* and *in vitro* using head kidney leukocytes (Cabas et al., 2012; Rodenas et al., 2015). Interestingly, the estrogenic response of EE_2 disappears when exposure to it ceases, although the adaptive immune response remains altered to some extent (Rodenas et al., 2015, 2016). To increase our knowledge of the effect of EE_2 on the immune response of gilthead seabream, we focus our study on its effects on peritoneal cells. The peritoneal exudate of gilthead seabream, as it has been described in other teleost fish, consists of granulocytes, macrophages, lymphocytes and mast cells (Gómez-González et al., 2014; Meseguer et al., 1993) and its cellular composition may vary depending on the extent of tissue damage or injury. Although some studies have revealed the effect of estrogens, both natural and synthetic, on peritoneal leukocytes, there is a noticeable lack of information about the effects of xenoestrogens on piscine peritoneal leukocytes, and, to the best of our knowledge, no information exists concerning how they may be modulated by EE_2. The aim of this work was, therefore, to evaluate the impact of dietary administration of EE_2 in leukocyte activation (respiratory burst and cytokine gene expression), and mast cell and acidophilic granulocyte abundance in the peritoneal exudate of fish upon intraperitoneal (i.p.) vaccination.

2. Material and Methods

2.1. Animals and experimental design

Healthy specimens of gilthead seabream *Sparus aurata* L. (Actinopterygii, Perciformes, Sparidae) were reared at the Centro Oceanográfico de Murcia (Instituto Español de Oceanografía, Mazarrón, Murcia, Spain), where they were kept in running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium vol/h) with a natural temperature and photoperiod, and fed three times per day with a commercial pellet diet (44% protein, 22% lipids; Skretting) at a feeding rate of 1.5% of fish biomass. Environmental parameters, mortality and food intake, as well as behaviour, were recorded daily. The experiments described were approved by the Consejería de Agua, Agricultura y Medio Ambiente of the Región de Murcia (approval number A13160507).

I*n vivo* EE_2 treatment was carried out with juvenile gilthead seabream specimens (n = 400) with a body weight of 26.6 ± 4.2 g in 170 L aquaria. Briefly, EE_2 (5 μg/g food, 98%

purity; Sigma) was incorporated in the commercial food using the ethanol evaporation method (0.3 L ethanol/kg of food), as described previously (Shved et al., 2007). The specimens were fed three times a day *ad libitum* with the pellet diet supplemented with EE_2 (treated fish) or the unsupplemented pellet diet (untreated fish) for 76 days (days of treatment, dot, after which they were fed with a commercial feed for a further 142 days). In order to evaluate the effect of EE_2 on an induced immune response, the specimens were i.p. injected with hemocyanin (45 µg/fish; Sigma-Aldrich) plus alum adjuvant (3.6 µg/fish; Thermo Scientific) (vaccinated fish) or phosphate buffered saline (PBS) (unvaccinated fish) 1 day after treatment (dat) (priming) and 92 dat (booster) (Fig. 1).

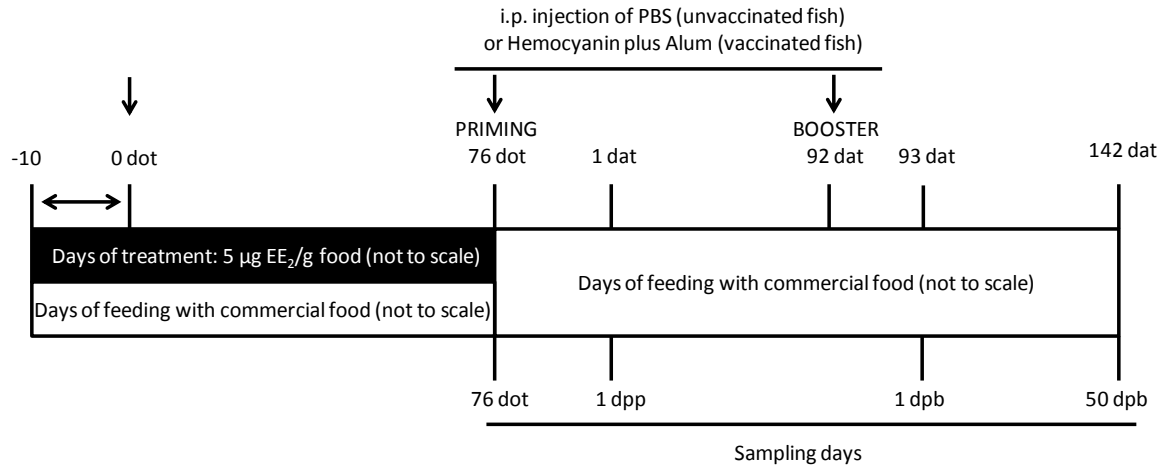


Figure 1. Schematic drawing of the experimental design. Animals were exposed to 0 (untreated fish) and 5 (treated fish) µg EE_2/g food for 76 days (days of treatment, dot), after which they were fed with commercial food for a further 142 days. Fish were i.p. injected with PBS (unvaccinated fish) or with hemocyanin plus imject alum adjuvant (vaccinated fish) at 76 dot (priming) and 92 days after treatment (dat) (booster). Samples were collected 1 (1 day post-priming, dpp), 93 (1 day post-booster, dpb) and 142 (50 dpb) dat.

Samples of liver and peritoneal exudate leukocytes were collected 1 (1 day post-priming, dpp), 93 and 142 (1 and 50 day post-booster, dpb, respectively) dat (Fig. 1). Specimens (n=6 fish/treatment/time of sampling) were fasted for 24 h before each sampling. They were tranquilized by 8 µl/l of clove oil and immediately anesthetized using 40 µl/l of clove oil and weighed. Two-three mL of sRPMI culture medium [RPMI culture medium (Sigma-Aldrich) with 2 mM glutamine (Sigma-Aldrich), 100 i.u./ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich) adjusted with 0.35% NaCl to seabream serum osmolarity (353.33 mOs)] were intraperitoneally injected at 1 dpp, 1 dpb and 50 dpb. Fish abdomens were massaged for 10 min to dislodge tissue-attached cells into the sRPMI culture medium. Then, incisions were made below of the lateral fin to access the peritoneum and the peritoneal exudates were

aspirated and collected into 15 mL Falcon tubes, after which the specimens were decapitated and the liver samples were keep on RNAlater (Sigma-Aldrich) and on ice until analysis.

2.2. Peritoneal exudate cell suspensions

Peritoneal exudates (approximately 2-3 mL) were passed through 70 µm cell strainers in order to remove large clumps of cells. Then, the cells were centrifuged at 600 x *g* for 5 min and resuspended in 5 mL of sRPMI. The total number of cells in peritoneal exudate was counted using an automatic cell counter (Bio-Rad).

2.3. Determination of percentage of acidophilic granulocytes and mast cells in peritoneal exudate and head kidney cell suspensions

The percentage of acidophilic granulocytes and mast cells was analyzed using 0.1 x 10^6 peritoneal exudate cell suspensions. Cells were incubated on ice with a specific monoclonal antibody (mAb) against gilthead seabream acidophilic granulocytes (G7, 1:100) (Sepulcre et al., 2002), to measure the percentage of acidophilic granulocytes, or with a mAb against seabream mast cells (GB10, 1/1) (Gómez-González et al., 2016), to determine the mast cell percentage in 100 µL of FACS buffer (PBS, 0.35% NaCl, 2% fetal bovine serum (FBS, Sigma-Aldrich) and 0.05% sodium azide (Sigma-Aldrich). After 30 min, cells were centrifuged at 600 x *g* for 5 min, the supernatant was removed by aspiration and the cells were washed twice with FACS buffer. Then, cells were labelled with the polyclonal antibody Alexa Fluor 488 F(ab')2 fragment (1:500) of goat anti-mouse IgG (H+L) (ThermoFisher Scientific) for 30 min at 4 °C. The supernatant was removed and cells were washed, as previously described. Fluorescence intensity was measured by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences) and data were analyzed with FlowJo v10.0.4 software.

2.4. ROS production assay

ROS production was measured using luminol-dependent chemiluminescence produced by 0.5 x 10^6 peritoneal exudate cell suspensions. Cells were incubated at 21 °C and 5% CO_2 during 20 min. Then, the ROS production was determined by adding 100 µM luminol (Sigma-Aldrich) and 1 µg/mL phorbolmyristate acetate (PMA) (Sigma-Aldrich) in Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich) to the cell culture. Chemiluminescence was recorded every 127 s for 1 h using a FLUOstartluminometer (BGM, LabTechnologies). Three experimental replicates were performed for all samples (6). The values reported are the

average of the replicate samples, expressed as maximum of the reaction curve from 127 to 1016 s, from which the background (only culture medium) was subtracted.

2.5. Gene expression analysis

Gene	Accesion number	Name	Sequence (5'-3')	Used
actb	X89920	F3	ATCGTGGGGCGCCCCAGGCACC	PCR
		R3	CTCCTTAATGTCACGCACGATTTC	
rps18	AM490061	F1	AGGGTGTTGGCAGACGTTAC	RT-qPCR
		R1	CTTCTGCCTGTTGAGGAACC	
vtg	AF210428	F1	CTGCTGAAGAGGGACCAGAC	RT-qPCR
		R1	TTGCCTGCAGGATGATGATA	
erα	AF136979	F1	GCTTGCCGTCTTAGGAAGTG	RT-qPCR
		R1	TGCTGCTGATGTGTTTCCTC	
gper1	HG004163	F1	GGCTGCCAGAGAATGTCTTC	RT-qPCR
		R2	GAGGCAGCTGTTGGAGAAAG	
csf1r	AM050293	F3	CTGCCCTACAATGAGAAG	RT-qPCR
		R4	TCAGACATCAGAGCTTCC	
ighm	AM493677	F1	AACCTCAGCGTCCTTCAGTG	RT-qPCR
		R1	GCACGTATCAGGGACGTTCA	
ight	FM145138	F1	TGGCAAATTGATGGACAAAA	RT-qPCR
		R1	CCATCTCCCTTGTGGACAGT	
il1b	AJ277166	F2	GGGTCTGAACAACAGCACTCTC	RT-qPCR
		R3	TTAACACTCTCCACCCTCCA	
il10	JX976621	F1	TGCTTCGTAGAAGTCTCGGATGT	RT-qPCR
		R1	TGGAGGGCTTTCCTGTCAGA	
hrh1	LN875558	F3	CATGCCTCTGAACCTGGTGT	RT-qPCR
		R3	AAATTGAGGCTGTGCTTGCC	
hrh2	KP728255	F1	CCTAACACGCTTCACTCCGT	RT-qPCR
		R1	AGCTGCAGTTTTCTGTGGGA	
hrh3	KP728256	F1	CTGTTTCAGCACACGGCTTC	RT-qPCR
		R1	GGCACACACGTACCACTACA	

Table 1. Gene accession numbers and primer sequences used for gene expression analysis. The gene symbols followed the Zebra Fish Nomenclature Guidelines (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines) (F and R are forward and reverse respectively).

Liver samples were defrosted and RNAlater removed. Total RNA was extracted from liver and peritoneal exudate cell suspensions of 1, 93 and 142 dat (1 dpp, 1dpb and 50 dpb, respectively) with TRIzol Reagent (ThermoFisher Scientific) as indicated by the manufacturer's instructions, and the RNA concentration was quantified by spectrophotometry (NanoDrop, ND-1000). RNA was treated with DNase I (amplification grade, 1 unit/µg RNA, Invitrogen) to remove genomic DNA traces that could interfere with the PCR reactions. SuperScript III RNase H- Reverse Transcriptase (Invitrogen) was used to synthesize first strand cDNA with oligo $(dT)_{18}$ primer (Invitrogen) from 1 µg of total RNA, at 50 °C for 50 min. The quality of cDNA was analyzed by performing a semi-quantitave PCR of ß-actin (*actb*) housekeeping gene using an Eppendrof Mastercycle Gradient Instrument (Eppendorf). The reaction mixture was incubated for 2 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 55 °C (the specific annealing temperature), and 45 s at 72 °C and finally holding at 72 °C for 10 min. Real-time PCRs were performed with an ABI PRISM 7500 (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) to analyze the gene expression of: i) the hepatic vitellogenin, *vtg*, ii) estrogen receptor α and the G protein-coupled estrogen receptor 1, *erα* and *gper1*, iii) the pro-inflammatory cytokine interleukin 1β, *il1b*, iv) gene markers of macrophages (colony stimulating factor 1 receptor, *csf1r*), and IgM^+ B lymphocytes (immunoglobulin M heavy chain, *ighm*) and IgT^+ B lymphocytes (immunoglobulin T heavy chain, *ight*), and v) the histamine receptor 1, *hrh1*, 2, *hrh2*, and 3, *hrh3*. Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C. For each mRNA, gene expression was corrected by the ribosomal protein S18 gene (*rps18*) content in each sample. The gilthead seabream specific primers used are shown in Table 1. In all cases, each PCR was performed with triplicate samples. Less than 3% variation in the *rps18* gene expression was observed among samples.

2.6. Determination of IgM and IgT specific titer

The hemocyanin specific IgM and IgT titers were determined by ELISA from peritoneal supernatant of all groups 1dpp, 1dpb and 50dpb. 96-well ELISA plates were pre-coated for 45 min. Then, plates were incubated with 10 µg/ml of the antigen hemocyanin diluted in PBS o/n. After that, plates ware washed three times with low salt buffer (2.42 g TRIS, 22.22 g NaCl and 2.5 mL Tween 20 dissolved in 1 L of distilled water, pH was 7.3 adjusted). In order to block unspecific bindings, plates were added PBS 1% bovine serum albumin (BSA,Sigma-Aldrich) and shacked for 2 h. Plates were washed once with low salt buffer. Then, doubling-

dilutions of fish peritoneal supernatants were added to the plate and shacked for 2 h. Plates were washed three times with high salt buffer (2.42 g TRIS, 29.22 g NaCl and 5 mL tween 20 dissolved in 1 L of distilled water, pH was 7.7 adjusted) followed by the incubation with a mAb specific to gilthead seabream IgM produced in mouse (Aquatic Diagnostics Ltd) or with a polyclonal antibody specific to gilthead seabream IgT produced in rabbit (GenScript), respectivelly. Plates were rinsed three times with high salt buffer and incubated with an anti-mouse IgG (whole molecule)-peroxidase antibody produced in goat (Sigma-Aldrich) or an anti-rabbit IgG (whole molecule)-peroxidase antibody (Sigma-Aldrich), respectivelly. Plates were washed three times with high salt buffer. Finally, the chromogen tetramethylbenzidine (TMB) (Sigma-Aldrich) was added, the reaction was stopped with 2M H_2SO_4 and the absorbance was read at 450 nm using a FLUOstart luminometer (BGM, Lab Technologies).

