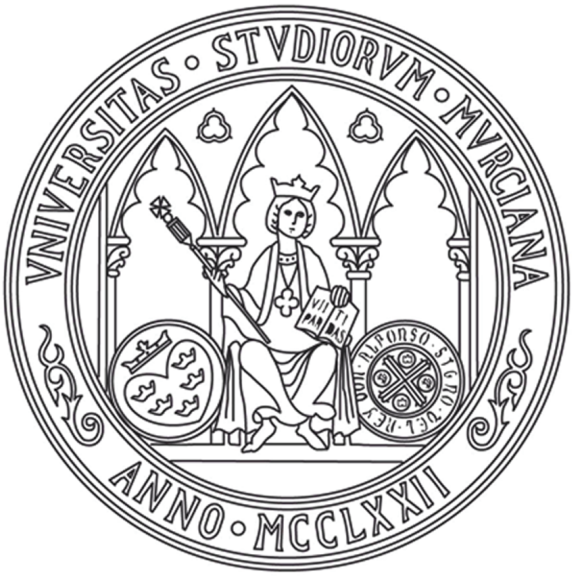

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Estrogens Regulate the Innate and the Adaptive Immune Response

Regulación de la Respuesta Inmunitaria Innata y Adaptativa por los Estrógenos

Dña. María del Carmen Rodenas Bleda

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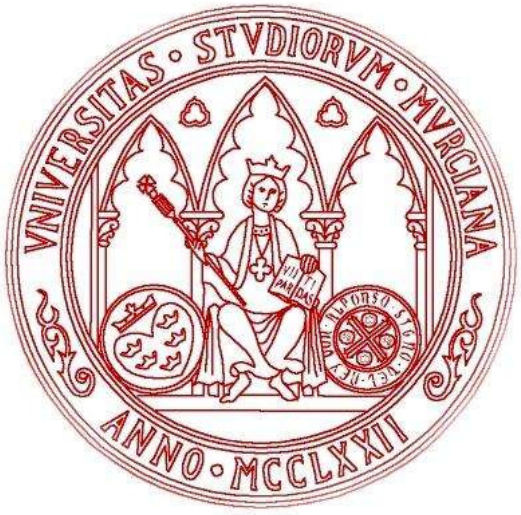

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A mis padres

A mis tutores

A mis compañeros

¡Gracias!

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List of Abbreviations

Ab	Antibody
Actb	β-actin
AGs	Acidophilic granulocytes
ATP	Adenosine triphosphate
BCR	B cell receptor
BP	Blocking peptide
BSA	Bovine serum albumin
BW	Body weight
cAMP	Cyclic adenosine monophosphate
CCL	C-C motif chemokine ligand
CD	Cluster of differentiation
cDNA	Complementary DNA
CFOS	Proto-oncogen *cfos*
CO_2	Carbon dioxide
COX	Cyclooxygenase
CREB	cAMP-response element binding protein
Ct	Cycle threshold
CXCL	C-X-C motif chemokine ligand
CX3CL	C-X3-C motif chemokine ligand
DBD	DNA binding domain
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease

Dot	Days of treatment
Dpb	Days post-booster
Dpp	Days postpriming
Dpt	Days post-treatment
EDCs	Endocrine disrupting chemicals
E_2	17β-estradiol
EdU	5-ethynyl-2'-deoxyuridine
EE_2	17α-ethinylestradiol
EGF	Epidermal growth factor
ER	Estrogen receptor
ERE	Estrogen-responsive element
ERK	Extracellular signal-regulated kinases
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal serum
FITC	Fluorescein isothiocyanate
fMLP	N-Formylmethionyl-leucyl-phenylalanine
FSC	"Forward scater", cell size
G1	Specific agonist of GPER
G-CSF	Granulocyte colony-stimulating factor
GDP	Guanosine diphosphate
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GPCRs	G protein-coupled receptors
GPER1	G protein-coupled estrogen receptor 1
GSI	Gonadosomatic index
GTP	Guanosine triphosphate

HEK293	Human embryonic kidney cells 293
Hf-FBS	Hormone free-fetal bovine serum
HK	Head kidney
HL-60	Human promyelocytic leukemia cells
HSI	Hepatosomatic index
IFN	Interferon
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IL	Interleukin
IL-1RA	Interleukin-1 receptor antagonist
Ig	Immunoglobulin
IPCS	International Programme on Chemical Safety
LDB	Ligand binding domain
LPS	Lipopolysaccharide
Ly	Lymphocytes
M	Monocytes
MAPK	Mitogen-activated protein kinase
MCSF	Macrophage colony-stimulating factor
MB	Body mass
MFI	Mean fluorescence intensity
MG	Gonad mass
ML	Liver mass
MS	Spleen mass
NF-κB	Nuclear factor kappa beta
NL	Natural ligand
mRNA	Messenger ribonucleic acid

O_2^-	Superoxide anion
PAMPs	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PKA	Protein kinase A
PMA	Phorbol 12-myristate 13-acetate
PRRs	Pattern recognition receptors
PTGDS	Prostaglandin D2 synthase
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen intermediate
Rps18	Ribosomal protein S18
RT	Reverse transcription
SEM	Mean
SERM	Selective estrogen receptor modulator
SKBR3	Human breast cancer cell line
SOCS3	Suppressor of cytokine signaling 3
SSI	Splenosomatic index
sRPMI	RPMI-1640 culture medium with 0.35% NaCl
TLR	Toll like receptors
TMB	Tetramethylbenzidine
Tmx	Tamoxifen
TNF	Tumor necrosis factor

U	Unit
VaDNA	*Vibrio anguillarum* genomic DNA
Vtg	Vitellogenin
XCL	X-C motif chemokine ligand
WHO	World Health Organization
WWTP	Wastewater treatment plant

Abstract

Endocrine disrupting chemicals (EDCs) are a class of heterogeneous chemicals present in the environment that bind to estrogen receptors (ERs) and interfere with the estrogen-dependent control of body homeostasis. In the present thesis, we present evidence that EDCs modulate the immune system of gilthead seabream (*Sparus aurata* L.) even when exposure to these chemicals has ended. We evaluate the effect of 17α-ethinylestradiol (EE_2), a bio-active estrogen used in oral contraceptive pills, and tamoxifen (Tmx), a drug used worldwide for the treatment of ER-positive breast cancer. Both have become a widespread problem in the environment due to their high resistance to degradation. The gilthead seabream is a marine teleost fish of significant economic value in the Mediterranean area and has been extensively used in research as a protandrous hermaphrodite fish. Furthermore, during the development of the thsesis we identified a membrane-anchored receptor called G protein-coupled estrogen receptor (GPER1) in human neutrophils. The thesis was carried out in four stages.

First of all, we confirmed that EE_2 and Tmx, incorporated in the diet, promote an estrogenic response in adult fish although the effect of Tmx was seen to be much less pronounced. We also investigated the capacity of both compounds to modulate the immune response induced by an antigenic challenge and its capacity to recover its functionality when the treatments ceased. Both EE_2 and Tmx transiently inhibited the induction of *interleukin-1β* (*il-1β*) gene expression and transiently increased reactive oxygen species (ROS) in head kidney leukocytes from vaccinated fish. Moreover, EE_2 and Tmx increased the antibody titer but only the fish exposed to Tmx showed a higher IgM titer and a higher percentage of IgM^+B lymphocytes during the recovery period.

After observing that EE_2 and Tmx affected the immune response of adult specimens, we attempted to ascertain whether these effects are also produced in juveniles. Moreover, it was seen that treatment with G1, a GPER-selective agonist, also altered the immune response of adult specimens. Only EE_2 and Tmx differently increased *vtg* mARN levels during the treatment and the expression returned to basal levels during recovery. Although none of the three compounds affected ROS production, they all inhibited the induction of *il1b* gene expression after priming. Significantly, Tmx increased the percentage of IgM^+ cells in both head kidney and spleen during the recovery period. More importantly, the antibody response of vaccinated fish was modified by the three compounds but the exact effects depended on the time when the immunization was performed.

The previous results led us to investigate the influence of EE_2 on the innate immune response, focusing on lymphocytes and in the humoral activity when exposure to this EDC ceased. Significantly, EE_2 affects the percentage and proliferation of T and IgM^+-B lymphocytes. For the first time it was seen that EE_2 significantly induces the production of antibodies, an effect mediated through GPER1 signalling pathway.

Finally, we functionally characterized GPER1 in human neutrophils. These cells express a functional GPER1 in the plasma membrane and stimulation of the neutrophils with G1 resulted in a dose-dependent increase in transcript levels of *CFOS*, a marker of GPER1 activation. Besides, G1 significantly primed the production of O_2^- and drastically altered their gene expression profile. G1 treatment regulated the activation and life span of human neutrophils through several signaling pathways. To sum up, our results show for the first time that GPER1 activation promotes the polarization of human neutrophils towards a pro-inflammatory phenotype and identify GPER1 as a potential therapeutic target in immune diseases where neutrophils play a key role.

General introduction

RESEARCH OVERVIEW

At present, the global community faces multiple and interlinked challenges ranging from the impacts of the ongoing financial and economic crisis to greater climate change vulnerabilities and extreme weather events. At the same time, it must also reconcile meeting the pressing food and nutrition needs of a growing population with finite natural resources (FAO, 2016). Fisheries is a finite resource that in recent decades has been subjected to exploitation levels that in many cases have put at risk its sustainability. The development of industrial-scale aquaculture since the early 80's has provided an alternative source of fish products. In this context, fisheries and aquaculture make crucial contributions to the world's well-being and prosperity due to the sector provides livelihoods and income, both directly and indirectly, for a significant share of the world's population. Aquaculture is set to remain one of the fastest-growing animal food-producing sectors and, in the next decade, total production from both capture and aquaculture will exceed that of beef, pork or poultry (FAO, 2016). However, the practice of aquaculture means the confinement of a large number of individuals in confined spaces and their subjection to a more or less regular handling, which triggers stress in animals affecting the different developmental stages. These conditions cause the development of infectious diseases that are responsible for substantial economic losses. Therefore, knowledge of the immune system of fish has become one of the primary objectives in aquaculture research. Currently, gilthead seabream, *Sparus aurata* L., is one of the two most important species of fish farming in Spain and in the Región de Murcia. In recent years, increased supply from other Mediterranean countries such as Greece and Turkey has increased competition in the market. Competitiveness problem that prevents Spanish companies from reduce their production costs and increase their productivity. Therefore, it seems appropriate and useful for Spanish gilthead seabream companies the development of research with the aim of identifying the determinant factors of their competitiveness at production process, and contributing to improved efficiency focusing our interest in avoid diseases (Llorente, 2013).

Fishes are the most diverse of all vertebrate groups. They were the first creatures with brains and bony jaws. Due to their incredible diversity, fishes make up half of all vertebrate species alive today and bony fishes (osteichthyes) is represented with over 25.000 living species. If we focus on teleostei, they are the later species emerged since 450 million years ago when bony fishes (osteichthyes) appeared. Teleost fishes have adapted to diverse ecological habitats ranging from fresh water over seawater to environmental extremes. Teleost fishes are of high interest for humans in two large areas, namely as part of the diet and as model organisms for research purposes. From the point of view of nutritional resource, fish and fishery products represent a very valuable source of protein and essential

micronutrients for balanced nutrition and good health (Fao, 2012). Therefore, aquaculture is among one of the fastest-growing sectors in world food production: for the last decade it has supplied one-third of seafood consumed worldwide (Reverte et al., 2014).

Fish possess both innate and acquired/adaptive immunity. However, the innate immunity is stronger than adaptive immunity. The adaptive immune system is thought to have arisen early in vertebrate evolution, 450 million years ago, in the first jawed vertebrates. The basal position of fish in vertebrate phylogeny makes them very attractive for comparative immunological studies. We have to consider that most of the current knowledge on the immune system and fish´s pathogens comes from aquaculture species (Rauta et al., 2012).

Aquatic organisms have being exposed to a multitude of chemicals, acting as endocrine disrupting compounds or endocrine disrupting chemicals (EDCs), which are discharged by the human population. EDCs exert their effects via agonistic/antagonistic interactions with hormone receptors or by interfering with the normal synthesis, transport, metabolism, and secretion of endogenous hormones (Segner, 2006). The list of EDCs in freshwater and marine environments that mimic or block endogenous hormones is expanding at an alarming rate. Among EDCs, the most studied are the compounds that interfere with estrogen receptors (ERs) (Jobling et al., 2002), such as 17α-ethinilestradiol (EE_2).

The gilthead seabream is a marine, seasonally breeding, protandrous teleost with a great commercial value in the Mediterranean area. It is one of the main aquaculture species which has contributed to improve our knowledge in immune response and in avoiding fish diseases. The head kidney, it's the main hematopoietic organ and is composed by acidophilic granulocytes (AGs), lymphocytes (Ly), macrophages and precursors cells, where Ly are the responsible of adaptive immunity. This cell type expresses nuclear ERα, one of the ER type, and membrane-anchored G protein-coupled estrogen receptor 1 (GPER1), while AGs, the equivalent to human neutrophils, express only GPER1 (Cabas et al., 2013a; Liarte et al., 2011a) Cabas et al., 2015). ERs expression in immune cells made them susceptible to estrogens, both natural and synthetic.

The research group "Innate Immune System of Teleost Fish", where this PhD thesis has been developed, has a long history in the study of the immune system of Mediterranean aquacultured species, such as gilthead seabream and European seabass (*Dicentrarchus labrax* L.). Moreover, the effects of estrogens on the immune response of gilthead seabream have also been long studied by this group.