2.7. Statistical analysis

Normal distribution of the data was analyzed with the Statgraphics Centurion XVI v16.1.15 software. Statistical differences were analyzed by independent-samples Student's t-tests or Mann-Whitney test (unvaccinated fish *vs*. vaccinated fish and untreated fish *vs*. EE_2 treated fish), using the software GraphPad Prism v5.01.

3. Results

3.1. EE_2 modulates the expression of hepatic vitellogenin

The expression of the hepatic *vtg* gene was analyzed 76 dot and 93 dat to assess the endocrine disruption activity of dietary intake of EE_2. Hepatic *vtg* mRNA levels of EE_2-treated fish were much increased 76 dot compared with the untreated fish (Fig. 2A). However, no statistical differences were found at 93 dat between EE_2-treated and untreated fish (Fig. 2B).

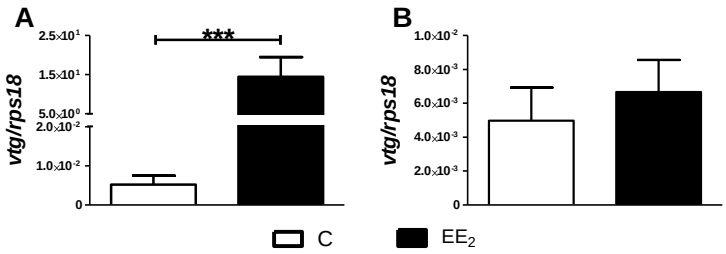


Figure 2. EE_2 increases the expression of the gene encoding vitellogenin (*vtg*) in gilthead seabream liver. RT-qPCR analysis of hepatic *vtg* mRNA levels in untreated (C, open bars) and dietary EE_2-treated fish (EE_2, solid bars) at 76 days of treatment (dot) (A) and 93 days after treatment (dat) (B).

Data are mean ± SEM of 6 individuals. The asterisks denote statistically significant differences between groups according to Student's t-test. ***p<0.001.

3.2. EE_2 modulates the estrogen receptors expression in peritoneal leukocytes

Total peritoneal leukocytes were found to express *erα* and *gper1*, assayed 1 dat (1dpp) by *RT-qPCR* (Fig. 3). Moreover, we observed that vaccination decreased the *erα* transcript levels, but not those of *gper1* (Fig. 3A), while EE_2 treatment increased the *era* and *gper1* mRNA levels in both unvaccinated and vaccinated fish (Fig. 3A, B).

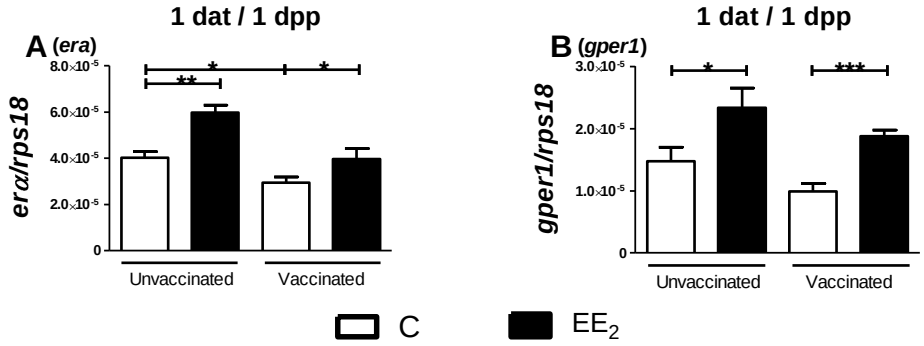


Figure 3. EE_2 alters the mRNA expression levels of the estrogen receptors *erα* and *gper1* in peritoneal exudate. RT-qPCR analysis of *erα* (A) and *gper1* (B) mRNA levels at 1 dat (1dpp) in the peritoneal exudate leukocytes of fish i.p. injected with PBS (unvaccinated) or hemocyanin plus imject alum adjuvant (vaccinated) previously exposed (EE_2, solid bars) or not (C, open bars) to dietary EE_2. dat: days after treatment; dpp: days post-priming; dpb: days post-booster. Data are mean ± SEM of 6 individuals. The asterisks denote statistically significant differences between groups according to Student's t-test. *p < 0.05, **p<0.01 and ***p>0.001.

3.3. EE_2 modulates the leukocyte recruitment of the peritoneal cavity and peritoneal leukocyte populations

Changes in the number of peritoneal exudate leukocytes and leukocyte populations produced by EE_2-treatment and vaccination were studied 1 dat (1dpp) (Fig. 4A-F). As expected, vaccination increased the number of peritoneal leukocytes (Fig. 4A). Surprisingly, dietary intake of EE_2 increased leukocyte recruitment in both vaccinated and unvaccinated fish (Fig. 4A). In addition, changes in leukocyte populations were also observed in peritoneal exudate 1 dpp (Fig. 4B-F). Firstly, the percentage of mast cells was substantially lower in vaccinated fish (Fig. 4B), while dietary EE_2 further reduced it in vaccinated fish (Fig. 4B). Secondly, neither vaccination nor dietary EE_2 promoted any modification in the percentage of acidophilic granulocytes (Fig. 4C), although it was slightly higher in EE_2-treated vaccinated than in unvaccinated fish (Fig. 4C). Finally, dietary EE_2 increased the transcript levels of

genes encoding specific markers for macrophages and IgM^+ and IgT^+ B lymphocytes (*csf1r*, *ighm* and *ight*, respectively) in both unvaccinated and vaccinated fish (Fig. 4D-4F).

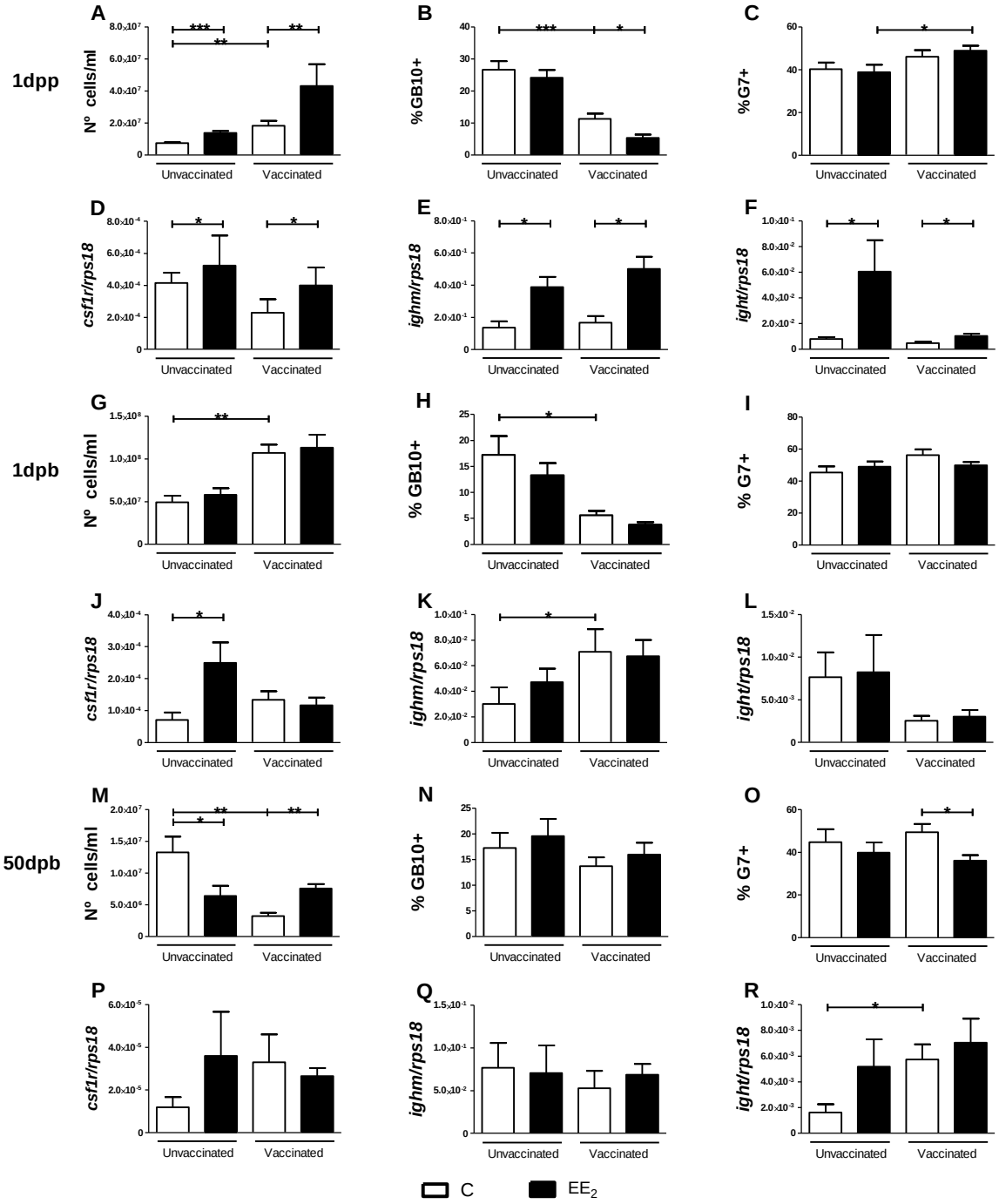


Figure 4. Modulation of peritoneal leukocyte recruitment and populations by dietary intake of EE_2. The total leukocyte numbers (A, G and M), the percentage of mast cells (MC, $GB10^+$ cells) (B, H, N), the percentage of acidophilic granulocytes (AG, G7+ cells) (C, I and O),the *csf1r* mRNA levels (D, J, P), the *ighm* mRNA levels (E, K, Q) and the *ight* mRNA levels (F, L, R) at 1, 93 and 142 dat (1 dpp, 1 dpb and 50 dpb, respectively) in the peritoneal exudate leukocytes of fish i.p. injected with PBS (unvaccinated) or with hemocyanin plus imject alum adjuvant (vaccinated) previously exposed to

dietary EE_2 (EE_2, solid bars) or not (C, open bars). dat: days after treatment; dpp: days post-priming; dpb: days post-booster. Data are mean ± SEM of 6 individuals. The asterisks denote statistically significant differences between groups according to Student's t-test. *p < 0.05, **p<0.01 and ***p>0.001.

Ninety-three days after suspending the EE_2 treatment, which coincided with 1 dpb, the picture was rather similar to 1 dat (1dpp) (Fig. 4G-4L). The most noticeable differences were that EE_2 increased the expression of *csf1r* only in unvaccinated fish (Fig. 4J), while the effect of dietary EE_2 in the transcript levels of *ighm* (Fig. 4K) and and *ight* (Fig. 4L) were no longer observed.

Finally, the total leukocyte numbers were found be reduced in vaccinated and unvaccinated and EE_2-treated fish at 142 days after EE_2 treatment, which coincided with 50 dpb (Fig. 4M). EE_2 did not produce any change in MC percentage, and *csfr1*, *ighm* and *ight* expression (Fig. 4N, P-R) but a slight decrease in the percentage of acidophilic granulocytes was found in vaccinated and EE_2-treated fish (Fig. 4O). On the other hand, vaccination induced an increase in the *ight* expression (Fig. 4R).

3.4. EE_2 alters the respiratory burst of peritoneal exudate leukocytes

The respiratory burst was analyzed 1, 93 and 142 dat (1dpp, 1dpb and 50dpb, respectively) in peritoneal exudate leukocytes (Fig. 5). ROS production robustly increased 1 dat (1 dpp) and 142 dat (50dpb) in vaccinated fish, while was slightly inhibited at 93 dat (1dpb) (Fig. 5A-5C). In addition, dietary intake of EE_2 significantly induced ROS production 1 dat in unvaccinated fish (Fig. 5A), while had no effect at 93 and 142 dat (Fig. 5B and 5C). However, dietary EE_2 slightly decreased ROS production in vaccinated fish 93 and 142 dat (Fig. 5B and 5C).

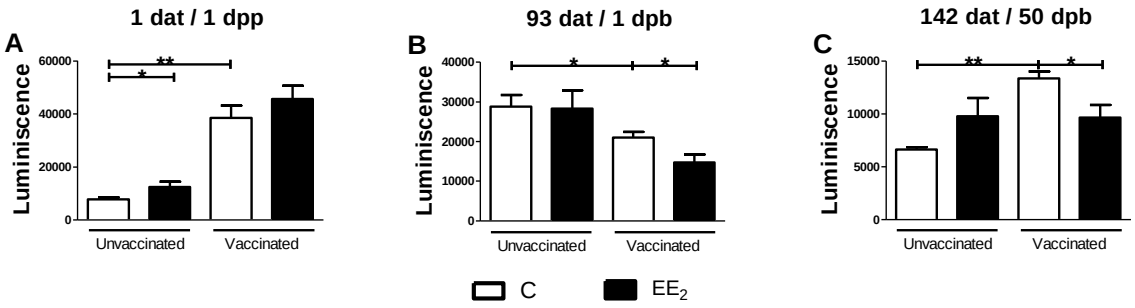


Figure 5. Dietary intake of EE_2 modulates the respiratory burst activity of peritoneal exudate leukocytes. ROS production triggered by PMA was measured as maximum values of luminol-dependent luminescence at 1 (A), 93 (B) and 142 (C) dat (1 dpp, 1 dpb and 50 dpb, respectively) generated by total peritoneal exudate leukocytes of fish i.p. injected with PBS (unvaccinated) or with hemocyanin plus imject alum adjuvant (vaccinated) previously exposed to dietary EE_2 (EE_2, solid

bars) or not (C, open bars). dat: days after treatment; dpp: days post-priming; dpb: days post-booster. Data are mean ± SEM of 6 individuals. The asterisks denote statistically significant differences between groups according to Student's t-test. *p < 0.05 and **p<0.01.

3.5. EE_2 alters peritoneal *il1b* and *il10* mARN gene expressions

The mRNA levels of the genes encoding the pro-inflammatory IL-1β and the anti-inflammatory IL-10 were analyzed by RT-qPCR in total peritoneal exudate leukocytes 1, 93 and 142 dat (1 dpp, 1 dpb and 50 dpb, respectively) (Fig. 6). At 1 dpp (Fig. 6A) and, to some extent at 50 dpb (Fig. 6C), *il1b* expression was found to be higher in vaccinated fish. Strikingly, dietary intake of EE_2 strongly increased *il1b* transcript levels 1 (Fig. 6A) and 142 dat (Fig. 6C) in unvaccinated fish, while had no effect in vaccinated fish at any of the time analyzed (Fig. 6A-6C). On the other hand, *il10* expression was not altered at 1 (Fig. 6D) and 93 dat (Fig. 6E), but increased at 142 dat in vaccinated fish and decreased in vaccinated and EE_2 treated fish (Fig. 6F).

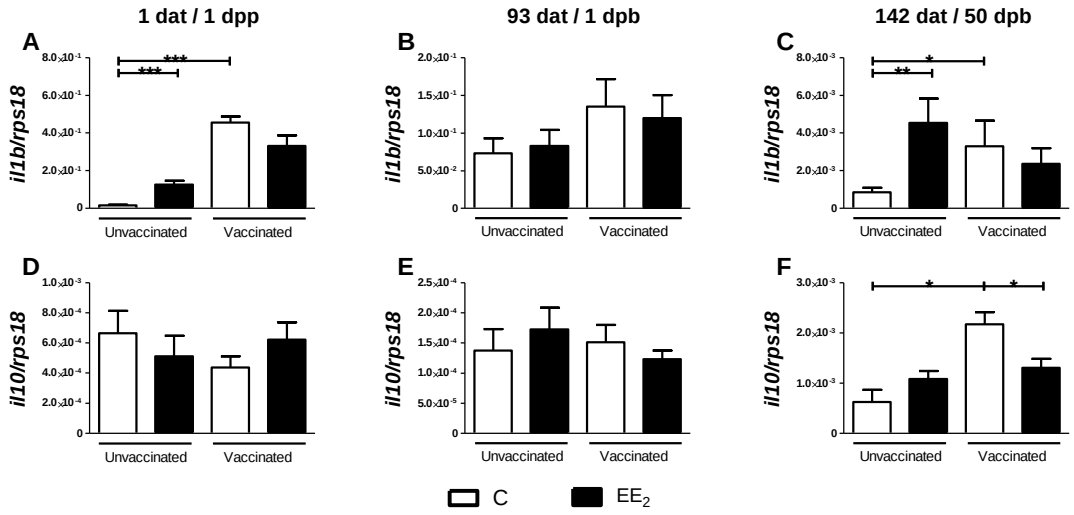


Figure 6. Dietary intake of EE_2 alters *il1b* and *il10* gene expressions in peritoneal exudate cells of unvaccinated fish. The mRNA levels of the genes encoding IL-1β and IL-10 was analyzed by RT-qPCR at 1 (A and D), 93 (B and E) and 142 (C and F) dat (1 dpp, 1 dpb and 50 dpb, respectively) in total peritoneal exudate leukocytes of fish i.p. injected with PBS (unvaccinated) or with hemocyanin plus imject alum adjuvant (vaccinated) previously exposed to dietary EE_2 (EE_2, solid bars) or not (C, open bars). dat: days after treatment; dpp: days post-priming; dpb: days post-booster. Data are mean ± SEM of 6 individuals. The asterisks denote statistically significant differences between groups according to Student's t-test. *p < 0.05, **p<0.01 and ***p<0.001.

3.6. EE_2 diminishes IgT natural antibody levels specific to hemocyanin

The IgM and IgT antibody titer specifically against hemocyanin was measured in the culture supernatant of total PE cells at 1, 93 and 142 dat (1dpp, 1dpb and 50dpb, respectively)

(Fig. 7). The statistical analysis of the IgM antibody levels against hemocyanin in PE supernatant at all studied time points pointed out that EE_2 or vaccination do not alter the IgM antibody titer in PE supernatant (Fig. 7A, C and E). However, IgT antibody was diminished in vaccinated fish at 1dat (1dpp) (Fig. 7B). Surprisingly, and although not changes were found before (Fig. 7B and 7D), the IgT natural antibody levels against hemocyanin were decreased in unvaccinated EE_2-treated fish at 142 dat (50dpb) (Fig. 8F)).

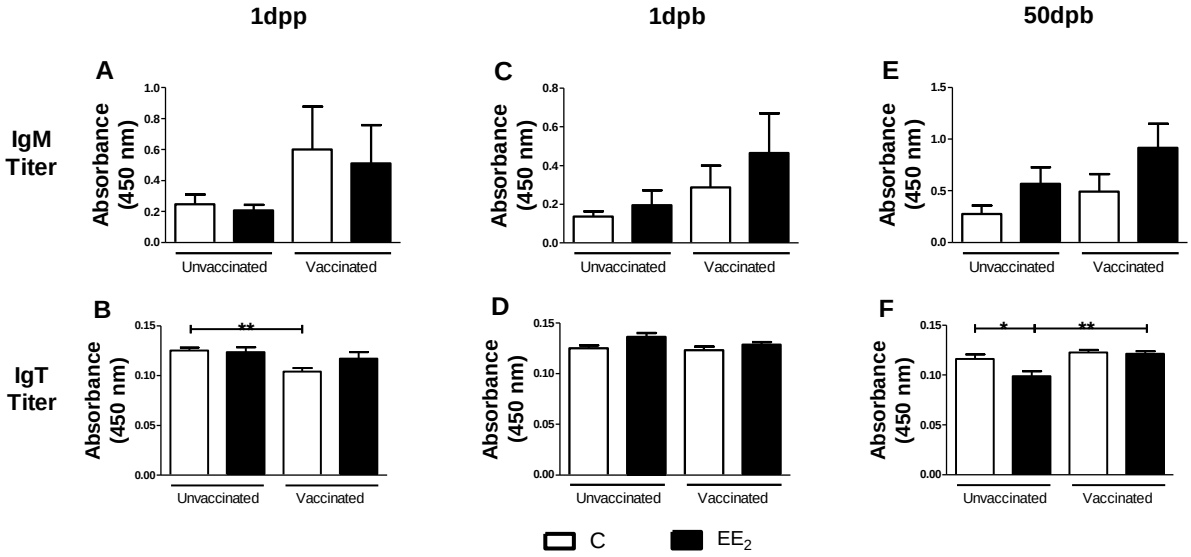


Figure 7. Dietary intake of EE_2 reduces the IgT natural antibody levels against hemocyanin. The specific IgM and IgT titer against hemocyanin was measure cells at 1 (A and B), 93 (C and D) and 142 (E and F) dat (1dpp, 1dpb and 50dpb, respectively) in the supernatant of total peritoneal exudate of fish i.p. injected with PBS (unvaccinated) or with hemocyanin plus imject alum adjuvant (vaccinated) previously exposed to dietary EE_2 (EE_2, solid bars) or not (C, open bars). dat: days after treatment; dpp: days post-priming; dpb: days post-booster. Data are mean ± SEM of 6 individuals. The asterisks denote statistically significant differences between groups according to Student's t-test. *$p < 0.05$, **$p<0.01$ and ***$p<0.001$.

3.7. EE_2 alters the expression of histamine receptors

The mRNA expression levels of the genes encoding for *hrh1*, *hrh2* and *hrh3* were analyzed at 1 dat (1dpp) (Fig. 8A-C), 93 dat (1dpb) (Fig. 8D-F) and 142 dat (50 dpb) (Fig. 8G-I) in peritoneal exudate leukocytes. On the one hand, at 1 dat (1dpp), the EE_2 treatment (only in unvaccinated fish) and vaccination were seen to have significantly decreased the *hrh2* expression levels (Fig. 8B), while vaccination did the same with the expression of *hrh3* (Fig. 8C). On the other hand, *hrh1* (Fig. 8D) and *hrh3* (Fig. 8I) expression was significantly up-regulated by vaccination, at 1dpb and 50 dpb, respectively.

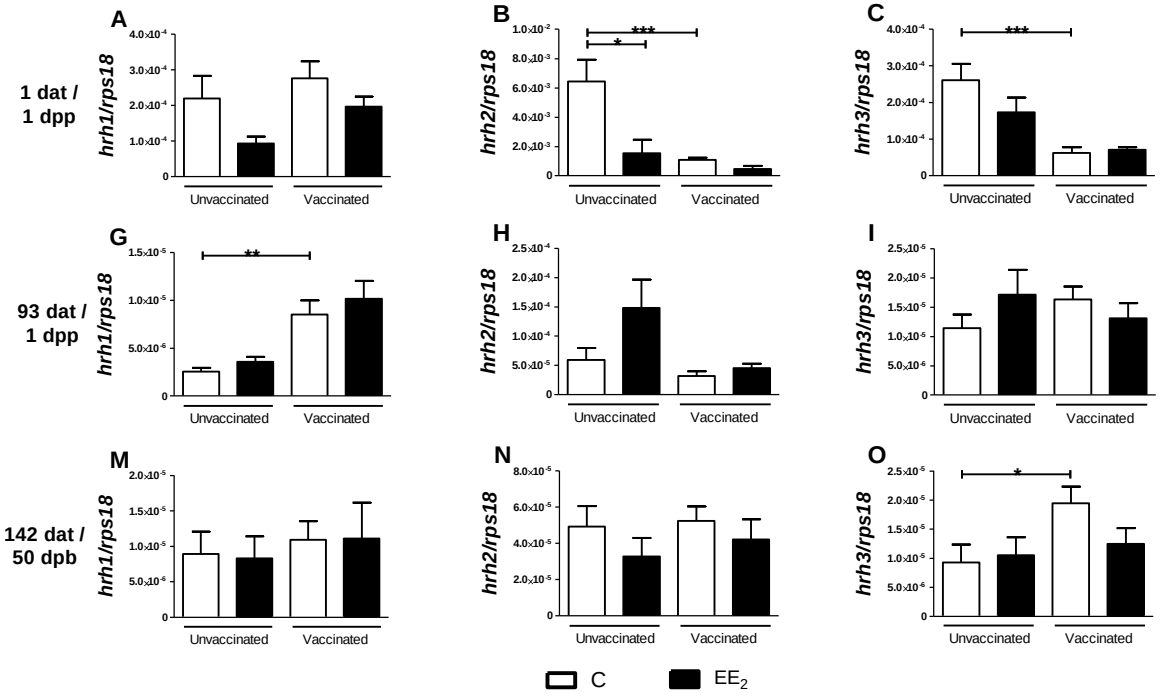


Figure 8. EE_2 and vaccination modulate histamine receptors expression in peritoneal leukocytes. Histamine receptors 1, 2 and 3 expression were analyzed in peritoneal exudate leukocytes at 1 (A, B and C), 93 (D, E and F) and 142 (G, H and I) dat (1 dpp, 1 dpb and 50 dpb, respectively) in total peritoneal exudate leukocytes of fish i.p. injected with PBS (unvaccinated) or with hemocyanin plus imject alum adjuvant (vaccinated) previously exposed to dietary EE2 (EE2, solid bars) or not (C, open bars). dat: days after treatment; dpp: days post-priming; dpb: days post-booster. Data are mean ± SEM of 6 individuals. The asterisks denote statistically significant differences between groups according to Student's t-test. *p < 0.05, **p<0.01 and ***p<0.001.

4. Discussion

Xeno-estrogens EDCs are able to mimic or antagonize the action of natural estrogens. That, as well as other sex steroids, have demonstrated effects beyond the reproductive context, with drastic effects along molecular to organism levels, particularly on aquatic animals (Castillo-Briceno and Kodjabachian, 2014). Despite, most fish studies on these compounds have focused on the alterations they produced on the reproductive system, it is also known that they can also alter other aspects of fish biology, including immunity (Filby et al., 2007). In this study, we analyze the effect that EE_2 has on the immune response of a marine hermaphrodite species, the gilthead seabream, focusing on peritoneal exudate leukocytes and the histamine signaling pathway.

First of all, EE_2 promotes a significant increase in hepatic *vtg* gene expression in gilthead seabream, which is considered as a maker of endocrine disruption for estrogenic exposure in male fish (Sumpter and Jobling, 1995), as previously demonstrated in gilthead seabream, both in adults and juveniles, and in other fish species (Cabas et al., 2012; Kausch et al., 2008; Rodenas et al., 2016). Notably, this increase disappeared when the treatment ceased, as we also previously described (Rodenas et al., 2015, 2016). Moreover, we have observed that ERs, both nuclear ERs and membrane-anchored GPER1, are expressed in peritoneal exudate leukocytes, as described for both mammal and fish immune cells (Blasko et al., 2009; Cabas et al., 2013b; Liarte et al., 2011c; Straub, 2007), and that EE_2 increased the expression of both receptors. The present results extend these earlier observations by demonstrating that (i) EE_2 would exert its effect on gilthead seabream peritoneal leukocyte biology not only through the classical nuclear receptor pathway but also through the novel GPER1 and (ii) EE_2 would increase estrogen signaling in these cells by inducing ER receptors. In addition, it is also interesting that vaccination decrease the expression of gene encoding ERα and does not affect that of GPER1 in peritoneal exudate leukocytes, while increases the expression of both genes in the head kidney of this species (Cabas et al., 2012, 2013b). Such differences may be due to the alterations of different leukocyte populations after an immune challenge (Chaves-Pozo et al., 2004, 2005a; García-Castillo et al., 2002).