Given the above, this thesis focuses on the study of effects of two pharmacological compounds found in the aquatic systems, EE_2 and tamoxifen, and

G1, a GPER1 selective agonist, on the innate and adaptive immune response of gilthead seabream at different reproductive stages. Moreover, we analyzed its capacity of recovery when the exposition to these chemicals ends. In line with these aims, we analyze the presence of GPER1 on human neutrophils and its possible implication in the regulation of the immune system as target for immunological disorders, taking into account that these cells are the most abundant circulating leukocytes in human and play a critical role in different pathologies. In addition, although human neutrophil abundance and function are regulated by estrogens, since they express ERs, the relevance of GPER1 in the functions of these cells remains unknown.

1. IMMUNE SYSTEM

The immune system is composed of a complex network of molecules, cell types and tissues that collectively serve to protect the host from bacterial, fungal, and viral infections, as well as from the growth and dispersal of tumor cells. The collective and coordinated response of the immune system against these foreign substances and pathogens constitutes the immune response (Janeway, 2001). Generally, the immune system can be divided into two major layers of defense: innate or natural and adaptive or acquired immune responses (Figure 1). The fundamental difference between them is that the adaptive immune response is highly specific for a particular pathogen and is most effective with each successive encounter with the same pathogen (Roitt, 1994). While the innate immune system is more ancient, the adaptive immune system first arose more recently with the appearance of cartilaginous fish (Dooley and Flajnik, 2006).

The innate system is the earliest immune mechanism that defends the host from infection by other organisms in a non-specific manner. The innate immune response include physical barriers, phagocytic cells and eosinophils, natural killer cells and various blood molecules (complement and acute phase proteins) (Abbas et al., 2001). Among these, polymorphonuclear neutrophils are the most abundant leukocytes in the blood. They are "professional" phagocytic cells of the innate immune system that act as the first line of defense against invading pathogens, principally bacteria and fungi but also viruses. Therefore, they are powerful effector cells that destroy infectious threats through phagocytosis, degranulation, reactive oxygen species (ROS) and neutrophil extracellular traps (Borregaard, 2010; Kolaczkowska and Kubes, 2013). Because of their powerful microbicidal equipment, they have a major role in inflammatory responses other than anti-infectious defenses and they lead the first wave of host defense against infection or tissue damage. However, numerous *in vitro* and *in vivo* studies, focusing on novel aspects of the neutrophil biology and function, have recently shed a new light on the

potential role that neutrophils can exert in the modulation of innate and adaptive immune responses (Drifte et al., 2013). Besides several preformed or rapidly generated inflammatory mediators, neutrophils display the capacity to *de novo* synthesize and release also several chemokines and cytokines with immunoregulatory properties (Cassatella, 1999; Mantovani et al., 2011).

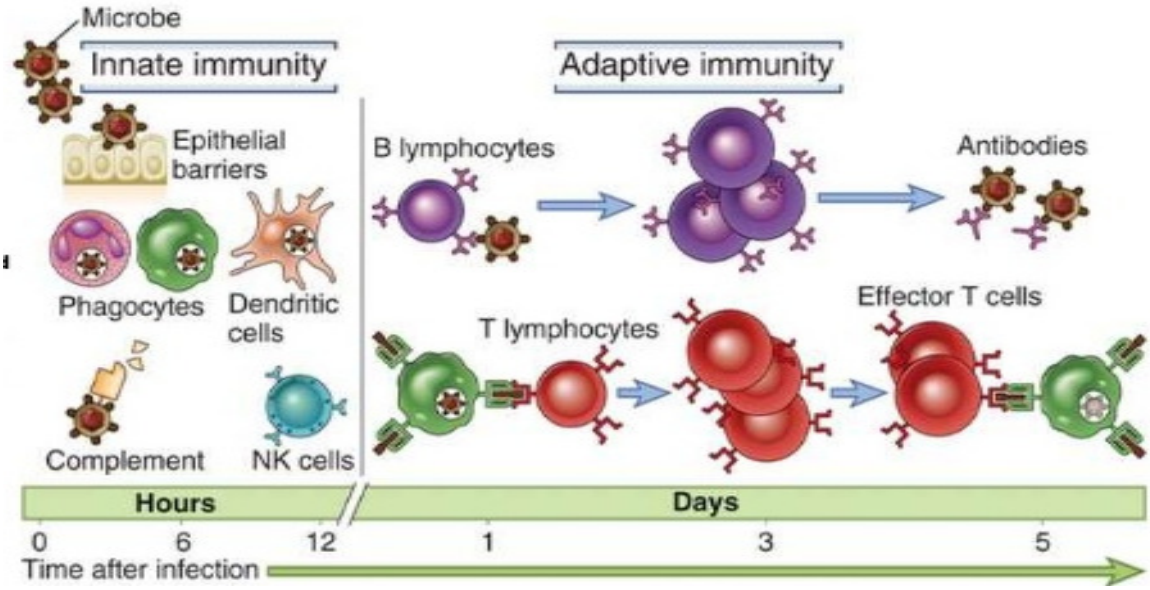

Figure 1. Main intervenient in innate and adaptive immune systems (Adapted from Abbas and Lichtman, 2009).

The adaptive immune response has two key features: specificity to the invasive agent and immunological memory (Roitt, 1994). The adaptive immune response includes Ly (B and T cells) and secreted antibodies (immunoglobulins, Ig) (Abbas et al., 2001). These types of Ly collectively define the adaptive immune response, however, they each have focused roles and function through distinct types of receptors (Rauta et al., 2012). Whereas B cells are responsible for the production of antibodies (Abs) and in their mature form are referred to as plasma cells, T cells can develop into effector cells in response to an activating antigen and are responsible for cell-mediated immunity. The functions of effector cells fall into one of three broad classes: killing, activation, and regulation. For example, cytotoxic T cells serve the purpose of killing cells that have been infected with intracellular pathogens, such as viruses. While helper T cells provide essential intercellular signals that influence the behavior and activity of other immune cells (including B cells and macrophages), regulatory T cells mediate the activity of other Ly and help regulate immune responses (Figure 2). During the course of the immune response, a number of those B and T cells that have survived past infections can also serve to differentiate into the long-living Ly, known as memory cells, responsible for immunological memory (Rauta et al., 2012).

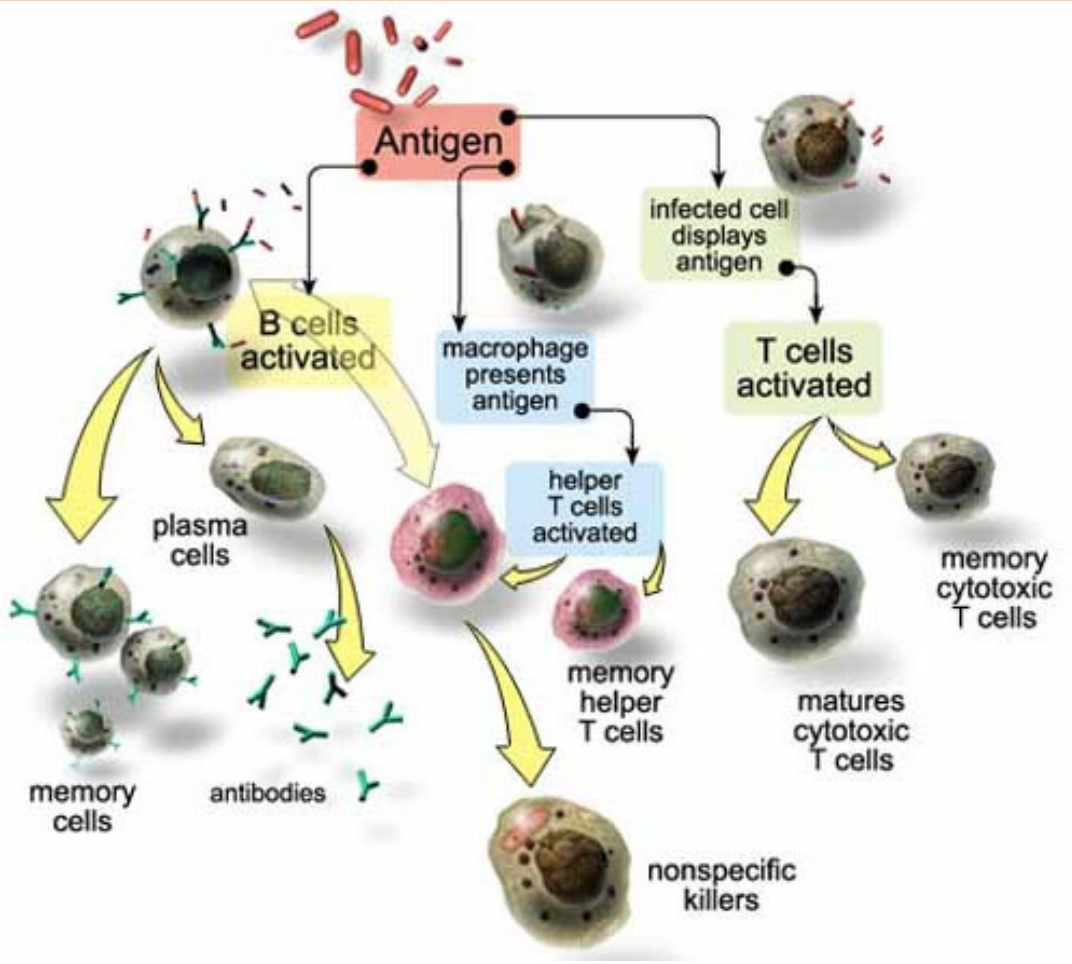

Figure 2. Cell-mediated immune response (source: www.uta.edu/chagas/images/immunSys.jpg).

IgM is the most evolutionary conserved Ab isotype, present in all vertebrates, and it is also the earliest isotype to be expressed during immune development (Figure 3). While the largest population of mature B cells (also termed B-2 cells), are primarily responsible for T-cell dependent immune responses, B-1 cells represent mature Ly subsets that are involved in innate-like responses, with more rapid responses to non-protein antigens. These cells are a major source of Abs that are constitutively secreted without prior immune activation (Baumgarth et al., 2005; Holodick et al., 2010). These secreted Ig have been termed "natural antibodies" as may be present from birth without external antigenic exposure, which stand in contrast to "immune" Ab that arise after specific immune exposure or antigenic challenge. In mice, secreted natural IgM is generated by B-1 cells predominantly in the spleen, and to a lesser extent in the bone marrow. Host defenses have also been shown to benefit from B-1 cell responses to common microbial pathogens (Baumgarth et al., 2005). Yet, there is mounting evidence that natural IgM Ab also contribute to critical innate immune functions involved in the maintenance of tissue homeostasis. These functions include augmenting the clearance of apoptotic cells

and the reinforcement of mechanisms involved in protection from the development of autoimmune disease (Silverman et al., 2009).

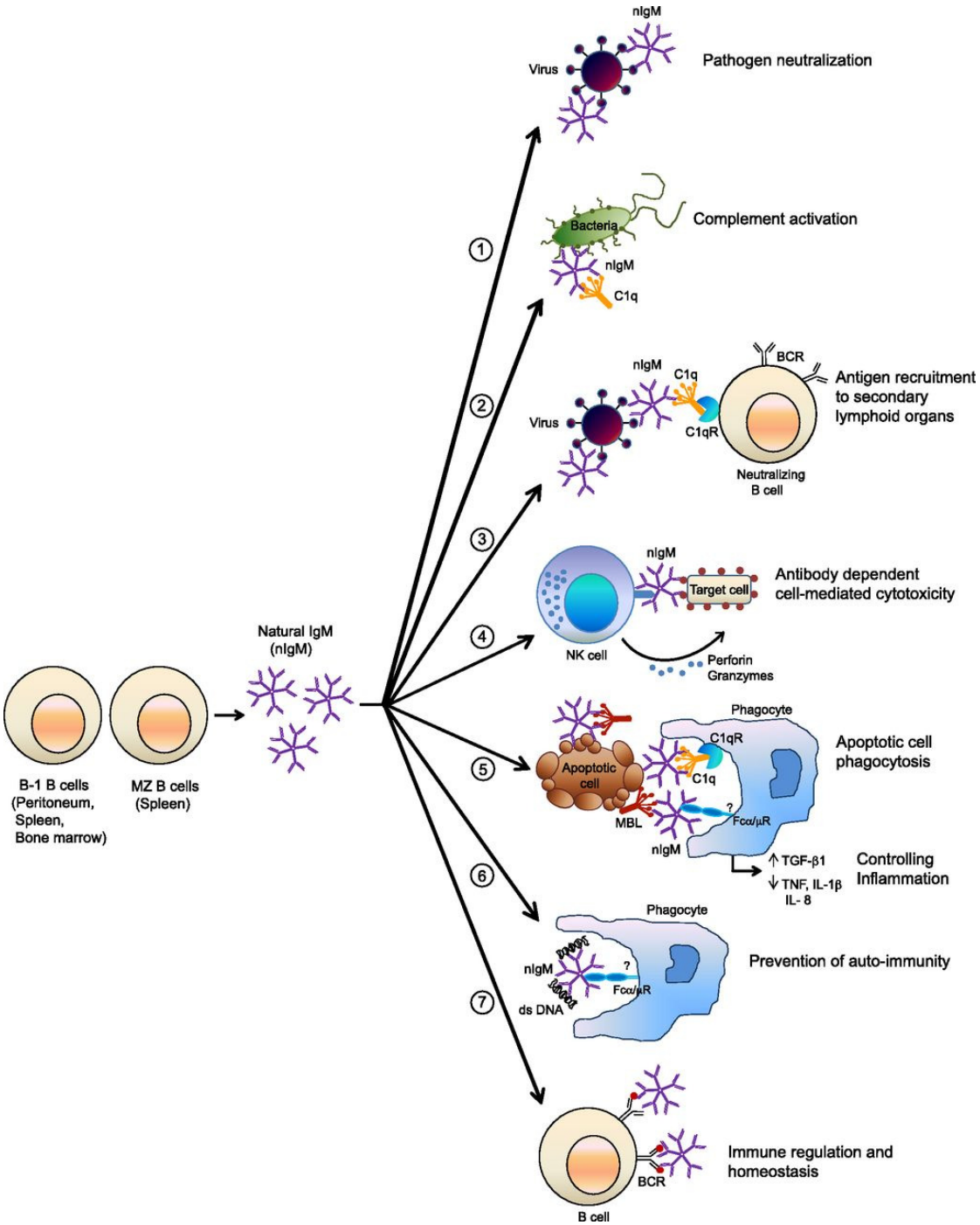

Figure 3. Diverse roles of natural IgM in immunity (Panda and Ding, 2015).

Natural IgM Abs are reported to recognize a wide range of different microbial components, which include viral antigens and bacterial toxins (Ochsenbein et al., 1999). The broad reactivity pattern of the natural Ab may therefore help to protect against a variety of pathogens not previously encountered. Due in part to the high avidity of polymeric IgM, these may contribute to the initial immune defense and to the control of invading pathogens until the

immune system has time to launch a specific adaptive response (Baumgarth et al., 2005). In certain cases, the B-1 expressed IgM Ab have been shown to directly neutralize or inhibit pathogens as well as aid the initiation of adaptive immune responses from B-2 cells, which together play critical roles in protection against bacterial and viral infections (Baumgarth et al., 2005; Boes et al., 1998; Haas et al., 2005; Ochsenbein et al., 1999; Zhou et al., 2007). The repertoire of natural Abs also enables recognition of non-protein self-antigens such as phosphorylcholine, phosphatidylcholine, and carbohydrate determinants, and that in some cases these ab are capable of cross-reactivity with structurally related epitopes on microbes. Due to the importance of auto-reactive ab in apoptotic cell clearance, the self-recognition properties of these Abs may be just as important for the host as pathogen recognition.

Ly and other cells from the immune system, such as macrophages and dendritic cells, produce a large array of cell signaling proteins that are collectively referred to as cytokines, which are responsible for the intercellular communications necessary for the accurate and efficient performance for both innate and adaptive immune responses. Cytokines are glycoproteins produced, usually as the result of an activating stimulus, by various cells of the body that induce signaling by binding to specific cell surface receptors. In general terms, cytokines are responsible for much of the activation and regulation of the body's response to disease and infection, and can directly addict the activity of most regulation of the body's response to disease and infection, and can directly affect the activity of most immune cells. Playing a crucial role in the functioning of Ly, cytokines can serve to recruit other cells in the body's response to invasion and act to mediate normal cellular processes (Zhang and An, 2007). Thus, deciphering the action of cytokines is central to understanding various aspects of the immune system.

Cytokines can modulate immune responses through an autocrine or paracrine manner upon binding to their corresponding receptors. Cytokines are derived from macrophages, Ly, AGs, dendritic cells, mast cells, and epithelial cells. (Savan and Sakai, 2006). In innate immunity, macrophages can secrete interleukin (IL)-1, IL-6, IL-12, tumor necrosis factor-alpha (TNF-α), and chemokines such as IL-8 and monocyte chemoattractant protein-1, all of which are indispensable for neutrophil and Ly recruitment to the infected tissues and their activation as pathogen eliminators (Svanborg et al., 1999).

The body produces several different classes of cytokines, including:

- Proinflammatory cytokines: promote systemic and site specific inflammation. The typical proinflammatory cytokines are IL-1β, IL-6 and TNF-α.
- Antinflammatory cytokines: the functional definition of an antiinflammatory cytokine is the ability of the cytokine to inhibit the synthesis of IL-1, TNF-α,

and other major proinflammatory cytokines. Cytokines with anti-inflammatory activities are IL-1 receptor antagonist (IL-1RA) or IL10.

- Interferons (IFNs): are involved in the antiviral response (type I) and immune regulation (type II).
- Colony stimulating factors: cause proliferation and differentiation of specific target cells, such as macrophage colony-stimulating factor (MCSF), granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF).
- Growth and differentiation factors: subfamily of transforming growth factor beta (TGF-β) like proteins that play an important role during prenatal and postnatal development, and the maintenance of various tissues.
- Chemokines: a group of structurally related cytokines that can induce chemotaxis of specific nearby cells. Its members can be divided into four main categories, X-C motif chemokine ligand (XCL1 and XCL2), C-C motif chemokine ligand (CCL-1), C-X-C motif chemokine ligand (CXCL8), C-X3-C motif chemokine ligand (CX3CL1).

The innate and adaptive immune responses are closely related, as the innate immune responses play an instructive role in the development of acquired immune response. These two components are connected in many ways, which confirms interlinking relationship (Dixon and Stet, 2001; Fearon and Locksley, 1996; Medzhitov, 2007; Medzhitov and Janeway, 1997). The basic principle of innate control of adaptive immunity is based on establishing an association between the antigens recognition by Ly and the pathogen associated molecular patterns (PAMPs) recognition by pattern recognition receptors (PRRs) (Janeway, 1989). For example, some phagocytes capture and degrade antigens and present them to T cells attached to the surface major histocompatibility complex in a form suitable for them to recognize. In response, the Ly secrete soluble factors which activated phagocytes to destroy the pathogens that they have phagocytized. The result of these interactions is that the majority of immune responses against pathogens consisting of a wide variety of innate and adaptive components. In the early stages of infection, the innate response predominates but Ly subsequently begin to generate the adaptive response (Figure 4).

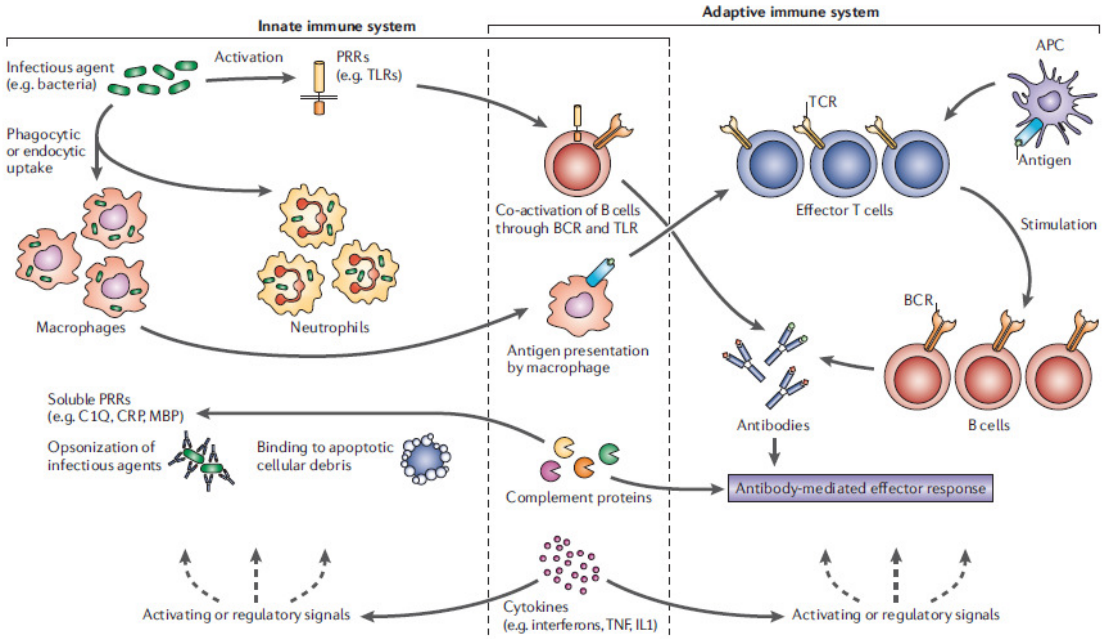

Figure 4. The innate and adaptive immune systems crosstalk. Innate immune mechanisms generally involve inmediate, nonspecific responses to foreing infectious agents, and include cellular functions sucha as phagocytosis and endocytosis by macrophages and neutrophils. Adaptive immune mechanisms involve the engagement of receptors that are selected for reactivity with specific antigens (T-cell receptors, TCRs, and Ig receptors on B cells). Innate and adaptive immune systems crosstalk occurs by means that have not been fully understood (Adapted from Gregersen and Behrens, 2006).

1.1 Immune system of teleost fish

Teleost fish constituted a large zoological group with more than 20.000 identified species which are ubiquitous throughout almost all aquatic environments with diverse oxygen concentrations, water pressures, temperatures, and salinities (Rauta et al., 2012). Related representatives from the same group can be found in different ecosystems. For instance, Perciformes are adapted to both freshwater and marine habitats. Thus, it is expected that a considerable diversity of host/pathogen interactions characterize fish immune defense mechanisms (Fillatreau et al., 2013). Teleosts are the first animal group with an innate and adaptive immune system well structured and differentiated. Its innate response comprises physical barriers (epithelium and mucosa), cellular effectors (phagocytic cells and nonspecific cytotoxic cells) and humoral factors (complement and other acute phase proteins) and adaptive response comprises a cellular (Ly) and humoral (Abs) components. However, despite their similarities with other vertebrate immune system, there are clear differences as fish depend more heavily on innate defense mechanisms, mainly in low temperature conditions (the fish are poikilotherms) since the adaptive immune response is dependent on the temperature (Abruzzini et al., 1982; Avtalion and Wishkovsky, 1987; Bly and Clem, 1991; Cuchens and Clem, 1977)

Research on immune system in lower vertebrates will become indispensable for better understanding the evolutionary history of immune systems throughout vertebrates as a whole. As an important link to vertebrate evolution, fish is believed to be an excellent model and an indispensable component of comparative immunology. Therefore, fishes in general and teleost fishes in particular are of importance for humans, is their usage in research as model organisms. The most popular fish model species are also increasingly used to analyze human diseases like genetic disorders (Ota and Kawahara, 2014), brain disorders (Kalueff et al., 2014; Stewart et al., 2014), or toxicological (Gibson et al., 2005) and immunological (Goody et al., 2014) aspects, among others (Lieschke and Currie, 2007; Löhr and Hammerschmidt, 2011). Teleost fishes share not only developmental aspects with their mammalian counterparts, but also the endocrine system including hormones, receptors, and signaling cascades displays a striking homology (Löhr and Hammerschmidt, 2011).

1.1.1 Lymphoid tissues and organs

The secondary lymphoid organs present in mammals are also found in fish, except the lymph nodes and the bone marrow (Press and Evensen, 1999). Therefore, kidney (anterior or cephalic/head, HK), thymus and spleen are the largest lymphoid organs in teleosts (Zapata et al., 2006). The HK in teleost fish is the equivalent of the bone marrow in vertebrates and is the largest site of haematopoiesis until adulthood (Zapata et al., 2006).

Kidney

The entire kidney contains immune cells. The HK has the highest concentration of developing B cells, and also contains low levels of ab-secreting cells (Bromage et al., 2004; Zwollo et al., 2005; Zwollo et al., 2008; Zwollo et al., 2010). The HK (aglomerular) assumes hemopoietic functions (Meseguer et al., 1995; Zapata et al., 2006) and unlike higher vertebrates it is the principal immune organ responsible for phagocytosis (Danneving et al., 1994), antigen processing (Brattgjerd and Evensen, 1996; Kaattari and Irwin, 1985) and formation of IgM and immune memory (Whyte, 2007). The HK is a well innervated organ and serves as an important endocrine organ, homologous to mammalian adrenal gland and release corticosteroids and other hormones. Thus, the HK is an important organ with key regulatory functions and the central organ for immune–endocrine interactions and neuroimmuno-endocrine connections. In contrast, posterior kidney, basically with excretory function, possesses both renal and immune tissues (Zwollo et al., 2005; Zwollo et al., 2008).

The main cells found in the HK are macrophages, which aggregate into structures called melanomacrophage centres, and granulocytes. Moreover, it is composed by Ly, which are found at all developmental stages and exist mostly as Ig positive cells (B cells) (Press et al., 1994). Also, reticular cells play an important role in supplying the interactions necessary for the function of Ly (Press et al., 1994) and endothelial cells of sinusoids (Uribe et al., 2011). In gilthead seabream, HK is formed by macrophages, Ly and AGs as well as precursor cells. AGs are the major cell type participating in innate host responses, while the HK is the central immune organ that provides a source for AGs (Chaves-Pozo et al., 2007; Sepulcre et al., 2002).

Thymus

Produces T cells involved in allograft rejection, and stimulation of phagocytosis and ab production by B cells (Zapata and Amemiya, 2000). Blood filtration and erythrocytic destruction is performed by the melanomacrophagic centers by accumulation of associated to ellipsoid capillaries. These centers may retain antigens as immune complexes for long periods.

Spleen

Fish erythrocytes, neutrophils and granulocytes are believed to be present in the spleen whereas Ly are the major cell type found in the thymus. The spleen functions as major secondary immune organ, as found in mammalian species, with abundant IgM positive mature B cells. It also plays a major role in the clearance of blood-borne antigens and immune complexes in splenic ellipsoids, and in the antigen presentation and the initiation of the adaptive immune response (Whyte, 2007; Zabotkina, 2005). It plays a vital role in haematopoiesis, antigen degradation and Ab production processing. As fish have no lymph nodes, the spleen plays an essential role in antigen trapping (Press et al., 1994; Rauta et al., 2012).