One of the most interesting observations of this study is that dietary intake of EE_2 was able to promotes leukocyte recruitment to the peritoneal cavity and the expression of *csf1r*, *ighm* and *ight*, what strongly suggests that macrophages and B lymphocytes were recruited to the peritoneal cavity of unvaccinated fish, as occurred in the gonad of gilthead seabream exposed to dietary EE_2 (Cabas et al., 2011) and in agreement with the changes observed in leukocyte populations of Japanese sea bass blood after E_2 treatment (Thilagam et al., 2009). Nevertheless, once treatment had ceased, *cfsr1* gene expression remained at high levels for 3 months in EE_2-unvaccinated fish, despite the number of total peritoneal exudate leukocyte returned to basal levels. Moreover, EE_2 increased the respiratory burst and the expression of the gene encoding IL-1β of peritoneal exudate leukocytes in unvaccinated fish, even 3 months after ceasing the treatment. These data are in agreement with the induction of ROS production in blood leukocytes of Japanese sea bass promoted by E_2 (Thilagam et al., 2009) and the induction of *il1b* expression levels in the head kidney of gilthead seabream exposed to dietary EE_2 (Cabas et al., 2012). Similarly, other xeno-estrogens, such as bisphenol A, nonylphenol and different phthalates, were found to increase the production ROS production by common carp phagocytic cells (Gushiken et al., 2002; Watanuki et al., 2003). The biological

consequences of the long lasting effects of dietary intake of EE_2 shown here for the first time deserve further investigations. It is tempting to speculate that epigenetic mechanisms are involved (Bhandari et al., 2015), since the endocrine disruption effect of EE_2 is over at this time, assayed as hepatic *vtg* expression.

The effect of dietary intake of EE_2 in vaccinated fish was also interesting, although weaker than the observed in their unvaccinated counterparts. EE_2 exerted its greatest effects in the total number of leukocytes after 1 dpp, as occurred in unvaccinated fish, but further decreased the percentage of mast cells. Therefore, EE_2 showed a synergistic effect with vaccination increasing the total number of leukocytes and reducing mast cell abundance. In contrast, other EDCs, such as PCB 126, have been shown to increase the abundance of mast cells in gills and intestine of gilthead seabream (Lauriano et al., 2012).

Surprisingly, we also observed that EE_2 reduced the hemocyanin-specific IgT levels in peritoneal leukocyte supernatant several months after ceasing the treatment in unvaccinated fish, once the EE_2-mediated endocrine disruptor effect had disappeared. This result suggests that EE_2 decreases the capability of fish to regulate the production of natural antibodies, as has been observed for others xeno-estrogens. For instance, the ER antagonist PCB 126 increased the titer of specific antibodies secreting cells against *Edwardsiella ictaluri* in channel catfish (Rice and Schlenk, 1995) but decreased the antibody number against *Vibrio anguillarum* in Chinook salmon (Regala et al., 2001) and E_2 increased the antibody response in mammals (Sthoeger et al., 1988).

Finally, dietary of EE_2 reduced the *hrh2* mRNA levels after 76 days of treatment in unvaccinated fish, while no modulation was observed once the treatment had ceased or in EE_2-treated and vaccinated fish. These results indicate that EE_2 could alter the histamine signaling pathway in MC by modulating their functions. Recent studies have demonstrated that xenoestrogens induce mast cell degranulation and activation in rodent mast cells (Mizota and Ueda, 2006; Narita et al., 2007; O'Brien et al., 2014; Rajkovic et al., 2014; Uchida et al., 2003) and human mast cells (Kennedy et al., 2012; Narita et al., 2007). Moreover, in this study we confirmed that EE_2-treated fish showed reduced growth (data not shown), as has been previously described by others authors (Baumann et al., 2014; Rodenas et al., 2016; Shved et al., 2008), which seems to suggest that EE_2 modulates the appetite through alterations in the histamine signaling pathway, as previous related data pointed out. For example, the estrogenic EDCs endosulfan and atrazine modulate appetite through brain histamine receptors (Giusi et al., 2010). In addition, the HRH1 and HRH2 agonist cimetidine

commonly used as a treatment of gastrointestinal disorders can also acts as an EDC (García-García et al., 2016; Lee et al., 2015).

Vaccination also induces changes in the expression of the gene encoding for the HR. After priming, vaccination induced a reduction in the *hrh2* expression (as it happened in Chapter II), similar to that produced by the dietary intake of EE_2, and in the *hrh3* expression. However, some months after the treatment, vaccination increased the expression of *hrh1* and *hrh3*. Further studies must be done to determine the modulation of the HRs after an immunity challenge.

To conclude, our data point to a very complex role of estrogens in fish immunity, as it is widely accepted in mammals (Straub, 2007). We found that dietary intake of EE_2 promotes a long lasting inflammatory response in the peritoneal cavity of unvaccinated gilthead seabream juveniles after ceasing the treatment and even though its endocrine disruption effect was over. However, the impact of dietary EE_2 in vaccinated fish was rather minor and transient. These results, therefore, paves the way to futures studies aimed at understanding the molecular mechanisms involved in the long lasting effect of EDCs in fish immunity.

CONCLUSIONS

1. The protocol developed to isolate gilthead seabream peritoneal mast cells allowed 95% pure mast cell fractions to be obtained.

2. Gilthead seabream peritoneal mast cells contain histamine and compound 48/80 induces its release. Moreover, genomic DNA of *Vibrio anguillarum* induces the expression of the genes encoding for IL-1β and IL-8 in these cells.

3. Histamine and compound 48/80 modify the peritoneal and head kidney leukocyte populations when intraperitoneally injected, but only compound 48/80 induces peritoneal leukocyte ROS production. In addition, histamine reduces the production of IgM levels, when combined with the bacterium *V. anguillarum*.

4. Gilthead seabream peritoneal leukocytes express the genes encoding the histamine receptors Hrh1, Hrh2 and Hrh3. Both histamine and compound 48/80, when intraperitoneally injected, inhibit the expression of *hrh2*.

5. Although histamine regulates the innate and adaptive immunity of gilthead seabream, neither histamine nor compound 48/80 can be regarded as an efficient as adjuvant for gilthead seabream intraperitoneal vaccination.

6. GB10, a monoclonal antibody, is able to recognize a surface receptor of gilthead seabream peritoneal mast cells and, besides, rapidly induces their death via oncosis.

7. Gilthead seabream peritoneal leukocytes express the genes encoding for nuclear estrogen receptor α and GPER1, the expression of both being induced by the dietary intake of EE_2.

8. The dietary intake of EE_2 induces an inflammatory response in gilthead seabream peritoneal exudate, in which an increase in peritoneal leukocyte numbers and in the expression of *csf1r*, *ighm*, *ight* and *il1b* is observed together with an enhancement of ROS production. Furthermore, the dietary intake of EE_2 decreases the expression of *hrh2* and modulates the production of specific IgT antibodies against hemocyanin in unvaccinated fish.

9. Some of the effects of the dietary intake of EE_2 were largely maintained for several months after cessation of treatment.

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RESUMEN EN CASTELLANO

a. RESUMEN.

La dorada (*Sparus aurata* L.) es un pez teleósteo marino de gran valor comercial en el sector de la acuicultura en el área Mediterránea. Nuestro grupo de investigación ha realizado numerosos estudios sobre la respuesta inmunitaria de dorada que se han centrado desde la caracterización funcional de tejidos y células del sistema inmunitario (Cabas y col., 2013b; Roca et al., 2006, entre otros) hasta el estudio de los mecanismos de regulación de la inflamación (Angosto y col., 2012; Candel y col., 2014, 2016; Castillo-Briceno y col., 2009, 2010; Cuesta y col., 2007b; Chaves-Pozo y col., 2004, 2005a; Esteban y col., 2013, 2015; López-Castejón y col., 2007, 2008; Mulero y col., 2007b, 2008a, 2008b; Pelegrín y col., 2004; Roca y col., 2006, 2007; Sepulcre y col., 2007, 2009, entre otros).

Entre los resultados más innovadores e interesantes para el desarrollo de esta Tesis Doctoral destaca que la dorada fue la primera especie, no mamífero, en la que se ha encontrado histamina en los gránulos de sus células cebadas y que esta participa en la regulación de la respuesta inflamatoria (Mulero y col., 2007a). En este sentido, y teniendo en cuenta que el exudado peritoneal de dorada está compuesto principalmente por granulocitos acidófilos, macrófagos, linfocitos y células cebadas y que en él se puede encontrar un número elevado de células cebadas (Meseguer y col., 1993) como sucede en rata (Diamant, 1990), podríamos llevar a cabo ensayos *in vivo* e *in vitro*, con número suficiente de células cebadas, que nos permitiesen avanzar en el conocimiento de su implicación en la respuesta inflamatoria de peces. Sin embargo, carecemos de herramientas que permitan la caracterización de las células cebadas y desconocemos la relevancia funcional de la histamina en peces. Otro de los aspectos, en los que se ha desarrollado una amplia actividad investigadora en dorada, ha sido el de determinar el efecto de los estrógenos, naturales (17β-estradiol, E_2) y sintéticos (17α-etinilestradiol, EE_2), componente activo de la mayoría de píldoras anticonceptivas y terapias hormonales de reemplazamiento presente en medios acuáticos), sobre la respuesta inmunitaria haciendo especial hincapié en la respuesta de granulocitos acidófilos y macrófagos de riñón cefálico (Cabas y col., 2011, 2012, 2013b; Liarte y col., 2011a, 2011b, 2011c; Rodenas y col., 2015, 2016).

Con estos antecedentes, la presente Tesis Doctoral se ha centrado en la caracterización de las células cebadas del exudado peritoneal y en el papel de la histamina en dorada. La investigación de la Tesis Doctoral se ha llevado a cabo secuencialmente en cuatro bloques:

1) En el primer trabajo diseñamos un protocolo de aislamiento de células cebadas peritoneales de dorada. Teniendo en cuenta que el exudado peritoneal de dorada está

compuesto principalmente por granulocitos acidófilos, macrófagos, linfocitos y células cebadas, el protocolo consistió en realizar: i) gradientes de densidad discontinuos y centrifugación, aprovechando las diferencias de tamaño celular de estos tipos celulares, ii) cultivo celular, gracias a las distintas capacidades de adhesión celular que presentan estas células y iii) separación celular activada magnéticamente (MACS), utilizando un anticuerpo específico contra granulocitos acidófilos de dorada. Así obtuvimos poblaciones enriquecidas en células cebadas del exudado peritoneal de dorada con una pureza superior al 95%. Estas poblaciones fueron analizadas por citometría de flujo y microscopía electrónica de transmisión. A continuación, en la fracción enriquecida de células cebadas se analizó la presencia de histamina, la capacidad de liberarla y el perfil de expresión génica de factores mediadores clave en la inmunidad innata. Así se demostró, mediante citometría de flujo, la presencia de histamina en las células cebadas peritoneales y, mediante ELISA, la capacidad para liberar dicha histamina en presencia del compuesto 48/80, conocido activador de las células cebadas. Además, mediante RT-qPCR, se comprobó que el ADN bacteriano, ADN genómico de *Vibrio anguillarum* (*Va*DNA), induce la expresión de los genes que codifican la interleuquina (IL) 1β e IL-8. El protocolo de aislamiento de células cebadas funcionales diseñado supone una herramienta metodológica muy importante que nos permitirá el estudio de los mecanismos inflamatorios en vertebrados.

2) A continuación analizamos el papel de la histamina en la inmunidad innata y adaptativa de dorada. Para ello, ejemplares de dorada fueron inyectados intraperitonealmente con histamina o con el compuesto 48/80 solos o en combinación con la bacteria atenuada *Vibrio anguillarum*. Pasados 49 días, se volvió a realizar el mismo protocolo de inyección de histamina o compuesto 48/80, combinados o no, con la bacteria atenuada *V. anguillarum*. Un día después de las primeras inyecciones, analizamos las poblaciones celulares del exudado peritoneal y del riñón cefálico mediante citometría de flujo y microscopía electrónica de transmisión. Treinta días después de las segundas inyecciones (79 días tras el inicio del experimento), analizamos la producción sérica de inmunoglobulina (Ig) M específica contra *V. anguillarum* mediante ELISA. Así, comprobamos que la histamina y el compuesto 48/80 modulan las poblaciones celulares presentes en la cavidad peritoneal y en el riñón cefálico pero solo el compuesto 48/80 induce la producción de reactivos intermediarios de oxígeno (ROS) de los leucocitos peritoneales. Por otra parte, la histamina, pero no el compuesto 48/80, altera la respuesta inmunitaria adaptativa humoral al disminuir la producción de IgM específica contra *V. anguillarum*. Además, los leucocitos del exudado peritoneal expresan los genes que codifican los receptores de histamina (HRs) 1, 2 y 3 (*hrh1*, *hrh2* y *hrh3*) y tanto la

histamina y el compuesto 48/80 como la inmunización inhiben la expresión del gen que codifica *hrh2*. En conjunto, estos resultados indican que la histamina juega un papel importante en la inmunidad innata y adaptativa de dorada y que tanto la histamina como el compuesto 48/80 son seguros pero no son eficientes como adyuvantes en la vacunación de dorada.

3) En el tercer trabajo generamos una nueva herramienta para el estudio de las células cebadas de dorada, en concreto, un anticuerpo monoclonal específico de células cebadas de dorada. El anticuerpo fue obtenido en ratones BALB/c inmunizados repetidamente con poblaciones purificadas de células cebadas de dorada, obtenidas siguiendo el protocolo establecido en el primer trabajo de esta tesis. La línea mieloide celular SP2/0-Ag14 fue fusionada con esplenocitos de los ratones inmunizados y la producción de anticuerpos fue analizada mediante citometría de flujo. Mediante diluciones seriadas de los hibridomas capaces de producir anticuerpos contra células cebadas de dorada se llegó al aislamiento de una línea de hibridomas capaz de producir anticuerpos monoclonales específicos de células cebadas de dorada, GB10. Mediante citometría de flujo determinamos que GB10 producía la muerte de las células cebadas de exudado peritoneal. Posteriormente, y mediante citometría de flujo y microscopía electrónica de transmisión, identificamos al mecanismo de muerte celular oncosis como la causa de la mortalidad celular producida por GB10 en las células cebadas de dorada. La generación de este anticuerpo no sólo facilitará la caracterización de las poblaciones celulares de exudado peritoneal sino que también podrá ser utilizado para la generación de ejemplares de dorada deficientes en células cebadas. Hemos iniciado la obtención de nuevos anticuerpos monoclonales con cierto éxito y ya disponemos de un nuevo anticuerpo que específicamente reconoce células cebadas y que estamos caracterizando.