Gut-associated lymphoid tissue

It consists principally of different sized Ly, plasma cells and macrophages, as well as several types of granulocytes and mast cells (Zapata and Amemiya, 2000). In teleosts, gut intraepithelial Ly are largely considered T cells, whereas lymphoid cells present in the lamina propria are mainly B cells (Zapata et al., 2006).

1.1.2 Innate immune response

Innate or non-specific immunity is a fundamental defense mechanism in fish. It is commonly divided into three compartments: the epithelial/mucosal barrier, the humoral parameters and the cellular components. It plays a key role in the acquired immune response and homeostasis through a system of receptor proteins which

identify molecular patterns that are typical of pathogenic microorganisms, including polysaccharides, lipopolysaccharide (LPS), peptidoglycan, bacterial DNA, viral RNA and other molecules that are not normally on the surface of multicellular organisms (Uribe et al., 2011).

Innate cellular response of the fish includes a variety of leukocytes, which include phagocytes (monocytes/macrophages and granulocytes) and nonspecific cytotoxic cells (Secombes, 1996). Phagocytes are more important in innate immunity by its capacity to eliminate viruses, bacteria and parasites (Rowley et al., 1988; Sepulcre et al., 2002) and, moreover, can be the initiator of activation and regulation of the specific immune response (Vallejo et al., 1991; Vallejo et al., 1992). The main cells involved in phagocytosis in fish are neutrophils and macrophages (Secombes and Fletcher, 1992). These cells remove bacteria mainly by the ROS production during a respiratory burst. In addition, neutrophils possess myeloperoxidase in their cytoplasmic granules, which in the presence of halide and hydrogen peroxide kills bacteria by halogenation of the bacterial cell wall. Moreover, these cells have lysozymes and other hydrolytic enzymes in their lysosomes (Fischer et al., 2006). AGs of HK gilthead seabream is the more active and abundant phagocytic cell of the species (Chaves-Pozo et al., 2004; Sepulcre et al., 2002). Moreover, AGs might be considered as functionally equivalent to mammalian neutrophils, since they are the most abundant circulating granulocytes (Sepulcre et al., 2002), 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 Toll like receptors (TLRs), with the exception of TLR3 (Sepulcre et al., 2007).

The recognition of microbial pathogens mediated by PRRs is critical to the initiation of innate immune responses. PRRs sense the conserved molecular structure of a pathogen and induces subsequent host immunity through multiple signaling pathways that contribute to the eradication of the pathogen (Janeway and Medzhitov, 2002). Activation of PRR promotes the production of all cytokines previously described.

IL-1 is an important early response pro-inflammatory cytokine that mediates immune regulation in both innate and adaptive immunity. IL-1 could be secreted by monocytes, activated macrophages, granulocytes, endothelial cells, activated T cells, and many other cell types. There are 10 ligand proteins in the IL-1 gene family, the main members of which include IL-1α, IL-1β, and IL-18. IL-1α and IL-1β share the same receptor on target cells and exert similar biological functions, although IL-1β shows more potent function in humoral immune response. Over the years, IL-1β genes have been identified in various teleost fish species, including rainbow trout (*Oncorhynchus mykiss*), gilthead seabream, carp (*Cyprinus carpio*), European sea

bass, channel catfish (*Ictalurus punctatus*) and yellowfin seabream (*Acanthopagrus latus*). IL-1 activates target cells by binding to IL-1 receptors on the cell surface and ultimately triggering inflammation to cope with pathogen infection (Zhu et al., 2013).

The IL-10 family of cytokines consists of nine members: IL-10, IL-19, IL-20, IL-22, IL-24, IL-26 (Ouyang et al., 2011). The central functions of IL-10 family cytokines converge on protection of organs and tissues from damage caused by infections and by inflammatory responses. They are essential for host defense against various infections and in the development of many autoimmune diseases. IL-10 itself inhibits the innate and adaptive immune responses from leukocytes and limits the potential tissue damage caused by inflammation (Ouyang et al., 2011). The IL-10 gene has been identified in a number of teleost fish. In general, the fish IL-10s, similar to the mammalian orthologues, act as a suppressor and exert a conserved role in dampening inflammatory responses (Zou and Secombes, 2016).

1.1.3 Adaptive immune response

Besides the direct relevance of the study of the immune system of fish, it is also of interest to understand the evolution of the adaptive immune system in vertebrates. Although fish lack bone marrow and lymph nodes, fish infectious by bacterial or viral pathogens can dead to the production of specific Abs, which in some cases correlates perfectly with protection against re-infection by these pathogens (Fillatreau et al., 2013). Such a protection may persist for more than one year. Acquired immunity is mediated by Ly and affected mainly through Abs which can neutralize viruses, facilitate phagocytosis of pathogens by opsonization, and activate classical complement pathway (Sakai, 1984).

Immunological memory in the B cell linage consists of long-live plasma cells and memory B cells. Moreover, Abs appears in serum faster and the affinity of the Ab generated is higher than in the primary response. Therefore, immunological memory resides in the Ab which are continuously made by long-lived plasma cells as well as in the expanded B and T memory cells which can cooperate to generate additional Ab with higher affinity, improving the immune response (Frasca et al., 2016). The generation of memory B cells is maintained, but the Ab response is not, in the elderly after repeated influenza immunizations.

Igs are pivotal factors in adaptive immunity and are specifically produced by B cells. Two forms of Igs have been identified: B cell receptor (BCR), a membrane-bound molecule that acts as an antigen receptor on the B cell surface; and the widely known Ab secreted by plasma cells, which is an important molecule mediating humoral immune responses. Although in mammals Igs could be divided into five categories according to the constant region: IgM, IgD, IgG, IgA, and IgE, it

was believed for a long time that only one of them, IgM, exists in fish. However, recent studies overturned this view as several other functional Igs such as IgD and IgT (also known as IgZ), have been discovered in teleost fish. The first unexpected discovery was IgD in channel catfish (*Ictalurus punctatus*) (Bengten et al., 2002) . Since then, a third novel Ig identified in bony fish served as evidence of the complexity of the fish Ig family. Simultaneously, IgT, similar to zebrafish (*Danio rerio*) (Danilova et al., 2005), was identified in rainbow trout (*Oncorhynchus mykiss*) (Hansen et al., 2005) and pufferfish (*Tetraodon biocellatus*) (Savan et al., 2005). Further research demonstrated that IgT is a mucosal immune-related Ig that has anti-pathogenic function only in mucosal tissues (namely gut, gills and skin), similar to IgA in warm-blooded animals and IgX in amphibians (Zhang et al., 2010; Zhu, 2012).

T cells play important roles in the adaptive immune system. Cytotoxic and helper T cells have been found to be present in fish (Fischer et al., 2013) . Both $CD8^+$ and $CD4^+$ cells have been reported using specific Abs. Abs against T cell specific surface antigens that are well conserved throughout vertebrates have been also used to identify fish T cells. For example, CD3ε and ZAP70 are well conserved and Abs against the intracellular domain of human CD3ε and ZAP70 have been used to identify T cells in fixed cells and tissues of several fish species, such as CD3ε for Atlantic salmon (Koppang et al., 2010), ZAP70 for carp (Piazzon et al., 2015) and zebrafish (Yoon et al., 2015). Mature T cells are distributed throughout the body, particularly in lymphoid tissues such as the thymus, kidney and spleen. More recently, abundant T cells were identified in mucosal tissues, such as the intestine, gill and skin. It is well known in mammals that T cells play a central role in adaptive immune response and the several subsets of T cells have a distinct function involved in both humoral and cell-mediated immune responses. In fish similar functions of T cells known for mammals have been reported by mean of *in vivo* and *in vitro* experiments (review by Nakanishi et al., 2015).

1.1.4 Sex esteroid as regulators of the immune response

Immune system is regulated by many factors taking into account that immune, endocrine, and central nervous systems are integrated through a network of signal molecules (cytokines, hormones, and neurotransmitters) that act on a common set of receptors. The immune system is amply modulate by the reproductive system (Tokarz et al., 2015) , being attributed to the sex hormones and the presence of their receptors on immune cells (Khan and Ansar Ahmed, 2015) . The regulation of immune response is different in males and females due to the presence and prevalence of different hormones. In males, testosterone plays a major role as sex hormone; however, in females the predominant role is for estrogens and progesterone. This difference in hormone levels in both sexes leads

alterations in the outcome of immune responsiveness. In general, females had superior humoral and cell-mediated immunity. Females tend to have a reduced incidence of certain tumors and generally resist a variety of bacterial and viral infections and parasitic infestations more successfully than males, which correlated with their greater longevity. Complex hormonal interactions affect both developing lymphocytes and regulate mature immune cells. Therefore, it has been shown a difference in the vulnerability to autoimmune diseases in two sexes where women have more prevalence. It is believed that the autoimmune diseases development depends upon estrogen-induced immune suppression both in human autoimmune diseases and in their experimental animal model counterparts (Gourdy et al., 2016). Due to the presence of immune cell specific receptors for sex hormones, estrogen-based therapies have been considered for many years as promising strategies in autoimmune diseases such as multiple sclerosis, and also its administration likewise exerts protective effects against autoimmune diabetes (Gourdy et al., 2016). In previous studies it has been shown that 17β-estradiol (E_2), the main fish estrogen, promotes the mobilization of AGs from the HK to the gonad (Chaves-Pozo et al., 2005a; Liarte et al., 2007). Moreover, it modulate fish leukocyte trafficking during an inflammatory process by activating endothelial cells (Liarte et al., 2011a, 2011b). E_2 also orchestrated profound alterations in the macrophage immune-related processes and pathways (Liarte et al., 2011c) . On the other hand, testosterone and the main fish androgen 11-ketotestosterone plasmatic levels correlate with the expression of extracellular matrix-related molecules that take part in inflammatory processes (Castillo-Briceño et al., 2009, 2011). Physiological concentrations of testosterone exerted a pro-inflammatory effect on both AGs and macrophages, while 11-ketotestosterone showed an anti-inflammatory effect in AGs and a strong pro-inflammatory effect in macrophages. Interestingly, both androgens modulated the expression of toll-like receptors in these two immune cell types, suggesting that androgens might regulate the sensitivity of phagocytes to pathogens and damage signals (Águila et al., 2013). Furthermore, testosterone enhanced the phagocytic activity and the production of ROS of leukocytes (Castillo-Briceño et al., 2013).

2. ESTROGEN RECEPTORS

It has been recognized for over 40 years that estrogens, in addition to their classic genomic actions mediated through activation of nuclear ERs, can also elicit rapid and cell surface-mediated responses that are often non-genomic (Szego and Davis, 1967). Therefore, other receptors must also be involved because E_2 actions have been described in cells lacking ERs (Gu et al., 1999; Nadal et al., 2000; Qiu et al., 2003). For example, estrogens cause rapid activation of second messengers in a human breast cancer cell line, SKBR3 cells, that lack ERα and ERβ, but express the orphan, at this moment, GPER1 (Filardo et al., 2000). Despite previously rapid

effects of estrogens had been identified, it was not until 2005, when an orphan GPCR was identified as an estrogen-binding intracellular membrane (Revankar et al., 2005; Thomas et al., 2005) . In 2013, previous works in our laboratory demonstrated that gilthead seabream AGs, which are the functional equivalent of mammalian neutrophils, express GPER1 at both mRNA and protein levels (Cabas et al., 2013a). Nevertheless, there are no studies about the presence of this ER in human neutrophils and its action in this immune cell type.

2.1 Nuclear estrogen receptors: ERs

The nuclear hormone receptor superfamily is characterized by an impressive functional diversity of its members despite a remarkable overall structural unity. A variety of ligands bind specifically to them and these receptors control gene networks that have profound effects on growth, development, and homeostasis. The ligand-receptor complexes recognize transcriptional enhancer DNA sequences, the hormone response elements, resulting in induction or repression of gene activity. The similarity between all these hormone response enhancer elements, as well as between the receptors themselves, indicates a conserved general strategy for the hormonal control of transcription by steroids. The activated receptors bind to responsive promoters and most likely mediate the assembly of stage- and tissue-specific transcription factor complexes that stimulate or inhibit gene expression (Wahli and Martínez, 1991).

The members of the superfamily have a common functional domain structure (Figure 5). This includes a variable N-terminal domain, often important for transactivation of transcription; a well conserved DNA-binding domain, crucial for recognition of specific DNA sequences and protein:protein interactions; and at the C-terminal end, a ligand-binding domain, important for hormone binding, protein:protein interactions, and additional transactivation activity (Kumar and Thompson, 1999). The DNA-binding domain has the most conserved amino acid sequence among the members of the steroid-thyroid-retinoid receptor superfamily (Kumar, 1999).

To date, in most vertebrates have been isolated two distinct types of ER, ERα and ERβ. ERs are expressed in different cell types including immune cells (Ascenzi et al., 2006; Phiel et al., 2005; Pierdominici et al., 2010) and the presence of one ER subtype over the other might change estrogen effects, promoting or dampening inflammation (Straub, 2007). Several studies with mouse models of systemic lupus erythematosus have suggested a prominent pro-inflammatory role for ERα, contributing to disease progression (Bynote et al., 2008; Li and McMurray, 2007; Svenson et al., 2008). On the other hand, ER appears to have an anti-inflammatory and immunosuppressive effect on lupus mice models and administration of the ERβ-

selective agonist diarylpropionitrile leads to a reduction of autoantibody production and an amelioration of albuminuria (Li and McMurray, 2007). In 1989, a full sequence for the nuclear ER was reported in rainbow trout (Pakdel et al., 1989). Up to now, the impacts of the endogenously regulated natural estrogen, E_2, are mediated by at least three known ER subtypes, ERα, ERβ1 and ERγ (Katsu et al., 2013; Nelson and Habibi, 2013). The ERγ-form in fish appears to be closely related to ERβ1, suggesting that it reflects a gene duplication event that has occurred within the teleosts. As a consequence, ERβ and ERγ have been named ERβ1 and ERβ2, respectively (Hawkins et al., 2000). Thus, the ancestral condition for the jawed vertebrates (Gnathostomata) is considered to have two forms of ERs, corresponding to ERα and ERβ (Thornton, 2001). These are the two forms of ERs that have been found in mammals, fishes, birds, reptiles and amphibians (Katsu et al., 2013).

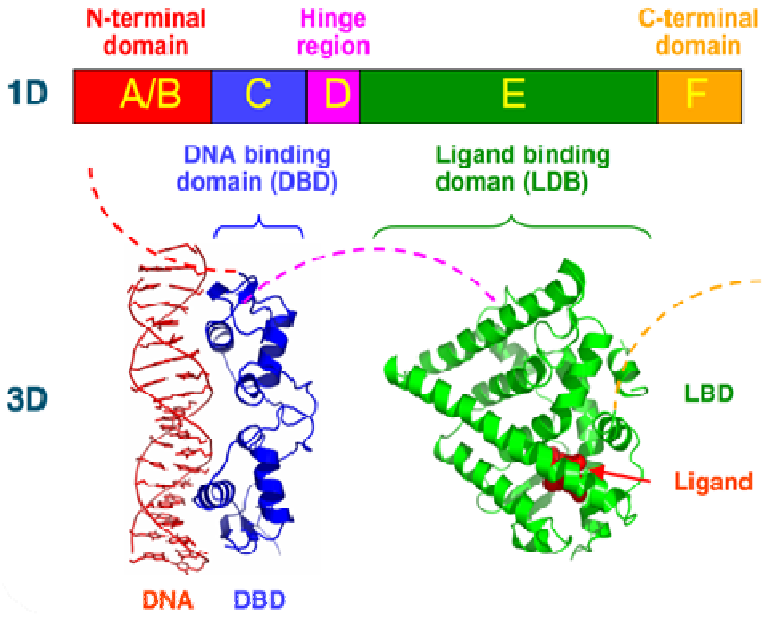

Figure 5. Schematic illustration of structure of nuclear ERs. Schematic 1D amino acid sequence of a nuclear receptor and the schematic 3D structures of the DBD (bound to DNA) and LBD (bound to hormone) regions of the nuclear receptor. The structures shown are of the estrogen receptor. Experimental structures of N-terminal domain (A/B), hinge region (D), and C-terminal domain (F). Source:https://en.wikipedia.org/wiki/Nuclear_receptor#/media/File:Nuclear_Receptor_Structure.png.

Moreover, a fourth isoform (ERα2) has been found in rainbow trout (Nagler et al., 2007). Focus on our model, gilthead seabream, ERα, ERβ1 and ERβ2 are expressed in reproductive and non-reproductive organs such as liver, heart, ovary, skin, testis and kidney (Pinto et al., 2006). In HK cell populations, macrophages and Ly constitutively express ERα gene. Moreover, macrophages are important in the immune modulatory role of E_2, as suggested by its ability to induce ERβ2 gene expression and up-regulate the expression of genes coding for ERα, ERβ1, pro-

inflammatory cytokines, chemokines and tissue remodeling molecules. Furthermore, the soluble factors produced by E_2-treated macrophages decreased in HK phagocytes, their phagocytic ability and capacity. Nevertheless, AGs do not express any of the three ER genes (ERα, ERβ1 and ERβ2) (Liarte et al., 2011a). As it has been mentioned before, these cells are considered as neutrophilic phagocytes with some morphologic similarities to their human counterparts (Weinreb, 1963) .

2.2 G protein-coupled estrogen receptors

G protein-coupled receptors (GPCRs) are the largest and most diverse group of membrane receptors in eukaryotes that respond to a variety of external signals. In fact, researchers estimate that between one-third and one-half of all marketed drugs act by these receptors. Binding of a signaling molecule to a GPCR results in G protein activation which, in turn, triggers the production of second messengers. The ligands that bind and activate these receptors include light-sensitive compounds, pheromones, hormones, and neurotransmitters. Through this sequence of events, GPCRs help regulate an incredible range of functions, from sensation to growth to hormone responses. They share a common architecture that has been conserved over the course of evolution. GPCRs consist of a single polypeptide that is folded into a globular shape and embedded in a cell's plasma membrane. Seven segments of this molecule span the entire width of the membrane, explaining why GPCRs are sometimes called seven-transmembrane receptors, and the intervening portions loop both inside and outside the cell. The extracellular loops form part of the pockets at which signaling molecules bind to the GPCR.

GPCRs interact with G proteins in the plasma membrane. When an external signaling molecule binds to a GPCR, it causes a conformational change in the GPCR. This change then triggers the interaction between the GPCR and a nearby G protein. These specialized proteins have the ability to bind the nucleotides guanosine triphosphate (GTP) and guanosine diphosphate (GDP) (Figure 6). The G proteins that associate with GPCRs are heterotrimeric, they have three different subunits: an alpha subunit, a beta subunit, and a gamma subunit. Two of these subunits (alpha and gamma) are attached to the plasma membrane by lipid anchors. The G protein alpha subunit binds either GTP or GDP depending on whether the protein is active (GTP) or inactive (GDP). In the absence of a signal, GDP attaches to the alpha subunit, and the entire G protein-GDP complex binds to a nearby GPCR. When a signaling molecule joins with the GPCR, a change in the conformation of the GPCR activates the G protein, and GTP physically replaces the GDP bound to the alpha subunit. As a result, the G protein subunits dissociate into two parts: the GTP-bound alpha subunit and a beta-gamma dimer. Both parts remain anchored to the plasma membrane, but they are no longer bound to the GPCR, so they can now diffuse laterally to interact with other membrane proteins. G proteins remain active as long

as their alpha subunits are joined with GTP. However, when this GTP is hydrolyzed back to GDP, the subunits once again assume the form of an inactive heterotrimer, and the entire G protein reassociates with the now-inactive GPCR. In this way, G proteins work like a switch-turned on or off by signal-receptor interactions on the cell's surface.

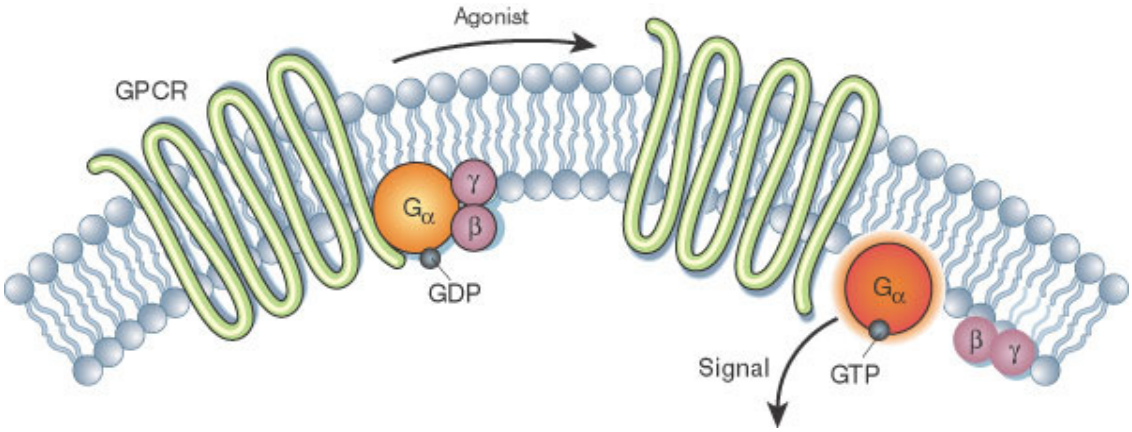

Figure 6. The diagram shows the activation of the G alpha subunit of a G-protein-coupled receptor (Li et al., 2002).

Whenever a G protein is active, both its GTP-bound alpha subunit and its beta-gamma dimer can relay messages in the cell by interacting with other membrane proteins involved in signal transduction. Specific targets for activated G proteins include various enzymes that produce second messengers, as well as certain ion channels that allow ions to act as second messengers. Some G proteins stimulate the activity of these targets, whereas others are inhibitory. Vertebrate genomes contain multiple genes that encode the alpha, beta, and gamma subunits of G proteins. The many different subunits encoded by these genes combine in multiple ways to produce a diverse family of G proteins.

Activation of a single G protein can affect the production of hundreds or even thousands of second messenger molecules (Figure 7). One especially common target of activated G proteins is adenylyl cyclase, a membrane-associated enzyme that, when activated by the GTP-bound alpha subunit, catalyzes synthesis of the second messenger cAMP from molecules of ATP. In humans, cAMP is involved in responses to sensory input, hormones, and nerve transmission, among others (Ritter and Hall, 2009).

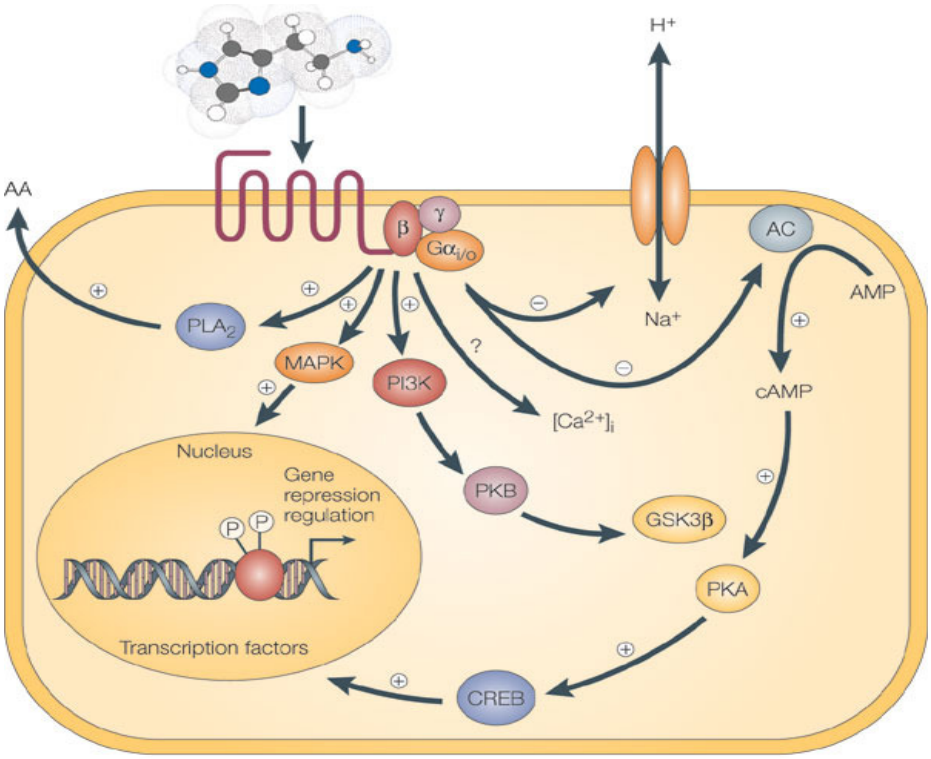

Figure 7. The diagram of a simplified cell shows how the activation of a G-protein-coupled receptor (GPCR) can affect multiple signaling cascades (Leurs et al., 2005).

2.2.1 GPER1

The former orphan receptor GPR30 was more recently referred to by its proposed functional designate GPER1. The receptor protein is localized to the plasma membranes of target cells and transfected cells (Filardo et al., 2007; Thomas et al., 2005) although there are reports that GPER1 is expressed predominantly in the endoplasmic reticulum (Revankar et al., 2005). The signaling mechanisms employed by GPER1 is shared with many other GPCR and allow for stimulation of adenylyl cyclase and release of membrane-tethered epidermal growth factor (EGF)-like polypeptides (Filardo and Thomas, 2005, 2012). Specific estrogen binding to plasma membranes of ER-negative cells expressing GPER1 was first demonstrated for the human wild-type and recombinant receptor in SKBR3 and HEK293 cells, respectively, and subsequently for GPER1 in teleost species like Atlantic croaker (*Micropogonias undulate*) (Pang et al., 2008; Thomas et al., 2005) and gilthead seabream (Cabas et al., 2013a). Human and teleost GPER1 were shown to have high affinity, limited capacity, displaceable, single binding sites for E_2 characteristic of steroid receptors (Filardo and Thomas, 2012). Another research group has demonstrated its binding to zebrafish GPER1 (Pang and Thomas, 2010). The rates of association and dissociation of E_2 binding to fish GPER1 are very rapid and are completed within a few minutes, which is typical of steroid membrane receptors (Liu et al., 2009; Pang et al., 2008; Thomas et al., 2005). The ligand binding of fish

GPER1 is specific for estrogens, because testosterone, cortisol, and progesterone have very low binding affinities for the receptor, approximately 0.1% that of E_2 (Pang et al., 2008; Thomas et al., 2005). GPER1 activation *in vivo* did not promot an evident estrogenic response, confirming the specificity of the G1 agonist over GPER1 in the gilthead seabream (Cabas et al., 2013a).