4) Finalmente estudiamos la capacidad del EE_2 de alterar la actividad de los leucocitos peritoneales de dorada. Para ello, el EE_2 se administró en la dieta de ejemplares juveniles de dorada durante 76 días y, a continuación, fueron alimentados con dieta comercial durante 142 días. Los ejemplares fueron inmunizados, vía intraperitoneal, con hemocianina en presencia de aluminio como adyuvante: i) 1 día después de finalizar el tratamiento y ii) a los 92 días de haber finalizado el tratamiento. Se tomaron muestras de hígado y de exudado peritoneal 1 día después de la primera inmunización y 1 y 50 días después de la segunda inmunización que fueron procesadas para citometría de flujo, luminiscencia, RT-qPCR y ELISA. El EE_2 actúa como disruptor endocrino ya que induce la expresión del gen que codifica para la vitelogenina (*vtg*) hepática y este efecto disruptor desaparece tras el cese del tratamiento, como ha sido

previamente descrito en dorada. El exudado peritoneal expresa los genes que codifican para receptores de estrógenos (ERs) y comprobamos que el EE_2 incrementa la expresión del ERα y del GPER1. El EE_2 induce una respuesta inflamatoria en el exudado peritoneal que consiste en un aumento: i) del número de leucocitos en la cavidad peritoneal, ii) en la expresión de los genes que codifican *csf1r*, *ighm*, *ight* e *il1b* y iii) en la producción de ROS. Además, el EE_2 provoca una disminución en el título de anticuerpos naturales, IgT. De forma interesante, algunos de estos efectos se mantuvieron meses después de la finalización del tratamiento. Por otro lado, observamos que el EE_2 disminuye la expresión del gen que codifica *hrh2*. Estos resultados indican que el EE_2 altera la respuesta inmunitaria de las poblaciones leucocitarias del exudado peritoneal en la dorada.

b. INTRODUCCIÓN.

La acuicultura es una de las fuentes de alimento de origen animal más importantes del mundo. Según datos de la FAO, el consumo de pescado supone un 16,7% de la proteína animal consumida por la población humana. Además, la gran producción de alimento, necesaria para una población de casi 10 millones de habitantes en 2050, supone un reto enorme para la humanidad teniendo en cuenta la limitación de los recursos naturales y la necesidad de proteger los ecosistemas naturales (APROMAR, 2016). De esta forma, la acuicultura ha surgido como una actividad necesaria para suplir las deficientes cantidades de pescado obtenidos mediante la pesca (APROMAR, 2016).

Como en cualquier sistema de producción, la eficiencia para conseguir las cantidades de pescado deseadas mediante la acuicultura depende de muchos factores, entre los que destacan la reproducción y la viabilidad de las poblaciones de peces. En este contexto, la mayoría de los estudios se han centrado en aspectos relacionados con los sistemas de reproducción, en parte olvidando el peso que supone, en términos de eficiencia, conseguir altas tasas de viabilidad en un sistema de producción animal. La producción de pescado de acuicultura supone el confinamiento de un gran número de individuos en un espacio limitado, condiciones que pueden provocar la aparición de enfermedades infecciosas y, por consiguiente, la pérdida de cantidades importantes de beneficios económicos. De esta manera, el estudio del sistema inmunitario de peces se vuelve imprescindible para alcanzar con éxito los objetivos planteados en acuicultura.

En términos de evolución, los peces tienen un papel clave ya que suponen el primer grupo animal que posee un sistema inmunitario innato y adaptativo, al igual que los vertebrados superiores (Rubio-Godoy, 2010; Uribe y col., 2011). A pesar de diferencias importantes entre peces y mamíferos, como el hecho de que los mamíferos hayan desarrollado fuertemente su respuesta adaptativa mientras que el sistema inmunitario de peces depende principalmente de su respuesta innata, en ambos grupos de animales tanto la respuesta innata como la adaptativa están constituidas por un componente celular, los leucocitos, y un componente humoral, los mediadores inflamatorios y los anticuerpos. Los principales leucocitos que median en la respuesta innata de peces son neutrófilos o granulocitos acidófilos, macrófagos, células citotóxicas no específicas y células cebadas o mastocitos mientras que en la respuesta adaptativa los principales leucocitos son linfocitos B y T. A diferencia de los mamíferos, los peces teleósteos no poseen médula ósea, siendo el riñón cefálico el principal órgano hematopoyético, en donde encontramos neutrófilos o granulocitos

acidófilos, macrófagos, linfocitos y células precursoras de estos y otros tipos de leucocitos. Por su parte, las células cebadas no se encuentran en los tejidos hematopoyéticos sino que sus precursores viajan desde éstos hasta los diferentes tejidos donde se diferencian para dar lugar a distintas clases de células cebadas. Por otro lado, la respuesta innata humoral está principalmente constituida por proteínas que encontramos en la sangre (como el complemento) y por citoquinas mientras que en la respuesta adaptativa las citoquinas y anticuerpos, capaces de reconocer específicamente a un patógeno, representan el componente humoral.

Las células cebadas, también llamadas células granulares eosinofílicas (EGCs), se han conservado evolutivamente en todas las especies de vertebrados. Tanto en mamíferos como en peces, estas células inmunitarias se encuentran en la mayoría de los tejidos del cuerpo y, especialmente, en lugares estratégicos donde existe contacto con una posible entrada de patógenos como son las mucosas de la piel, del tracto digestivo y de las vías respiratorias. Existe una gran heterogeneidad en las células cebadas debido a sus significativos cambios en tamaño celular, granularidad y contenido granular, sensibilidad a fijadores o respuesta a fármacos (Crivellato y col., 2015; Reite, 1998; Reite y Evensen, 2006). Se han descrito diferentes linajes de células cebadas (Dvorak, 2005) debido a que presentan diferentes funciones dependiendo del tejido en el que se encuentren (Crivellato y col., 2015). Además, los gránulos de estas células almacenan y secretan una gran variedad de compuestos como proteasas, serotonina o histamina (Dobson y col., 2008; Mulero y col., 2007a). Pese a que las células cebadas son consideradas propias de la inmunidad innata también pueden interferir en la respuesta inmunitaria adaptativa (Da'as y col., 2011).

La histamina es una amina ampliamente estudiada en mamíferos que es, principalmente, producida, almacenada y liberada por las células cebadas y que participa en una gran variedad de procesos biológicos como la respuesta inflamatoria, la neurotransmisión, la secreción de hormonas producidas en la glándula pituitaria y la regulación gastrointestinal y del sistema circulatorio sanguíneo (Leurs y col., 1995). La histamina participa en la progresión del proceso alérgico-inflamatorio mediante la inducción de la síntesis de IL-1, IL-6 e IL-8 y la disminución de la producción del factor de necrosis tumoral (TNF-α) (Bayram y col., 1999; Meretey y col., 1991; Vannier y Dinarello, 1994; Vannier y col., 1991). La histamina también afecta a monocitos (producción de IL-1, IL1-9, IL-12, IL-18 y TNF-α), a células dendríticas (cambios en la capacidad de polarización de las células T, cambios en Ca^{+2} intracelular, polimerización de actina, quimiotaxis, contenido de AMPc intracelular y producción de IL-12

e IL-10) y a células B y T (cambios en el balance de diferentes clases de linfocitos T y en la síntesis de Ig, proliferación celular, producción de interferón γ, IL-4 e IL-13) (Banu y Watanabe, 1999; Ferstl y col., 2012; Jutel y col., 2001, 2002; Osna y col., 2001).

En las rutas de señalización de la histamina participan los HRs 1, 2, 3 y 4 (Ash y Schild, 1966; Lovenberg y col., 1999; Oda y col., 2000). Los genes que codifican para los HRH1 y HRH2 se expresan en células nerviosas, células musculares de las vías aéreas, hepatocitos, condrocitos, células epiteliales, neutrófilos, eosinófilos, monocitos y linfocitos B y T (Leurs y col., 1995). Por su parte, el HRH3 se ha encontrado en neuronas del cerebro y en algunos tejidos periféricos (Jutel y col., 2002). Finalmente, el HRH4 ha sido descrito en células hematopoyéticas, incluyendo neutrófilos, eosinófilos, células T colaboradoras, basófilos y células cebadas (Liu y col., 2001; Zhu y col., 2001). Se ha descrito que, en ausencia de la enzima histidina descarboxilasa, necesaria para la síntesis de histamina, y los receptores HRH1 o HRH4, se inhibe la producción de IL-6 (Desai y Thurmond, 2011; Horvath y col., 2002). Además, la histamina induce la producción de IL-10 a través del receptor HRH2 tanto en células dendríticas como en células T (Jutel y col., 2002; Osna y col., 2001) y la activación del HRH4 induce la acumulación de células inflamatorias (eosinófilos y células cebadas) y la inflamación alérgica (Akdis y Simons, 2006). La histamina también modula el sistema inmunitario adaptativo, alterando la proliferación de las células B (a través de HRH1) y la producción de anticuerpos (a través de HRH1 e HRH2) (Banu y Watanabe, 1999; Jutel y col., 2001). En general, se considera que el HRH1 tiene un papel pro-inflamatorio en la inmunidad incrementando la migración de células al área de inflamación y que el HRH2 participa como mediador anti-inflamatorio mientras que todavía no se conoce bien qué papel, pro- o anti-inflamatorio, tendría el HRH3 y el HRH4 (Akdis y Simons, 2006).

Durante décadas se ha pensado que la histamina era exclusiva de vertebrados superiores aunque diferentes estudios habían demostrado cómo determinados agentes eran capaces de inducir la activación de las células cebadas en vertebrados inferiores. Por ejemplo, en presencia de toxinas de *Aeromonas salmonicida* se produce un descenso del contenido de histamina en el intestino y un aumento en sangre además de la degranulación de las EGCs de las paredes intestinales en trucha (Ellis, 1985). Posteriormente, se concluyó que *A. salmonicida*, el compuesto 48/80 (conocido activador de las células cebadas en mamíferos) y la concavalina A inducen la degranulación de las EGCs en el intestino posterior y en el recto de trucha mientras que los anti-histamínicos, prometacina y cimetidina, la inhiben (Vallejo y Ellis, 1989). Por otro lado, cuando ejemplares de trucha eran intraperitonealmente inyectados

con sustancia P y capsaicina se inducía la degranulación de las EGCs del intestino (Powell y col., 1991). Además, la sustancia P y el compuesto 48/80 aumentan la resistencia en los vasos sanguíneos branquiales en trucha, indicando su posible contracción (Reite, 1997). También se demostró que inyecciones de *Escherichia coli* inactivada, proteosa, pectona, compuesto 48/80 y, en menor medida, solución salina balanceada de Hanks (HBSS) en la vejiga natatoria de tilapia causan la degranulación de las EGCs y un rápido aumento de neutrófilos en la cavidad peritoneal (Matsuyama e Iida, 1999). Además, aunque se piensa que los peces no son capaces de producir IgE (Bengten y col., 2006), las células cebadas de peces expresan el receptor de alta afinidad FcεRI (Galli y col., 2005a) que, en mamíferos, es activado por IgE y cuya activación supone el inicio del proceso de liberación de histamina de las células cebadas.

Hace 9 años, y mediante técnicas immunohistoquímicas, se describió por primera vez, en nuestro grupo de investigación, que las células cebadas presentes en el intestino y en las branquias de dorada almacenan y liberan histamina y que esta es biológicamente activa y participa en la regulación de la respuesta inflamatoria de esta especie (Mulero y col., 2007a). Estos autores también demostraron que la histamina regula la capacidad fagocítica de los granulocitos acidófilos a través del Hrh1 y Hrh2 y que la supervivencia de la dorada se veía comprometida cuando eran inyectadas con dosis altas de histamina o del compuesto 48/80 y de un agonista del Hrh2. Desde entonces se han realizado algunos avances para determinar el papel de la histamina en vertebrados inferiores. Ese mismo año se describieron tres HRs en pez cebra, Hrh1, Hrh2 y Hrh3, que se expresan en numerosos tejidos (Peitsaro y col., 2007). También se ha comprobado que las células cebadas presentes en el intestino de diferentes especies de peces, incluida la dorada, se degranulan en presencia del compuesto 48/80 u otros compuestos activadores de células cebadas como la sustancia P o la capsaicina (Manera y col., 2011; Mulero y col., 2007a; Powell y col., 1991).

Los estrógenos, esteroides sexuales, actúan a través de los ER nucleares (Edwards, 2005) pero la relativamente reciente identificación de GPER1 (Filardo y col., 2002; Revankar y col., 2005; Thomas y col., 2005) ha abierto la posibilidad de explorar efectos adicionales. En mamíferos, existen dos ERs nucleares, ERα y ERβ (Thomas y col., 2010), y uno de membrana, GPER1 o GPR30 (Filardo y col., 2000). Por el contario, en peces encontramos el ERα y dos variantes del receptor ERβ, ERβ-I y ERβ-II (Nelson y Habibi, 2013), además del receptor de membrana GPER1 (Cabas y col., 2013b). Se sabe que los estrógenos modulan la respuesta inmunitaria (Straub, 2007). De hecho, los ERs, tanto los nucleares como el GPER1, se expresan en células del sistema inmunitario de peces, incluida la dorada (Cabas y col.,

2013b; Liarte y col., 2011b; Lynn y col., 2008; Pinto y col., 2006; Rodenas y col., 2015; Shved y col., 2009). Estudios realizados en peces han puesto de manifiesto que los estrógenos modulan la expresión de genes relevantes en inmunidad (Casanova-Nakayama y col., 2011; Tilton y col., 2006; Williams y col., 2007), la fagocitosis (Watanuki y col., 2002; Yamaguchi y col., 2001) y la producción de ROS (Watanuki y col., 2002), los niveles de Ig en sangre (Cuesta y col., 2007a; Hou y col., 1999; Saha y col., 2002; Suzuki y col., 1997) e incrementan la susceptibilidad contra patógenos (Casanova-Nakayama y col., 2011; Wang y Belosevic, 1994). En dorada se ha observado que el incremento endógeno de 17β-estradiol (E_2) en suero produce una migración de granulocitos acidófilos hacia la gónada (Chaves-Pozo y col., 2008a) y que el tratamiento con E_2 incrementa la actividad del complemento y la actividad peroxidasa y disminuye los niveles totales de IgM sanguíneos (Cuesta y col., 2007a), modula la producción de ROS y de IL-1β (Chaves-Pozo y col., 2003), inhibe la fagocitosis (Liarte y col., 2011b), modula la respuesta inflamatoria a través de la activación de células endoteliales (Liarte y col., 2011a) y modifica el perfil de expresión de genes relacionados con la inmunidad en macrófagos (Liarte y col., 2011c). Además, y aunque no se conocen publicaciones previas centradas en células cebadas de peces, diversas investigaciones han puesto de manifiesto que las células cebadas de mamíferos expresan ERs (Nicovani y Rudolph, 2002; Vliagoftis y col., 1992) y que su exposición a estrógenos modula su degranulación (Harnish y col., 2004; Spanos y col., 1996; Theoharides y col., 1993; Vliagoftis y col., 1992), la liberación de histamina (Cocchiara y col., 1990, 1992), β-hexosamininidasa (Zaitsu y col., 2007) o serotonina (Vliagoftis y col., 1992), el número de células cebadas, la concentración de histamina y la expresión de ERs en la glándula mamaria (Jing y col., 2012). También se ha detectado que en hembras, que presentan mayores niveles de estrógenos, aumenta el número de casos de enfermedades mediadas por células cebadas, como son el asma y las migrañas, en comparación con los machos (De Marco y col., 2002; Loewendorf y col., 2016; Mannino y col., 2002; Schatz y Camargo, 2003).