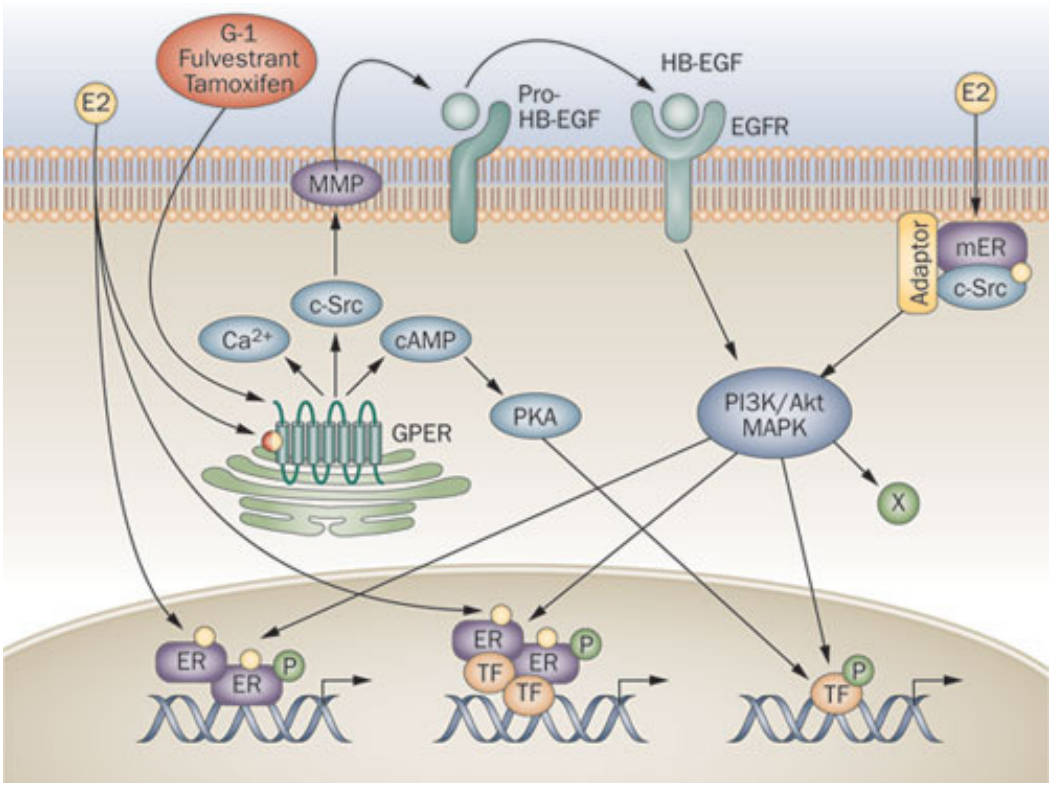

Figure 8. Endogenous estrogens including E2 are nonselective activators of the three known ERs, ERα, ERβ and GPER1. E2 activates nuclear ERs, inducing receptor dimerization and binding of receptor dimers to the promoters of target genes. Alternatively, activated ERs modulate the function of other classes of TFs through protein–protein interactions. E2, or selective agonists such as G-1, or selective estrogen receptor downregulators, such as fulvestrant, or selective estrogen receptor modulators, such as tamoxifen, also activate GPER1, which is predominantly localized intracellularly. GPER activation stimulates cAMP production, calcium mobilization and c-Src. Abbreviations: E2, 17β-estradiol; EGFR, epidermal growth factor receptor; ER, estrogen receptor; GPER, G-protein-coupled ER; MMP, matrix metalloproteinase; pro-HB-EGF, pro-heparin-binding-epidermal growth factor; TF, transcription factor (Prossnitz and Barton, 2011).

The development of a selective GPER1 agonist that does not activate ERs, named G1, has greatly facilitated research on GPER1 (Figure 8). To date GPER1 has been implicated in the development or progression of breast, endometrial, and ovarian cancers (Albanito et al., 2007; Filardo et al., 2006; Smith et al., 2007) , and in a broad range of physiological functions, including neurotransmitter and neuroendocrine regulation (Noel et al., 2009), protection against autoimmunity (Wang et al., 2009) and trauma-hemorrhage of the liver (Hsieh et al., 2007), lipid metabolism and cardiovascular tone(Haas et al., 2009), insulin secretion (Martensson et al., 2009), pulmonary vascular development (Pang et al., 2009),

primordial follicle formation (Wang et al., 2008b), regulation of oocyte meiotic arrest and maturation (Pang et al., 2008) and granulocyte activation in teleost fish (Cabas et al., 2013a).

Teleost fish and mammals, diverged from the vertebrate lineage about 200 million years ago. Therefore, a comparison of the functional characteristics of GPER1 in these two species should reveal whether estrogen binding and signal transduction are basic functions of these proteins in vertebrates (Thomas et al., 2010) . Typically for GPCRs only a small amount of the receptor protein in the cell is expressed on the plasma membrane and a large proportion remains in the endoplasmic reticulum (Filardo et al., 2007; Petaja-Repo et al., 2000). The mammalian GPER-specific agonist, G1, displays high affinity binding to croaker GPER1 and mimics the actions of E_2 in the fish oocyte maturation bioassay, indicating that G1 is a useful pharmacological tool for investigating GPER1-specific estrogen actions across a broad range of vertebrate species. Similarly, the nuclear ER antagonists, tamoxifen and ICI 182,780, act as estrogen agonists in both mammalian and fish bioassays, although their binding affinities for croaker Gper1 are lower than they are for human GPER1 (Pang et al., 2008). Recently, the estrogen binding characteristics of Gper1 has been confirmed in another teleosts species, zebrafish, using the membrane filtration assay (Liu et al., 2009). The finding that the estrogen binding functions of GPER1 have been retained in mammals and teleost fish, and are remarkably similar in these two vertebrate groups, suggests that its physiological role as a membrane estrogen receptor is a fundamental, conserved function in vertebrates. Both teleost and human GPER1 are coupled to and activate stimulatory G-proteins, resulting in increased cAMP production (Cabas et al., 2013a; Pang et al., 2008; Thomas et al., 2005).

Although from the identification of GPER1 there has been increasing attention to this receptor in recent years, there is not much information concerning the expression profile of GPER1 certain types of cells. Despite human neutrophils are the most abundant type of leukocytes and their physiology can be altered by estrogens (Cutolo, 2010; Nalbandian and Kovats, 2005; Stygar et al., 2006), it has not been described the presence of this receptor. However, it is known that they express ERα and ERβ as well as their various splice variants (Molero et al., 2002; Stygar et al., 2006). It has also been reported that ERs (ERα, ERβ) and GPER1 are functionally active in neutrophil-like HL-60 cells. Moreover, GPER1 is expressed in human eosinophils (Tamaki et al., 2014) , macrophages, dendritic and regulatory T cells. As it has been mentioned before, several lines of evidence indicate that the signaling functions of these receptors are also conserved in vertebrates. In gilthead seabream, AGs do not express any of the three nuclear ERs that have beendescribed in this specie (Liarte et al., 2011a; Pinto et al., 2006). By using G1, it was found that GPER1 is functional in AGs, as shown by the significant inductio on

cfos mRNA levels. Moreover, GPER1 activation *in vitro* slightly reduced the respiratory burst of AGs and drastically altered the expression profile of several genes encoding major pro- and anti-inflammatory mediators. Therefore, estrogens are able to modulate vertebrate granulocyte functions through a GPER1/cAMP/PKA/CREB signaling pathway. In addition, GPER1 activation *in vivo* promoted an anti-inflammatory response and modulated adaptive immunity (Cabas et al., 2013a).

3. ENDOCRINE DISRUPTION

Chemical active substances are ubiquitous in the environment and only recently they started to be considered hazardous pollutants. The main sources of pollutants are municipal (generated by households) and hospital wastes as well as inadequately utilized, expired medicines, supplements and personal care products. Another source is animal farms where steroid compounds (acting as growth promoters) and prophylactic antibiotics to prevent possible infections are commonly added to the food (Hirsch et al., 1999). The majority of pharmaceuticals are not removed during water treatment, due to their physicochemical properties. Active substances from medicines can accumulate in the tissues and be transferred to higher trophic levels with the food chain, which makes them extremely hazardous to health or life of organisms, including humans. Some of these substances are considered endocrine disruptors that can act directly on hormone receptors as hormone mimics or antagonists. Others can act directly on any number of proteins that control the delivery of a hormone to its normal target cell or tissue. Therefore they are named as EDCs. According to the definition that was developed in IPCS 2002: *An endocrine disruptor is 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*. Some of these chemicals can affect the endocrine system interfering with hormonally-controlled processes of humans and wild life, resulting in adverse effects on health, growth, development and reproduction.

The mechanism by which a chemical disrupts hormone action has a very large impact on the pattern of effects one would expect to observe. Generally, there are two pathways by which a chemical could disrupt hormone action:

A direct action on a hormone receptor protein complex. The effects could be quite complex and should be expected to follow the mechanisms for how hormones interact with receptors.

A direct action on a specific protein that controls some aspect of controlling hormone delivery to the right place at the right time. This could be a protein that is involved in hormone production (e.g. aromatase), an important

transporter (e.g. sodium/iodide symporter), or a carrier protein (e.g. cortisol binding protein).

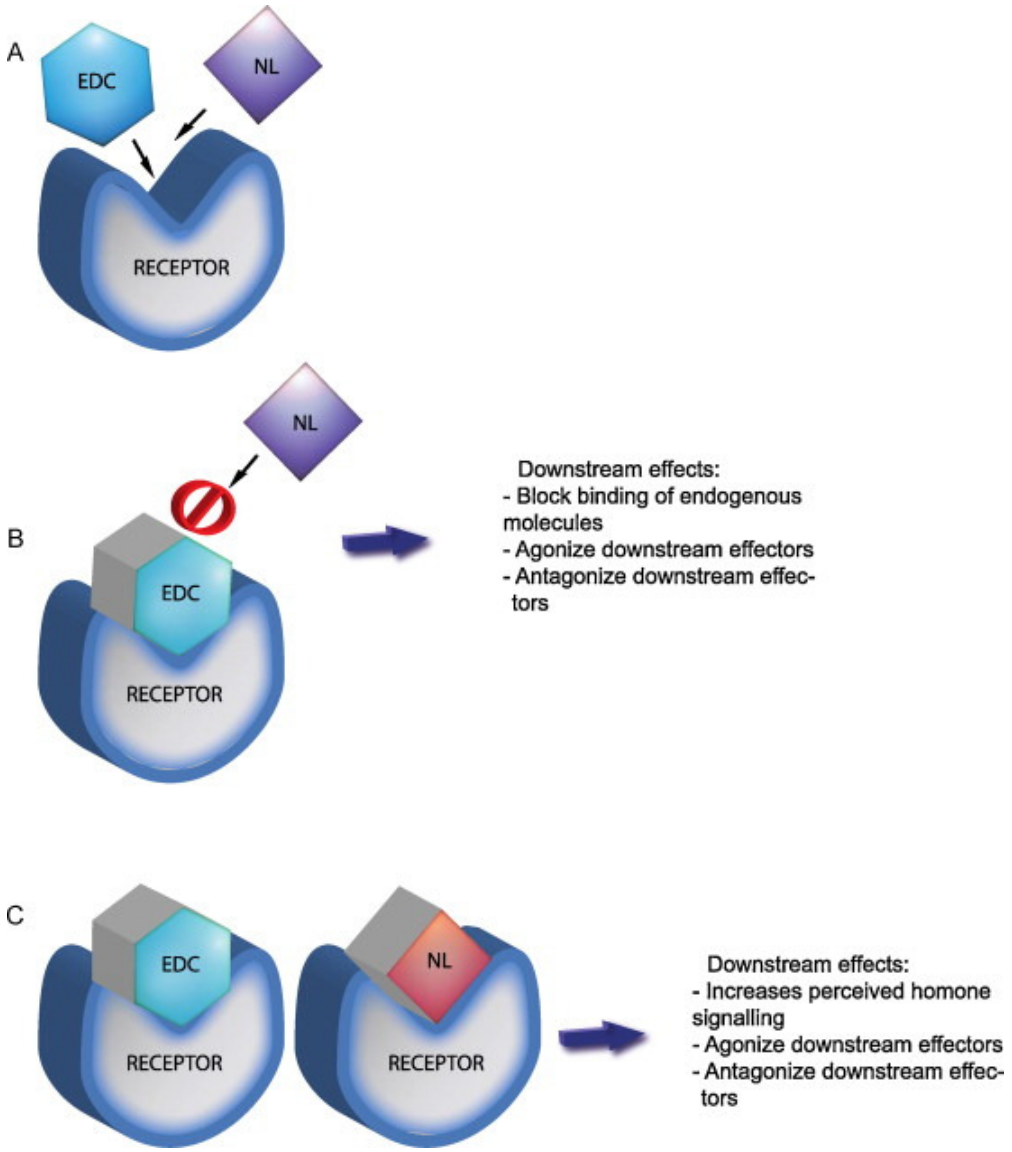

Figure 9. Representation of some of the interactions of endocrine disruptors (EDC) with natural ligands (NL) on receptor function. (A) EDCs can compete with NLs for receptor binding. (B) Binding of EDCs as opposed to NLs can result in altered downstream signaling of the receptor. (C) EDCs may bind unbound receptors while NLs are also bound which can result in agonistic or antagonistic downstream signalling (Rogers et al., 2013).

Thus, a chemical could block the synthesis of a hormone (Figure 9), with the result that the blood levels of the hormone would increase or decline. The impact on the downstream action of that hormone would likely be the same as the situation in which hormone levels are changed because of disease or genetic defect in which hormone synthesis is inhibited or stimulated (WHO, 2012).