En la actualidad existe una gran preocupación por la presencia y posibles efectos de disruptores endocrinos (EDCs) de origen antrópico en el medio acuático. Se conoce como EDCs a aquellas sustancias exógenas capaces de alterar funciones del sistema endocrino y, en consecuencia, causar daños en la salud de un organismo, de su progenie o de poblaciones de organismos (UNEP/WHO, 2013). Los EDCs son capaces de imitar a las hormonas endógenas a través de su interacción con receptores específicos o con proteínas específicas que controlan de alguna manera la producción de hormonas (UNEP/WHO, 2013). Se ha demostrado en peces que los EDCs estrogénicos imitan el comportamiento de los estrógenos a través de su

interacción con los ERs (Milla y col., 2011; UNEP/WHO, 2013) y elevan las concentraciones de vitelogenina (Kausch y col., 2008; Rodenas y col., 2015, 2016). Al igual que los estrógenos endógenos, los EDCs modulan la respuesta inmunitaria alterando el número de leucocitos (Liney y col., 2006; Rice y Schlenk, 1995; Schwaiger y col., 2000; Yin y col., 2007), la producción de ROS (Gushiken y col., 2002; Jin y col., 2010; Rice y col., 1996; Watanuki y col., 2003; Yin y col., 2007), la fagocitosis (Gushiken y col., 2002), la expresión de genes que codifican componentes humorales de la inmunidad innata (Baldwin y col., 2005; Jin y col., 2010; Quabius y col., 2005; Rodenas y col., 2015, 2016; Ruggeri y col., 2008) y los niveles de anticuerpos (Regala y col., 2001; Rodenas y col., 2016). Nuestro grupo de investigación ha publicado diferentes estudios en los que se demuestra cómo el 17α-etinilestradiol (EE_2) actúa como disruptor endocrino. En este sentido, se ha descrito que el EE_2 induce la producción de vitelogenina hepática (Cabas y col., 2012; García-Hernández y col., 2016; Rodenas y col., 2015, 2016), altera la capacidad de los peces para responder apropiadamente a una infección aunque no se comporta como una sustancia inmunosupresora (Cabas y col., 2012, 2013a; Rodenas y col., 2015, 2016). Además, el EE_2 induce la infiltración de granulocitos acidófilos y células B en la gónada e incrementa la expresión de moléculas relacionadas con la adhesión y con la infiltración de leucocitos (Cabas y col., 2011). Los EDCs también alteran la respuesta inmunitaria de las células cebadas de mamíferos. Se ha observado que diversos EDCs inducen la degranulación y activación de células cebadas (Kennedy y col., 2012; Mizota y Ueda, 2006; Narita y col., 2007; O'Brien y col., 2014; Rajkovic y col., 2014; Uchida y col., 2003) o aseveran la migraña (Vermeer y col., 2014). En peces se sabe que el PBC 126 incrementa el número de células cebadas en el intestino (Lauriano y col., 2012) y que el endosulfan y la atrazina modulan el apetito y el comportamiento motor a través de los HRs en las células del cerebro (Giusi y col., 2010). Sin embargo, no conocemos el efecto que el EE_2 tiene sobre los leucocitos peritoneales de dorada, incluyendo las células cebadas, y sobre la histamina.

La dorada es una especie de teleósteo marino de gran interés comercial en la acuicultura nacional y, especialmente, del área Mediterránea. Nuestro grupo de investigación ha trabajado intensamente con esta especie hermafrodita protándrica y ha comunicado importantes avances científicos en la respuesta inmunitaria, entre otros aspectos de su biología. Esta Tesis Doctoral supone un avance en el conocimiento de la respuesta inmunitaria de dorada, centrada en la caracterización de las células cebadas del exudado peritoneal y en los mecanismos de regulación de la liberación de histamina. Supone, además, una profundización en la línea de

trabajo que hace unos años demostró que las células cebadas de dorada contienen histamina y que esta regula la respuesta inmunitaria activando los granulocitos acidófilos

c. OBJETIVOS.

Los objetivos de esta Tesis Doctoral son los siguientes:

1. Desarrollar un protocolo de aislamiento de células cebadas del exudado peritoneal de dorada.

2. Analizar el papel de la histamina y del compuesto 48/80, activador de la liberación de histamina, como adyuvantes eficientes de vacunas intraperitoneales en dorada.

3. Producir un anticuerpo monoclonal específico de células cebadas de dorada.

4. Determinar los efectos de la administración en dieta de EE_2 en el exudado peritoneal de dorada y si estos posibles efectos desaparecen tras el cese del tratamiento.

d. PRINCIPALES RESULTADOS Y DISCUSIÓN.

i. Protocolo de aislamiento de células cebas del exudado peritoneal de dorada

La gran cantidad de estudios realizados sobre las células cebadas de mamíferos ha permitido conocer con detalle el papel de estos leucocitos en la inmunidad de vertebrados superiores (da Silva y col., 2014; Metcalfe y col., 1997; Urb y Sheppard, 2012; Yu y col., 2016). Sin embargo, las funciones y los mecanismos de activación de las células cebadas de animales vertebrados permanecen, todavía hoy, ampliamente desconocidos.

El objetivo de este trabajo fue diseñar un protocolo que permitiera el aislamiento de células cebadas del exudado peritoneal de dorada. Una vez se hubieran conseguido fracciones altamente purificadas de células cebadas, realizaríamos ensayos *in vitro* para analizar, por un lado, el mecanismo regulador de la liberación de histamina utilizando el efecto activador que el compuesto 48/80 puede ejercer en estas células y, por otro lado, la actividad funcional de estas células y para ello se utilizaría la capacidad estimuladora que el *Va*DNA tiene sobre la expresión de los mediadores inflamatorios (Sepulcre y col., 2002, 2007, 2011).

Meseguer y col. (1993) describieron que el exudado peritoneal de dorada presenta un alto número células cebadas lo que, en principio, permitiría obtener fracciones celulares con un número suficiente de células cebadas para el posterior estudio de estos leucocitos. Además, estos mismos autores determinaron que el exudado peritoneal de dorada está compuesto por granulocitos acidófilos, células cebadas, macrófagos y linfocitos. Con estos antecedentes, el protocolo de aislamiento de células cebadas consistió, en primer lugar, en someter todo el exudado peritoneal recogido de cada pez a un gradiente de ficol-paque mediante centrifugación por gradiente de densidad, generalmente utilizado para separar células mononucleares de células polinucleares, con el fin de separar por densidad las distintas poblaciones leucocitarias presentes en el exudado. De esta manera, las células de pequeño tamaño, mayormente linfocitos, formaron una interfase entre el ficol y el medio mientras que las células de gran tamaño, como granulocitos acidófilos, células cebadas y macrófagos, acabaron formando un precipitado celular. Posteriormente, y gracias a la capacidad de los macrófagos de comportarse como células adherentes, el total de células presentes en el precipitado celular fue sembrado en frascos para propiciar la adhesión de los macrófagos al frasco mientras que el resto de células (granulocitos acidófilos y células cebadas) permanecieron en suspensión, lo que facilitó su separación tras 16 horas de cultivo. A continuación, las células en suspensión fueron separadas en dos fracciones mediante MACS gracias a la utilización de un anticuerpo monoclonal específico contra granulocitos acidófilos

de dorada, G7 (Sepulcre y col., 2002). Además, y con el fin de eliminar posibles contaminaciones con linfocitos que no hubieran sido correctamente separados en el primer paso, un anticuerpo monoclonal contra células IgM de dorada, 9H8 (Aquatic Diagnostics), fue utilizado también en el MACS. Finalmente se obtuvieron dos fracciones celulares: i) una fracción $G7^+$ e IgM^+ compuesta mayoritariamente por granulocitos acidófilos y, en menor medida, por linfocitos B y ii) una fracción $G7^-$ e IgM^- negativa formada por células cebadas. La pureza de las poblaciones de células cebadas obtenidas fue analizada mediante citometría de flujo y microscopía electrónica de transmisión, arrojando valores superiores al 95%. Además, el número de células cebadas obtenido variaba de 5 a 20 millones por pez, número suficiente para la realización de ensayos funcionales.

Durante décadas se ha considerado que la histamina es un mediador pro-inflamatorio que únicamente estaba presente en vertebrados superiores. Sin embargo, recientemente, se ha comprobado que las células cebadas de intestino y branquia de dorada almacenan histamina y son capaces de liberarla cuando dichos órganos son tratados con el compuesto 48/80 (Mulero y col., 2007a). En nuestro trabajo, analizamos la presencia de histamina en fracciones enriquecidas en células cebadas del exudado peritoneal de dorada obtenidas con el protocolo que previamente hemos descrito. Para ello, las células cebadas purificadas fueron fijadas y permeabilizadas y, posteriormente, se sometieron a técnicas de inmunofluorescencia donde se utilizó, en primer lugar, un anticuerpo comercial contra histamina (Sigma-Aldrich, H7403) y, en segundo lugar, un anticuerpo secundario unido a un fluorocromo (Alexa Fluor 488 Goat Anti-Rabbit IgG). Mediante el análisis de la fluorescencia, determinamos que las células cebadas del exudado peritoneal también son capaces de almacenar histamina. Además, mediante ensayos de ELISA, se analizó la liberación de histamina en células cebadas purificadas tras incubarlas con 5, 10 y 20 µg/mL del compuesto 48/80 durante 15 minutos, comprobando que las células cebadas peritoneales de dorada son capaces de liberar histamina al ser tratadas con el compuesto 48/80 y que esta liberación es máxima con la concentración de 10 µg/mL. En un segundo ensayo, se analizó la liberación de histamina tras un tratamiento con 10 µg/mL de compuesto 48/80 durante 15, 30 y 60 minutos, observándose un máximo de liberación de histamina tras 15 minutos de estimulación. Por tanto, estos resultados han demostrado que las células cebadas del exudado peritoneal almacenan y liberan histamina *in vitro* y que son necesarios estudios *in vivo,* centrados en el papel de las células cebadas peritoneales y su capacidad para liberar histamina, para determinar específicamente el mecanismo de liberación de la histamina.

Las células cebadas de mamíferos expresan los genes que codifican para las citoquinas IL-1β, IL-8, CCL4 y TNF-α y dicha expresión es modulada en procesos inflamatorios (Ashraf y col., 1996; Bissonnette y col., 1995; Bradding y col., 1995; Buckley y col., 1995; Eklund y col., 1997; Gibbs y col., 2001; Gordon y Galli, 1990; Kandere-Grzybowska y col., 2003; Lorentz y col., 2000; Moller y col., 1993; Nigrovic y col., 2007; Rasheed y col., 2010; Sun y col., 2005; Tashiro y col., 1997; Williams y Coleman, 1995; Yano y col., 1997). En nuestro trabajo, analizamos la capacidad de las células cebadas para expresar los genes que codifican *il1β*, *il8*, *ccl4* y *tnfα* y su posible modulación con VaDNA, agente inmunoestimulante de granulocitos acidófilos y macrófagos de dorada (Sepulcre y col., 2002, 2007, 2011). Encontramos que las células cebadas de dorada expresan dichos genes y que la estimulación con ADN bacteriano incrementa los niveles de ARNm de *il1β* e *il8* pero no supuso cambios en la expresión de *ccl4* y *tnfα*. El estudio de la expresión de diferentes citoquinas o mediadores inflamatorios en células cebadas y de su modulación tras determinados estímulos en células cebadas sería de gran interés para poder entender el papel de estos leucocitos en peces.

El protocolo de aislamiento de células cebadas propuesto permite obtener fracciones altamente enriquecidas en células cebadas funcionales. Estas fracciones facilitarán la caracterización de las células cebadas peritoneales en dorada y ayudarán a conocer su papel en la respuesta inmunitaria. Pequeñas adaptaciones de este protocolo permitirá que sea utilizado en otros peces teleósteos. Además, las similitudes existentes entre las células cebadas de dorada y las de mamíferos hacen de esta especie un modelo animal de investigación ideal para el estudio de la evolución de las células cebadas.

ii. Papel de la histamina y del compuesto 48/80, activador de la liberación de histamina, como adyuvantes de vacuna de dorada

El papel de la histamina en el sistema inmunitario de peces y los mecanismos responsables de la liberación de histamina en células cebadas es bastante desconocido. En estudios previos se ha demostrado que los peces más avanzados evolutivamente, es decir, los que forman el orden de Perciformes, producen y almacenan histamina en los gránulos de sus células cebabas (Gómez-González y col., 2014; Mulero y col., 2007a). Además, la histamina es utilizada como un mediador inflamatorio por la dorada y es capaz de comprometer su supervivencia, producir contracción del músculo liso intestinal y participar en la producción de ROS de los granulocitos (Mulero y col., 2007a). Por otra parte, el compuesto 48/80 produce la degranulación de las células cebadas en especies de peces filogenéticamente

distantes (Manera y col., 2011, 2014; Vallejo y Ellis, 1989) e induce la liberación de histamina en intestino, branquias y células cebadas peritoneales de dorada tanto *in vivo* como *in vitro* (Gómez-González y col., 2014; Mulero y col., 2007a).

En este estudio se utilizó *V. anguillarum*, inactivado mediante calor, como modelo de inmunización en dorada, para investigar el papel de la histamina exógena y endógena en la respuesta inmunitaria de peces perciformes. Para ello, se realizaron inyecciones intraperitoneales no letales de histamina (10 mg/kg) y de compuesto 48/80, agente activador de las células cebadas, (0.25 mg/kg), solos o en combinación con 10^9 *V. anguillarum*. También se estudió la capacidad de la histamina exógena y endógena para modular a largo plazo la producción de anticuerpos contra el antígeno utilizado mediante una dosis de recuerdo, tanto de la histamina o del compuesto 48/80 como del patógeno, 49 días después.

Las inmunizaciones con *V. anguillarum* modularon la respuesta inmunitaria innata y adaptativa. Como se esperaba por estudios previos (Chaves-Pozo y col., 2005a), la primera inmunización con *V. anguillarum* provocó un reclutamiento de leucocitos en la cavidad peritoneal. Sorprendentemente, se produjo una disminución del porcentaje de células cebadas en la cavidad peritoneal mientras que los porcentajes de granulocitos acidófilos, equivalentes a los neutrófilos de humanos (Sepulcre y col., 2002), y de linfocitos B IgM^+ no se vieron alterados en la cavidad peritoneal. El incremento en el número de células totales en la cavidad peritoneal coindice con una disminución de granulocitos acidófilos en riñón cefálico, sugiriendo que estas células se movilizan desde el riñón cefálico hasta el lugar de inflamación, como había sido descrito previamente en doradas inmunizadas con *V. anguillarum* (Chaves-Pozo y col., 2005a). También incrementó la producción de ROS y el perfil de expresión de los genes que codifican para *il1b* e *il10*, tanto en riñón cefálico como en exudado peritoneal, tras la inmunización con el patógeno. Por último, la inmunización de recuerdo, aumentó la producción del título de anticuerpos IgM contra *V. anguillarum*. Todos estos datos indican la capacidad de *V. anguillarum* para inducir respuestas inflamatorias tanto locales como sistémicas. Como los granulocitos acidófilos son los principales leucocitos productores de ROS y de IL-1β en dorada (Mulero y col., 2008a; Sepulcre y col., 2007) y el porcentaje de estas células incrementó en exudado peritoneal de los peces tratados con histamina y *V. anguillarum*, parece claro que la histamina inhibe la producción de ROS y de IL-1β en respuesta a *V. anguillarum*. Estos resultados también podrían explicar el ligero cambio producido en la respuesta adaptativa, como la producción de IgM en respuesta a *V. anguillarum*, observada en peces inyectados con histamina y con *V. anguillarum*, como ya se

había descrito anteriormente en ratones en los que la histamina redujo el título de anticuerpos IgM específico contra eritrocitos de oveja (Tripathi y col., 2010). En cambio, otros artículos señalan que la histamina induce la producción de anticuerpos IgM en determinados tipos celulares (Banu y Watanabe, 1999; Falus, 1993) mientras que otros indican que se producen variaciones en la producción de IgE e IgG4 pero no en la de IgG1, IgG2, IgG3, IgA1 e IgA2 (Kimata y col., 1996).

El agente inductor de degranulación de células cebadas, el compuesto 48/80, también fue capaz de modular *in vivo* la respuesta inmunitaria innata de dorada como ya se había descrito previamente *in vitro* (Gómez-González y col., 2014; Mulero y col., 2007a). La combinación de la inyección de este agente y del antígeno redujo el porcentaje de granulocitos acidófilos en riñón cefálico aunque no se observó un aumento en exudado peritoneal. Estos resultados sugieren que la histamina endógena liberada promueve la movilización de los granulocitos acidófilos hacia otros órganos, como ha sido demostrado en ratón (McLachlan y col., 2008) . Estos cambios sistémicos inducidos por el compuesto 48/80 en dorada y el hecho de que se ha descrito que el compuesto 48/80 actúa como un potente adyuvante de vacunas mediante inyecciones subcutáneas o administración nasal (McLachlan y col., 2008), indican que deben realizarse más esfuerzos para determinar el papel de este compuesto en el sistema inmunitario de dorada y como adyuvante de vacunación de peces.

A diferencia de la histamina exógena, el compuesto 48/80 incrementó la producción de ROS de los leucocitos peritoneales aunque el porcentaje de granulocitos acidófilos no se vio incrementado, indicando que la liberación de la histamina endógena de las células cebadas incrementa la producción de ROS de los granulocitos acidófilos, como se ha demostrado *in vitro* utilizando dimaprit, un agonista del receptor Hrh2 (Mulero y col., 2007a). Sin embargo, el compuesto 48/80 fue incapaz de incrementar la expresión de los genes que codifican las citoquinas IL-1β o IL-10 ni la respuesta humoral adaptativa contra *V. anguillarum*.

En este estudio también identificamos el perfil de expresión de los HRs en leucocitos peritoneales y de riñón cefálico. En una reciente publicación, demostramos que los leucocitos de riñón cefálico de dorada expresan el gen que codifica *hrh1* (García-García y col., 2016). En nuestro trabajo hemos demostrado que este órgano también expresa *hrh2* y *hrh3* y, que además, estos tres genes son expresados en células de exudado peritoneal. Además, la expresión de *hrh2* fue fuertemente reducida en células de exudado peritoneal en ejemplares de dorada inyectados con histamina y el compuesto 48/80 mientras que *hrh1* e *hrh3* no vieron modificada su expresión. Estos resultados sugieren que el reto inmunitario provocado por las

inyecciones de histamina y compuesto 48/80, es decir, de histamina exógena e histamina liberada por las células cebadas, potencia los efectos inhibidores de la histamina en leucocitos peritoneales haciendo menos efectiva la señalización del Hrh2 (Mulero y col., 2007a).

En conjunto, este es el primer estudio que demuestra que tanto la histamina exógena como endógena son capaces de modular *in vivo* la respuesta inmunitaria de peces aunque no se haya identificado a la histamina ni al compuesto 48/80 como adyuvantes eficientes para la vacunación de dorada.

iii. Generación de un anticuerpo monoclonal, GB10, contra células cebadas de dorada que induce oncosis

Las células cebadas son un componente celular muy importante en la inmunidad innata y las funciones que desempeñan han sido ampliamente estudiadas en mamíferos. Sin embargo, el estudio de las células cebadas de peces teleósteos se ha visto dificultado por la falta de anticuerpos capaces de reconocer dichas células, utilizando, en la mayoría de los trabajos relativos a las células cebadas de peces, técnicas histológicas (Manera y col., 2014; Mulero y col., 2007a; Reite, 1998; Reite y Evensen, 2006). Con estos antecedentes, en este trabajo nos propusimos producir un anticuerpo monoclonal específico de células cebadas de dorada. Esta elección se fundamentaba en la experiencia previa en la obtención de anticuerpos monoclonales en el grupo de investigación (ej. G7, anticuerpo monoclonal específico contra granulocitos acidófilos de dorada) (Sepulcre y col., 2002) y en la posibilidad de obtener un número suficientemente elevado de células cebadas en fracciones altamente enriquecidas obtenidas tras el uso del protocolo previamente diseñado (Gómez-González y col., 2014) para poder realizar eficazmente las pertinentes inmunizaciones.

De esta manera, las poblaciones asiladas y purificadas de células cebadas peritoneales sirvieron de antígeno para inmunizar ratones BALB/c, cuyos esplenocitos fueron fusionados a la línea celular mieloide SP2/0-Ag14. Los hibridomas obtenidos tras la fusión fueron cultivados durante 14 días en un medio selectivo de hibridomas. A continuación, células totales del exudado peritoneal de dorada fueron incubadas con los sobrenadantes de las líneas de hibridomas obtenidas y se realizó entonces un primer análisis mediante inmunofluorescencia y citometría de flujo. Los hibridomas capaces de producir anticuerpos contra células cebadas peritoneales de dorada fueron clonados mediante diluciones seriadas (1/2) hasta conseguir pocillos monoclonales y cultivados durante 10-15 días en condiciones óptimas. Después, se volvió a realizar un análisis de producción de anticuerpos contra células cebadas peritoneales de dorada y se concluyó que la línea de hibridomas GB10 era capaz de

producirlos. Con el fin de evitar contaminaciones celulares con otras líneas de hibridomas, la línea de hibridoma GB10 se volvió a clonar una segunda vez y se analizó, por tercera vez, su capacidad de producción de anticuerpos contra células cebadas peritoneales de dorada. Se confirmó de nuevo su capacidad para identificar las células cebadas en el total de leucocitos presentes en el exudado peritoneal de dorada, ofreciendo unos porcentajes de células cebadas en el total del exudado peritoneal similares a los obtenidos en estudios previos, en los que las poblaciones de leucocitos del exudado peritoneal de dorada fueron analizados mediante microscopía electrónica de transmisión (Meseguer y col., 1993). Además, las células GB10+ en los análisis de citometría de flujo ocuparon una región similar a la obtenida cuando las células cebadas del exudado peritoneal de dorada son aisladas (Gómez-González y col., 2014). Una vez confirmada la capacidad de GB10 para producir anticuerpos contra células cebadas peritoneales de dorada, la línea celular GB10 fue expandida para conseguir grandes cantidades del anticuerpo.

GB10 produce la muerte de las células cebadas del exudado peritoneal, como se comprobó mediante el análisis por citometría de flujo y la incorporación de yoduro de propidio. Sin embargo, los demás tipos celulares presentes en el exudado peritoneal permanecían viables. Además, esta muerte celular se producía muy rápidamente, detectándose a partir de 10 minutos de exposición de las células al anticuerpo, alcanzándose el máximo nivel de mortalidad (100%) tras 20 minutos de exposición a GB10. También se comprobó que esta mortalidad es independiente de la temperatura, encontrando resultados similares de mortalidad cuando el exudado peritoneal era incubado a 4 °C y a 21 °C.

La clasificación de los mecanismos de muerte celular es un tema muy controvertido. En general, se considera que la muerte celular es producida por apoptosis, necrosis u oncosis (Balvan et al., 2015). Sin embargo, algunos autores consideran que apoptosis y oncosis son procesos de muerte celular que terminan en necrosis (Majno y Joris, 1995; Trump y col., 1997) mientras que otros consideran que tras el proceso de muerte celular por oncosis se produce la necrosis (Krysko y col., 2004; Zhang y col., 1998). En cualquier caso, en el proceso de apoptosis se produce activación de la ruta de las caspasas y la producción de ATP, fragmentación del ADN e irregularidad en el núcleo, contracción de la célula y cambio en la orientación de los lípidos de membrana y tiene lugar tras 12-24 horas después del estímulo que la induce (Balvan y col., 2015). La muerte celular a través de necrosis es independiente de caspasas, produciéndose reducción de ATP, fragmentación de ADN, lisis del núcleo e inflamación de la célula (Balvan y col., 2015). En cambio, en la muerte celular por oncosis se

produce una inhibición en la producción de ATP, dilatación del núcleo y acúmulos de cromatina, inflamación celular, pérdida de la integridad de la membrana celular y liberación del contenido celular (Balvan y col., 2015). Con estos antecedentes, apoptosis y oncosis parecían los mecanismos de muerte celular producidos por GB10 más factibles y por ello nuestra atención se centró en ellos. Por un lado, apoptosis es un mecanismo de muerte celular que requiere la activación de la ruta de las caspasas. Por este motivo, utilizamos un inhibidor de caspasas (capaz de inhibir la actividad de todas las caspasas involucradas en la muerte celular apoptótica) junto a GB10 para determinar si la utilización de este inhibidor y, por la tanto, la muerte celular mediada por apoptosis impedía la muerte de las células cebadas. Sin embargo, este inhibidor no fue capaz de bloquear la muerte de las células cebadas peritoneales expuestas a GB10. Por otro lado, se conoce que la muerte celular debida a oncosis se produce de una manera muy rápida y conlleva cambios morfológicos muy significativos tanto en el núcleo como en el citoplasma y en la membrana de la célula. Por este motivo, el exudado peritoneal de dorada fue incubado con GB10 durante 30 minutos o 2 horas y su morfología fue analizada mediante microscopía electrónica de transmisión. Se pudo observar que GB10 únicamente afectaba la morfología de las células cebadas, presentando cambios en el núcleo, citoplasma y límites celulares similares a los descritos en casos de muerte celular producida por oncosis entre los que observamos un aumento de la electronodensidad del núcleo, un cambio en el aspecto y contenido de los gránulos presentes en el interior de las células cebadas y un límite celular apenas visible.

GB10 no es el único ejemplo de anticuerpo monoclonal capaz de producir muerte celular mediada por oncosis. En mamíferos se han descrito algunos anticuerpos capaces de producir oncosis en determinados tipos celulares como linfocitos T y B (Bhat y col., 1996, 1997; Matsuoka y col., 1995), en células Jurkat (Zhang y col., 1998), en células tumorales de adenocardinoma gastrointestinal (Loo y col., 2007), en células madre embrionarias (Tan y col., 2009) o en células tumorales humanas y de roedores (Dorvignit y col., 2015; Hernández y col., 2011; Roque-Navarro y col., 2008). Sin embargo, no se ha descrito, hasta la fecha, la existencia de anticuerpos monoclonales específicos de algún tipo celular de peces capaz de producir muerte celular por oncosis.

En este estudio, hemos obtenido un anticuerpo monoclonal, GB10, contra células cebadas de dorada que permitirá detectar su presencia y porcentaje en ensayos *in vivo* de una manera rápida. Por otro lado, la capacidad de GB10 para inducir la muerte de las células cebadas deja abierta la posibilidad de obtener ejemplares de dorada deficientes en este tipo celular y poder

así avanzar en el conocimiento del papel de las células cebadas en la respuesta inmunitaria de dorada. Además, futuros estudios nos permitirán determinar cuál es el antígeno que GB10 es capaz de reconocer y si este antígeno está conservado evolutivamente y si GB10 puede actuar como agente degranulador capaz de inducir la liberación de factores pro-inflamatorios como la histamina. Por otra parte, hemos iniciado la producción de un nuevo anticuerpo contra células cebadas, BG4, que reconoce específicamente a las células cebadas de dorada, que estamos caracterizando y que, esperamos, nos sea de utilidad en el avance del conocimiento del papel de las células cebadas en la inmunidad de peces.

iv. Efectos del EE_2 en el exudado peritoneal de dorada

Los EDCs de carácter estrogénico son compuestos capaces de imitar o antagonizar a los estrógenos naturales. Al igual que otros esteroides, se ha demostrado que los EDC son capaces de inducir cambios no sólo en el sistema reproductor sino también en otros aspectos de la biología de peces, incluyendo su inmunidad (Filby y col., 2007) provocando cambios a nivel molecular, particularmente en organismos marinos (Castillo-Briceno y Kodjabachian, 2014). En este estudio, analizamos el efecto que el EE_2 produce sobre la respuesta inmunitaria de una especie hermafrodita, la dorada, centrándonos en los leucocitos peritoneales.