Considering that endocrine system is very similar across vertebrate species, endocrine effects manifest themselves independently of species. The effects are

endocrine system related and not necessarily species dependent. Effects shown in wildlife or experimental animals may also occur in humans if they are exposed to EDCs at a vulnerable time and at concentrations leading to alterations of endocrine regulation. Of special concern are effects on early development of both humans and wildlife, as these effects are often irreversible and may not become evident until later in life. They have the capacity to interfere with tissue and organ development and function, and therefore they may alter susceptibility to different types of diseases throughout life. Interdisciplinary efforts that combine knowledge from wildlife, experimental animal and human studies are needed to provide a more holistic approach for identifying the chemicals that are responsible for the increased incidence of endocrine-related disease and dysfunction (WHO, 2012). Endocrine disruption is no longer limited to estrogenic, androgenic and thyroid pathways. Chemicals also interfere with metabolism, fat storage, bone development and the immune system, and this suggests that all endocrine systems can and will be affected by EDCs (Diamanti-Kandarakis et al., 2009). EDCs represent a challenge, as their effects depend on both the level and timing of exposure, being especially critical when exposure occurs during development.

EDCs were originally thought to exert actions primarily through nuclear hormone receptors, including ERs, androgen receptors, progesterone receptors, thyroid receptors, and retinoid receptors, among others. Today, it has been shown that the mechanisms are much broader than originally recognized. Thus, endocrine disruptors act via nuclear receptors, non-nuclear steroid receptors (e.g., membrane ERs), non-steroid receptors (e.g., neurotransmitter receptors such as the serotonin receptor, dopamine receptor, nor-epinephrine receptor), orphan receptors (e.g., aryl hydro-carbon receptor), enzymatic pathways involved in steroid biosynthesis and/or metabolism, and numerous other mechanisms that converge upon endocrine and reproductive systems(Diamanti-Kandarakis et al., 2009).

3.1 Environmental estrogens or xenoestrogens

Despite EDCs represent a class of heterogeneous chemicals, we are focused on those which are known to bind ER and to interfere with many aspects of estrogen-dependent control of body homeostasis including the balance between cell growth/apoptosis (Diamanti-Kandarakis et al., 2009; Zoeller, 2010). The term xenoestrogens literally means “foreign estrogen”. These compounds come from chemical components that exhibit some degree of estrogen-like activity that are obtained from the environment. Therefore they are an important group of EDCs. They can be found in many things that our industrialized society relies so heavily upon, including food containing phytoestrogens, cleaning products, drugs such as birth control or hormone replacement treatment, electronics, plastics, our household products such as detergents, heavy metals such as lead and zinc.

Declines of natural populations of fish and other aquatic species attributed to chemical contamination have largely been thought to be due to direct actions on the exposed organisms.

3.1.1 17α-ethinylestradiol (EE_2)

More than 100 million women worldwide use contraceptive pills (Owen and Jobling, 2012). The active ingredient in most formulations is EE_2 (CAS Number: 57-63-6; C20H24O2; molecular weight = 296.403; Figure 10), a semisynthetic alkylated estradiol with a 17-alpha-ethinyl substitution which is a derivative of the natural sex steroid E_2. EE_2 was the first orally active synthetic steroidal estrogen synthesized in 1938 by Hans Herloff Inhoffen and Walter Hohlweg at Schering AG in Berlin (Nagpal and Meays, 2009). It is an orally bio-active estrogen used in almost all modern formulations of combined oral contraceptive pills and is one of the most commonly used medications. EE_2 is a component of combination oral contraceptives designed for women, of which approximately 16–68% of dose is excreted in the urine or feces (Johnson and Williams, 2004). It is ubiquitous in the aquatic environment worldwide, and high EE_2 concentrations (up to 41 ng/l) have been reported in surface waters (Hua et al., 2016).

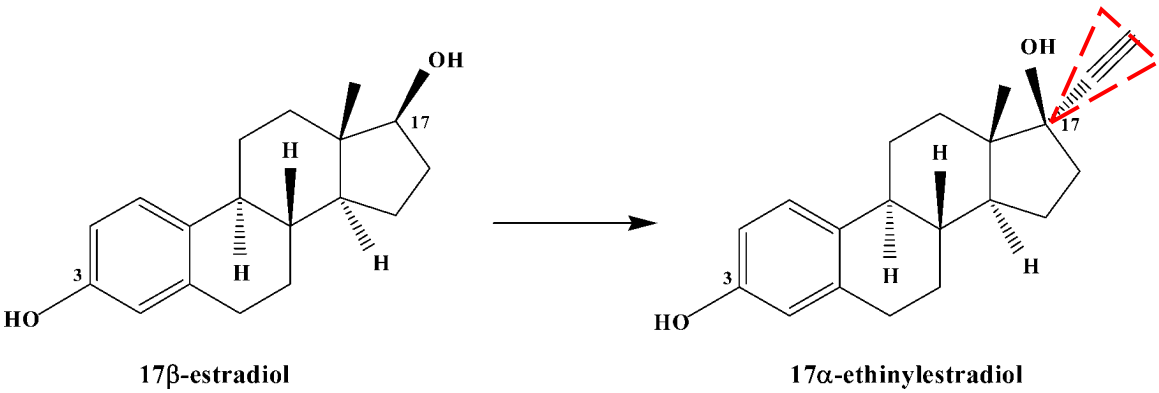

Figure 10. Structure of 17β-estradiol and 17α-ethinylestradiol.

It binds to the ER complex and enters the nucleus, activating DNA transcription of genes involved in estrogenic cellular responses. This agent also inhibits the enzyme involved in steroid metabolism 5-alpha reductase in epididymal tissue, which lowers testosterone levels and may delay progression of prostatic cancer. In addition to its anti-neoplastic effects, EE_2 protects against osteoporosis. In animal models, short-term therapy with this agent has been shown to provide long-term protection against breast cancer, mimicking the antitumor effects of pregnancy (Rajkumar et al., 2004). The biological half-life of a substance is the time it takes for a substance (drug, radioactive nuclide, or other) to lose half of its pharmacologic, physiologic, or radiologic activity. The half-lives of EE_2 compare with E_2 are approximately 33 ± 13 hours and 13 hours, respectively. In studying the

bioaccumulation potential of natural and synthetic estrogens, (Lai et al., 2002) predicted the bioaccumulation of steroidal estrogens in all organisms in the river systems. The bioaccumulation factor for EE_2 ranged from a low value of 33 for a benthic invertebrate to a maximum of 332 for a fish (Nagpal and Meays, 2009).

Current wastewater treatment systems are not sustainable options for removing pharmaceuticals. - It has proven to be inadequate at removing numerous pharmaceuticals creating the need for advanced treatment options. The oral contraceptive pill contains between 30 and 50 µg of EE_2 per pill (Rathner et al., 1979). As a large proportion of the EE_2 ingested was excreted as unmetabolized (Maggs et al., 1983). Reports from laboratory biodegradation studies indicated that EE_2 was highly stable and persistent in activated sludge, with no detectable degradation occurring after 120 hours of treatment. This fact is believed to contribute to the increased resistance of EE_2 to biodegradation as compared with natural steroidal estrogens (Desbrow et al., 1998).

Decades of research have shown that EE_2 and other estrogens cause widespread damage in the aquatic environment by disrupting endocrine systems in wildlife. This includes a condition called intersex: the irreversible development of eggs in the testes of male fish, which reduces their reproductive success (Arnold et al., 2014). When researchers introduced EE_2 into a Canadian lake in 2001 at the vanishingly low level of 5 parts per trillion (ppt), the population of one fish species collapsed (Caldwell et al., 2012). The potency of EE_2 as an endocrine disrupter makes it a serious threat to wildlife and fisheries (Owen and Jobling, 2012). Several models have relied on the utility of induction of the egg yolk precursor protein vitellogenin (VTG) as a sensitive and reliable biomarker for estrogen exposure in male and juvenile fish. For this, increase in *vtg* is considered a marker of estrogenic endocrine disruption (Sumpter and Jobling, 1995). In the gilthead seabream, EE_2 promotes the increased of *vtg* gene expression levels (Cabas et al., 2012; Cabas et al., 2013b). At low ppt, EE_2 induces feminisation of wild male fish (Jobling et al., 2006), diminishing reproductive success (Jobling et al., 2002; Harris et al., 2011). EE_2 exposure during development induces transgenerational phenotypes of reproductive impairment and compromised embryonic survival in fish of subsequent generations (Bhandari et al., 2015). In previous studies of our group, it has been shown that EE_2 promoted the infiltration of AGs and B cells in the testis as the same time that spermatogenesis was disrupted. Moreover, it also promoted a dose-dependent up-regulation of the expression of genes coding for cytokines, chemokines and adhesion molecules, what correlated with a leukocyte infiltration in the testis (Cabas et al., 2011). Moreover, it altered sex hormone serum levels, the expression profile of some steroidogenic-relevant molecules and hormone receptors, and the expression pattern of some immune molecules involved in testicular physiology. However, some of these effects varied between the reproductive stage of the specimens

(spermatogenesis and pre--spermatogenesis). This may be related to different threshold sensitivity to EE_2 in the gilthead seabream gonad at different stages (Cabas et al., 2013b) . Moreover, administration of EE_2 by bath was able to inhibit in a dose-dependent manner the induction of IL-1β gene expression, while *in vitro* administration inhibited the production of ROS and the phagocytic activity, and altered the immune gene expression profile in primary macrophages (Cabas et al., 2012).

3.1.2 Tamoxifen

Breast cancer is the most common cancer in women around the world. It can occur in both men and women, but it is very rare in the first one. In 1972, Craig Jordan demonstrated that Tmx (CAS Number: 10540-29-1; C26H29NO; molecular weight = 371.51) (Figure 11), a synthetic derivative of triphenylethylene, successfully prevent the formation of mammary tumors in mice. Since its introduction in 1997, endocrine therapy with this compound has been the first-line treatment for breast cancer in ER-positive men and women. In addition to being valuable as adjuvant treatment following surgery, radiation and/or chemotherapy (Colleoni et al., 2006), it is also used to treat metastatic breast cancer and to reduce the overall incidence in women identified as high-risk (Fisher et al., 2005).

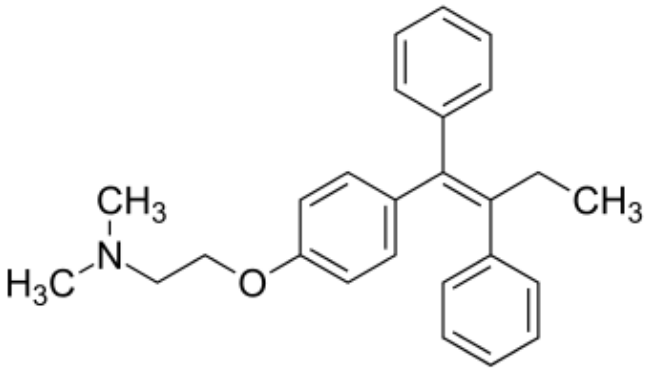

Figure 11. Structure of tamoxifen.

It is categorized as a classic "pro-drug" with a relatively low affinity for the ER. Tmx requires metabolic activation in order to elicit its pharmacological effects. The hepatic enzyme CYP2D6 is the responsible for converting the pharmacologically inactive Tmx to its metabolites endoxifen (4-OH-N-desmethyl-tamoxifen) and 4-hydroxytamoxifen. Interestingly, this metabolite has been shown to bind the ERs with an almost 100-fold greater affinity than tamoxifen. After these metabolites are generated, they interact with ERs in target tissues, including both breast and non-breast tissues, to produce a complex phenotype, resulting in both agonist and antagonist effects depending on the target tissue. Due to this dual action, Tmx is better described with the term selective estrogen receptor modulator (SERM).

Therefore, it has a complex mechanism of action owing to its molecular structure. It is chemically very similar to estrogen; however E_2 is a small carbon-rich steroid and Tmx has an extra chain which is important for its antagonistic action.

Tmx's pro- and anti-estrogenic actions are mediated by its competitive binding to the ERs which then undergo a conformational change. The nuclear complexes that form change the expression of estrogen-dependent genes to generate multiple growth-promoting signals both inside and outside the nucleus. In addition, Tmx acts as an agonist on GPER1 (Revankar et al., 2005; Vivacqua et al., 2006a).

As a SERM, however, it not only antagonizes the actions of estrogen at its receptor by blocking its binding, but mimics other actions through its own binding to the ER. For example, the prolonged binding of Tmx to specific genes can lead to a block in the proliferative actions of endogenous estrogen on mammary epithelium by reducing DNA polymerase activity, blocking E_2 uptake and eventually dampening the estrogen response. It acts as estrogen antagonists in some organs or tissues, such as the breast, but as partial estrogen agonists in other target tissues, like the uterus and bone. Tmx thus promotes uterine endometrial proliferation, endometrial hyperplasia, endometrial polyp formation, invasive endometrial cancer, uterine sarcoma, venous thromboembolism and its complications, such as stroke as well as bone growth, but restricts the growth of breast tissue (Figure 12).

Anticancer drugs such as Tmx have shown to be recalcitrant in wastewater. They are not removed by conventional wastewater treatments, and also have proven to be a challenge for the non-conventional technologies of water decontamination (Zhang et al., 2008). Therefore, there is a high probability that this compound reach the environment and their occurrence in wastewater, surface water and potential presence in drinking water is cause of concern (Booker et al., 2014). This kind of drug belongs to the group that is considered as emerging pollutants, which could be impacting the aquatic life in wastewater treatment plant (WWTP) effluents receiving waters. Most of the studies till date report relatively high levels of this compound in urban wastewaters (up to 42 ng/l) but also it has been found in natural waters up to 200 ng/l (review in Ferrando-Climent et al., 2014) In the north-east of Spain it has been detected in concentration between 25 and 38 ng/l.