Estudios previos, llevados a cabo en nuestro grupo de investigación, han demostrado que el EE_2 actúa como disruptor endocrino de dorada (Cabas y col., 2012; García-Hernández y col., 2016; Rodenas y col., 2015, 2016) y que, aunque no actúa como sustancia inmunosupresora, altera la capacidad de la respuesta inmunitaria de los ejemplares frente a una infección (Cabas y col., 2012; Liarte y col., 2011a; Rodenas y col., 2015; Rodenas y col., 2016). Estos trabajos se han centrado, mayoritariamente, en el análisis de la respuesta de los leucocitos de riñón cefálico (Cabas y col., 2011, 2012; Liarte y col., 2011a; Rodenas y col., 2015, 2016).

En nuestro estudio, ejemplares juveniles de dorada fueron tratados con EE_2 (5 µg/g comida) durante 76 días. A continuación, se realizaron dos inmunizaciones con 45 µg/fish de hemocianina y 3.6 µg/fish de aluminio, como adyuvante: i) tras finalizar el tratamiento y ii) 92 días después de la primera inmunización. Se tomaron muestras de hígado y exudado peritoneal 1 día después de la primera inmunización y 1 y 50 días después de la segunda inmunización.

En primer lugar, observamos que el EE_2 induce un incremento de la expresión del gen que codifica la *vtg*, considerado como un marcador de disrupción endocrina en peces machos

expuestos a compuestos estrogénicos (Sumpter y Jobling, 1995), como ya ha sido previamente demostrado en dorada (Cabas y col., 2012; García-Hernández y col., 2016; Kausch y col., 2008; Rodenas y col., 2015, 2016). Además, este incremento desaparecía tras el cese del tratamiento, como ya se ha demostrado anteriormente (Rodenas y col., 2015, 2016). En este trabajo se ha demostrado, por primera vez, que los leucocitos de exudado peritoneal de dorada expresan los genes codificantes de los ERs, el receptor nuclear ERα y el receptor acoplado a membrana GPER1, como ya se ha demostrado en mamíferos y en otros tipos de leucocitos de peces (Blasko y col., 2009; Cabas y col., 2013b; Liarte y col., 2011c; Straub, 2007), y que la expresión de dichos genes es inducida por el EE_2. Este estudio amplia observaciones previas demostrando que (i) el EE_2 podría ejercer un efecto en la biología de los leucocitos peritoneales de dorada no sólo a través de los clásicos ERs nucleares sino que también a través de GPER1 y que (ii) el EE_2 incrementaría la señalización de estrógenos en estas células a través de los ERs. Además, la inmunización con hemocianina no modula la expresión de *gper* pero disminuye la expresión de *era* mientras que incrementa la expresión de ambos genes en leucocitos de riñón cefálico de dorada (Cabas y col., 2012, 2013b). Estas diferencias pueden ser debidas a la alteración que un reto inmunitario produce en las poblaciones leucocitarias (Chaves-Pozo y col., 2004, 2005a; García-Castillo y col., 2002).

El EE_2 promueve un reclutamiento de leucocitos en la cavidad peritoneal e induce la expresión de *csf1r*, *ighm* e *ight*, lo que sugiere que macrófagos y linfocitos fueron reclutados en la cavidad peritoneal, como ocurre en la gónada de dorada tras un tratamiento de dieta suplementada con EE_2 (Cabas y col., 2011) y en línea con los cambios observados en las poblaciones de leucocitos sanguíneos de lubina japonesa después de un tratamiento con E_2 (Thilagam y col., 2009). Sin embargo, una vez que el tratamiento cesó, y a pesar de que el número de leucocitos peritoneales totales volvió a niveles basales, la expresión de *csf1r* permaneció elevada durante 3 meses más. Además, el EE_2 incrementó la explosión respiratoria y la expresión del gen codificante de IL-1β de leucocitos peritoneales, incluso tras 3 meses del fin del tratamiento. Estos datos coinciden con la inducción en la producción de ROS de leucocitos sanguíneos de lubina japonesa expuesta a E_2 (Thilagam y col., 2009) y con la inducción en la expresión de *il1b* en riñón cefálico de dorada expuesta a una dieta suplementada a EE_2 (Cabas y col., 2012). De manera similar, otros EDC de carácter estrogénico como el bisfenol A, nonilfenol o diferentes ftalatos incrementan la producción de ROS en células fagocíticas de carpa común (Gushiken y col., 2002; Watanuki y col., 2003). Las consecuencias biológicas de los efectos observados tras el tratamiento con EE_2 necesitan futuras investigaciones. De hecho, se podría especular que mecanismos de epigenética están

involucrados (Bhandari y col., 2015), ya que los efectos de disrupción endocrina, evaluados mediante la expresión de *vtg* en hígado, terminaron una vez cesó el tratamiento.

El EE_2 tiene efectos muy interesantes en peces vacunados siendo los más relevantes: i) el aumento del número total de leucocitos 1 días después de la inmunización, como ocurría en peces no vacunados y ii) el descenso en el porcentaje de células cebadas. Además, el EE_2 presentó efectos sinérgicos con la vacunación incrementando el número total de leucocitos y reduciendo la abundancia de células cebadas. Por el contrario, otros EDCs, como el PCB 126, incrementan la abundancia de células cebadas en branquias e intestino de dorada (Lauriano y col., 2012).

De manera muy interesante, se observó que, aunque la disrupción endocrina producida por EE_2 había desaparecido, se produce una reducción en la producción de anticuerpos IgT específicos contra hemocianina en el sobrenadante de células peritoneales varios meses después de finalizar el tratamiento. Estos resultados indican que el EE_2 disminuye la capacidad de la dorada para regular la producción de anticuerpos naturales. Existen otros ejemplos de EDCs capaces de modular la producción de anticuerpos de peces, es el caso de la producción de anticuerpos contra *Edwardsiella ictaluri* en individuos de pez gato americano (Rice y Schlenk, 1995) o *V. anguillarum* en salmón real (Regala y col., 2001) tratados con PCB 126. Además, también se ha descrito que la producción de anticuerpos en mamíferos aumenta en presencia de E_2 (Sthoeger y col., 1988).

El EE_2, además, disminuye la expresión del gen que codifica *hrh2* en los leucocitos del exudado peritoneal tras 76 días de tratamiento. Los xenoestrógenos son capaces de inducir degranulación y activación de células cebadas de roedores (Mizota y Ueda, 2006; Narita y col.., 2007; O'Brien y col., 2014; Rajkovic y col., 2014; Uchida y col., 2003) y de humanos (Kennedy y col., 2012; Narita y col., 2007). En este estudio se confirmó la capacidad del EE_2 para reducir el tamaño de individuos de dorada (datos no mostrados), como ya ha sido descrito por otros autores (Baumann y col., 2014; Rodenas y col., 2016; Shved y col., 2008). Además, se ha descrito que el endosulfan y la atrazina modulan el apetito y el comportamiento motor a través de los HRs presentes en la células del cerebro (Giusi y col., 2010). Los resultados obtenidos en nuestro trabajo y estas evidencias podrían indicar que el EE_2 modula el apetito a través de las rutas de señalización de la histamina. Además, se ha comprobado que la cimetidina, un agonista del HRH1 y del HRH2, tradicionalmente utilizada para desórdenes gastrointestinales, actúa como disruptor endocrino en dorada (García-García y col., 2016; Lee y col., 2015).

Finalmente, observamos que las inmunizaciones también fueron capaces de modular la expresión de los HRs. De hecho, tras la primera inmunización, al igual que en los peces tratados con EE_2, los peces vacunados también presentaban una disminución en la expresión de los genes que codifican *hrh2* y *hrh3*. Nuevos estudios son necesarios para entender con más detalle la expresión de estos receptores tras un reto inmunitario.

Para concluir, el EE_2 altera la actividad de los leucocitos del exudado peritoneal de dorada y algunas de estas modificaciones se mantienen aún a pesar del cese del tratamiento y de la desaparición del efecto disruptor, medido con el nivel de expresión génica de la *vtg* hepática.

e. CONCLUSIONES.

Los resultados de este trabajo conducen a las siguientes conclusiones:

1. El protocolo de aislamiento de células cebadas del exudado peritoneal de dorada puesto a punto permite conseguir una pureza superior al 95%.

2. Las células cebadas del exudado peritoneal de dorada almacenan histamina y el compuesto 48/80 induce su liberación. Además, el ADN genómico de *Vibrio anguillarum* induce la expresión de los genes que cifran la IL-1β y la IL-8 en dichas células.

3. La histamina y el compuesto 48/80 modulan las poblaciones de leucocitos del exudado peritoneal y de riñón cefálico de dorada cuando se inyectan intraperitonealmente, pero sólo el compuesto 48/80 induce la producción de ROS de los leucocitos del exudado peritoneal. Además, la histamina disminuye la producción de IgM específica cuando es administrada junto a una bacterina de *V. anguillarum*.

4. Los leucocitos del exudado peritoneal de dorada expresan los genes que cifran los receptores de histamina Hrh1, Hrh2 e Hrh3. Tanto la inyección intraperitoneal de histamina como la del compuesto 48/80 inhiben la expresión de *hrh2*.

5. Aunque la histamina regula la inmunidad innata y adaptativa de dorada, ni ella ni el compuesto 48/80 son eficientes como adyuvantes en la vacunación por vía intraperitoneal de dorada.

6. El GB10 es un anticuerpo monoclonal capaz de reconocer un receptor de membrana de células cebadas peritoneales de dorada que, además, induce su muerte rápidamente mediante oncosis.

7. Los leucocitos del exudado peritoneal de dorada expresan los genes que cifran el receptor nuclear de estrógenos α y el GPER1 y su expresión es inducida por la administración en la dieta de EE_2.

8. La administración en la dieta de EE_2 induce una respuesta inflamatoria en el exudado peritoneal de dorada que consiste en un aumento del número de leucocitos en la cavidad peritoneal y del perfil de expresión de *csf1r*, *ighm*, *ight* e *il1b* así como en la producción

de ROS. Además, también disminuye la expresión del gen *hrh2* y modula la producción de anticuerpos IgT específicos contra hemocianina en animales no inmunizados frente a hemocianina.

9. Algunos de los efectos de la administración en la dieta de EE_2 se mantuvieron hasta tres meses después de la finalización del tratamiento.

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ANEXXE I: Publications derived from the thesis

1) **NE Gómez-González**, E García-García, J Montero, A García-Alcázar, J Meseguer, A García-Ayala, V Mulero 2014. Isolation of mast cells from the peritoneal exudate of the teleost fish gilthead sea bream (*Sparus aurata* L.). Fish and Shellfish Immunology 40: 225-232.
2) **NE Gómez-González**, I Cabas, J, Montero, A García-Alcázar, V Mulero, A García-Ayala, 2017. Histamine and mast cell activator compound 48/80 are safe but inefficient systemic adjuvants for gilthead seabream vaccination. Developmental and Comparative Immunology (*in press*).
3) **NE Gómez-González**, I Cabas, MC Rodenas, A García-Alcázar, V Mulero, A García-Ayala, 2017. 17α-Ethinylestradiol alters the peritoneal immune response of gilthead seabream. Developmental and Comparative Immunology (*submitted*).
4) **NE Gómez-González**, M Arizcun, J Meseguer, V Mulero, MP Sepulcre, A García-Ayala. Generation of a mAb, GB10, against a cell surface receptor of fish mast cell that induces oncosis (*in preparation*).

ANNEXE II: Participation in publications during de PhD

1) E García-García, **NE Gómez-González**, J Meseguer, A García-Ayala, V Mulero 2014. Histamine regulates the inflammatory response of the tunicate *Styela plicata*. Developmental and Comparative Immunology 46: 382-391.

2) A López-Muñoz, S Liarte, **NE Gómez-González**, I Cabas, A García-Ayala, V Mulero 2015. Estrogen receptor 2b deficiency impairs reproductive function and anti-viral response in male zebrafish (*Danio rerio*). Developmental and Comparative Immunology 53: 55-62.

3) M García-García, S Liarte, **NE Gómez-González**, A García-Alcázar, J Pérez-Sánchez, J Meseguer, V Mulero, A García-Ayala, E Chaves-Pozo 2016. Cimetidine disrupts the renewal of testicular cells and the steroidogenesis in a hermaphrodite fish. Comparative Biochemistry and Physiology 189: 44-53.

4) ERM Martínez, MA de Oliveira, AAA Vigoya, **NE Gómez-González**, RW Schulz, J Bogerd, RH Nobrega. Molecular characterization and expression analysis of anti-Müllerian hormone (Amh) in the spermatogonial niche of common carp (*Cyprinus carpio*) (under review).

ANNEXE III: Participation in national and international conferences

1) **NE Gómez-González**, A García-Alcázar, V Mulero, MP Sepulcre, A García-Ayala. The role of histamine in the immune system of the gilthead seabream (*Sparus aurata* L.). I Jornadas Doctorales de la Universidad de Murcia (Murcia, Spain), 05/2015.

2) **NE Gómez-González**, I Cabas, J Montero, M. Carmen Rodenas, A García-Alcázar, MP Sepulcre, A García-Ayala, V Mulero. Histamine regulates the gilthead seabream (*Sparus aurata* L.) immunity. ISDCI (Murcia, Spain), 07/2015.

3) F Hermi, I Cabas, **NE Gómez-González**, V Gómez-Abellán, A García-Ayala, R Oueslati, V Mulero, MP Sepulcre. Identification and expression analysis of PGE2 receptor subtypes in gilthead seabream (*Sparus aurata* L.). ISDCI (Murcia, Spain), 07/2015.

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ANNEXE IV: Research stay in other laboratories during my PhD

- Host institution: **Reproductive and Molecular Biology Group, Department of Morphology, Institute of Bioscience of Botucatu, São Paulo State University, Botucatu, São Paulo, Brazil**.

  Responsible person in the host: **Rafael Henrique Nóbrega**.

  Stay period: **25th October 2014 – 24th January 2015**.