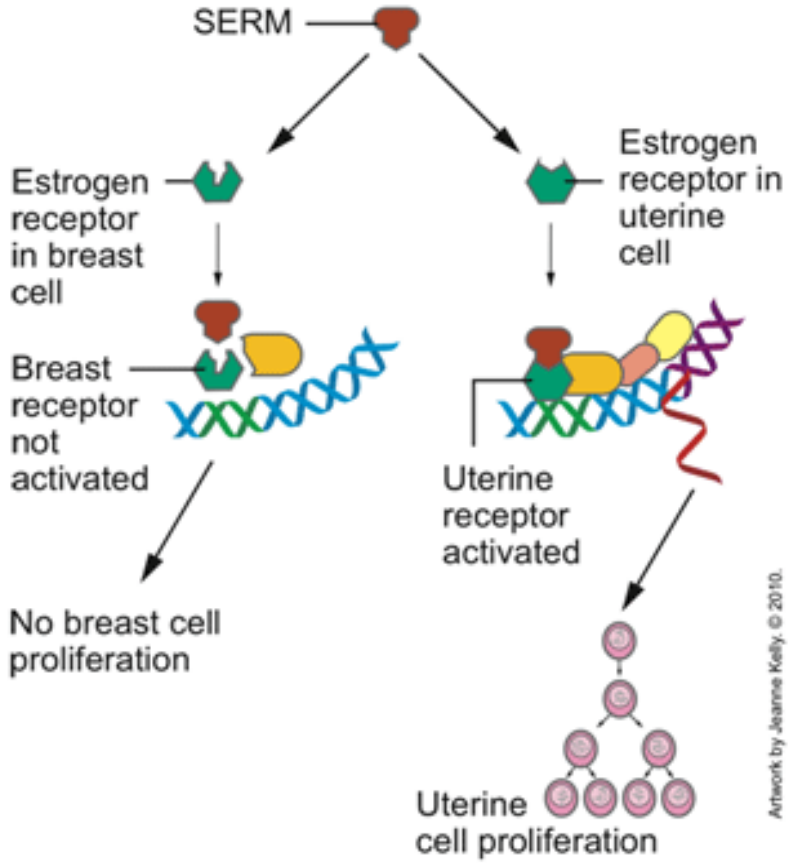

Figure 12. Pharmacodynamics of SERM such as tamoxifen (source: http://www.slideshare.net/alpatric/tamoxifen-presentation-10718324).

Since VTG synthesis is initially triggered by binding of estrogen or an estrogen mimic to the ER, it has been described that Tmx increased the *vtg* gene expression in some fish species (Benninghoff and Williams, 2008; Sun et al., 2007) and the VTG concentration in fish homogeneates of zebrafish males (Baumann et al., 2014) and affects the reproductive capacity detrimentally (Sun et al., 2007). Nevertheless, Tmx had no effect in other fish species (Leanos-Castañeda et al., 2004; Maradonna et al., 2009). It can be suggest that the estrogenic or anti-estrogenic properties of Tmx were dependent on the exposure concentration, and the pharmaceutical action of Tmx as SERM (Sun et al., 2011). More interestingly, Tmx act as estrogenic EDC in gilthead seabream males, since it up-regulate the expression of this gene (García-Hernández et al., 2016). Moreover, it altered the recruitment of leukocytes into the gonad, a process that has been characterized that is dependent of estrogens in this specie.

Objectives

Objectives

I. To determine the effect of the dietary administration of EE_2 and Tmx on the immune response of adult specimens of gilthead seabream and their capacity to recover once treatment ceases.

II. To determine the effects of the dietary administration of EE_2, Tmx and G1 on the immune response of gilthead seabream juveniles and their recovery capacity when treatment ceases.

III. To evaluate the effects of the dietary administration of EE_2 on lymphocyte activity and antibody production ingilthead seabream juveniles.

IV. To characterize the role of G protein-coupled estrogen receptor, GPER1, in human neutrophils.

Chapter 1

Tamoxifen persistently disrupts the humoral adaptive immune response of gilthead seabream (Sparus aurata L.)

There is increasing concern about the possible effect of pharmaceutical compounds may have on the fish immune system. Bath exposition of (EE_2), a synthetic estrogen used in oral contraceptives, altered the immune response of the gilthead seabream (*Sparus aurata* L.), a marine hermaphrodite teleost. Tamoxifen (Tmx) is a selective estrogen-receptor modulator used in hormone replacement therapy, the effects of which are unknown in fish immunity. This study aims to investigate the effects of dietary administration of EE_2 (5 μg/g food) and Tmx (100 μg/g food) on the immune response of gilthead seabream, and the capacity of the immune system to recover its functionality after a recovery period. The results show for the first time the reversibility of the effect of EE_2 and Tmx on the fish immune response. Tmx promoted a transient alteration in hepatic vitellogenin gene expression of a different magnitude to that produced by EE_2. Both, EE_2 and Tmx inhibited the induction of interleukin-1b gene expression while reversed the inhibition of ROI production in leukocytes following vaccination. However, none of these effects were observed after ceasing EE_2 and Tmx exposure. EE_2 and Tmx stimulated the antibody response of vaccinated fish although Tmx, but not EE_2, altered the antibody response and modulated the percentage of IgM^+ B lymphocytes of vaccinated fish during the recovery phase. Taken together, our results suggest that EE_2 and Tmx might alter the capacity of fish to appropriately respond to infection and show that Tmx has a long-lasting effect on humoral adaptive immunity.

Key words: 17α-ethinylestradiol, tamoxifen, immune response, fish, gilthead seabream.

1. INTRODUCTION

In mammals, it is well known that estrogens have an immune-modulatory role (Shved et al., 2007a; Straub, 2007). Although there are few studies on the effects of estrogens on the fish immune response (Iwanowicz et al., 2009; Milla et al., 2011; Shved et al., 2007a) it has been demonstrated that they can affect immune cells and modulate some of their activities, as has been seen in the case of the phagocytic activity of tilapia head kidney leukocytes (Law et al., 2001).

EDCs are natural or man-made substances present in the environment, which interfere with endocrine pathways, altering physiological functions in wildlife and humans (Tyler et al., 1998; Vos et al., 2000). Aquatic wildlife, in particular, appears to be at increased risk as many mechanisms of EDCs have not been well defined, accumulated results reveal that they can act via multiple action mechanisms (Tabb and Blumberg, 2006). EDCs act, in part, by mimicking or blocking the action of the natural steroids through both nuclear receptor-dependent and non receptor-dependent mechanisms, leading to differential target-gene transcription (Fisher, 2004; Tabb and Blumberg, 2006), affecting not only the endocrine or reproductive systems but also the immune system (Ahmed, 2000a; Chalubinski and Kowalski, 2006; Fisher, 2004). Although environmental chemicals are usually less potent than endogenous hormones, it is now clear that they act additively with them (Markey et al., 2002). One important group among EDCs, found in the aquatic environment, are estrogens, both natural such as E_2, estrone and estriol, and synthetic such as EE_2 (García-Reyero et al., 2011; Hinteman et al., 2006; Johnson et al., 2005; Ternes et al., 1999).

EE_2, a pharmacological compound with strong estrogenic activity, widely used as oral contraceptive and in hormone replacement therapy, is present in sewage treatment plant effluents in concentrations of 1-10 ng/L, although levels as high as 42 ng/L have been documented (Desbrow et al., 1988; Ternes et al., 1999), as it is poorly removed by conventional wastewater treatment methods (Karpova et al., 2007). It is well known that EE_2 possesses several characteristics that make it one of the most potent estrogenic compounds (Lai et al., 2002; Legler et al., 2002; Nash et al., 2004b; Thorpe et al., 2003; Tilton et al., 2005; Ying et al., 2003). In addition, EE_2 is bioaccumulated thanks to its resistance to degradation and, therefore, transfer throughout the food chain (Lai et al., 2002). Due to its environmental relevance, EE_2 has been widely used in fish reproductive performance analysis and, less frequently, in fish immune response studies (Filby et al., 2007; Jin et al., 2010; Law et al., 2001; Shved et al., 2009). Tamoxifen (Tmx), a nonsteroidal triphenylethylene derivative, is widely used in the treatment of primary and recurrent breast cancer (Carlson, 1997), where it acts as an antagonist competitor in the ERα (mammary tissues) and as an agonist competitor in

cholesterol metabolism (Coezy et al., 1982; Jordan and Koerner, 1975). The pharmacological targets of Tmx include ER, the multi-drug resistance gene product, permeability-glycoprotein (Callaghan and Higgins, 1995) and GPER (Revankar et al., 2005; Vivacqua et al., 2006b). Tmx has been detected in wastewater effluent and surface waters in concentrations ranging from 27 to 212 ng/L in UK (Hilton and Thomas, 2003; Thomas and Hilton, 2004) since its elimination in sewage treatment plants is often incomplete (Coetsier et al., 2009), reaching the aquatic environment and impacting on exposed organisms (Coetsier et al., 2009). Tmx has been recommended for use as a reference compound in fish testing in terms of understanding the potential impact of SERMs in aquatic life (Ankley et al., 2002). Previous studies in fish have analyzed the impact of dietary Tmx in fish reproduction (Mandiki et al., 2005; Singh et al., 2015).

The gilthead seabream (*Sparus aurata* L.) is a marine, protandrous hermaphrodite teleost. They are males, at least during the first two reproductive cycles in the western Mediterranean area and in the rearing conditions of the Oceanographic Centre of Murcia (Spain). The species has a great commercial value and, therefore, the impact of EDCs on its immune system is an important concern. We have previously demonstrated that 5 μg/g food of EE_2 acts as an EDC in gilthead seabream as it strongly promotes the hepatic *vtg* gene expression and alters reproductive performance (Cabas et al., 2011; Cabas et al., 2013b). Moreover, EE_2 bath-exposed specimens showed an altered capacity to appropriately respond to infection although the compound does not behave as an immunosuppressor (Cabas et al., 2012). Estrogens, both natural and synthetic, regulate the activities of acidophilic granulocytes, the functional equivalent of mammalian neutrophils (Cabas et al., 2011; Chaves-Pozo et al., 2007) and macrophages (Liarte et al., 2011a, 2011b). Curiously, acidophilic granulocytes do not express any of the three known ER, namely ERα, ERβ1 and ERβ2 (Liarte et al., 2011a; Pinto et al., 2006), while macrophages and lymphocytes constitutively express ERα (Liarte et al., 2011a). More recently, we have demonstrated that acidophilic granulocytes express GPER at both mRNA and protein levels (Cabas et al., 2013a).

The aim of this study was, therefore, to evaluate the effect of EE2 and Tmx taken in the diet on innate and humoral adaptive immunity of gilthead seabream and to analyze whether a limited exposure of EE_2 and Tmx inhibit the immune response of the specimens after the exposure.

2. MATERIALS AND METHODS

2.1 *In vivo treatments and sample collection*

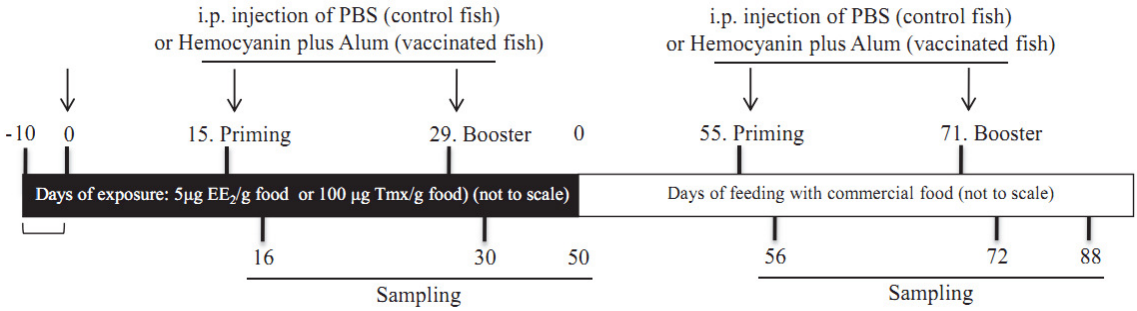

Figure 1. Schematic drawing of the experimental design: EE_2 or Tmx dietary exposure and vaccination schedule of gilthead seabream. Fish were exposed to 5 µg EE_2/g food or 100 µg Tmx/g food for 50 days, after which they were fed with commercial food for a further 88 days (recovery period). In these two experimental periods, fish were intraperitoneally injected with phosphate buffered saline (PBS) (control fish) or hemocyanin plus alum adjuvant (vaccinated fish) after 15 (priming) and 29 (booster) days of EE_2 or Tmx dietary intake and after 55 days (priming) and 71 days (booster) of the recovery period using specimens that were not vaccinated during the EE_2 or Tmx exposure. The samplings were carried out on days 16 (1 day postpriming, dpp) and 30 and 50 (1 and 21 days postbooster, dpb, respectively) and on days 56 (1 day dpp) and 72 and 88 (1 and 17 days dpb, respectively), respectively.

Healthy specimens of gilthead seabream were maintained at the Oceanographic Centre of 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 per day with a commercial pellet diet (44% protein, 22% lipids; Skretting) at a feeding rate of 1.5% of fish biomass. The environmental parameters, mortality and food intake as well as behavior and gross morphology were recorded daily and histopathology was recorded each fifteen days. Fish were fasted for 24 h before sampling. *In vivo* EE_2 or Tmx exposure experiments (Fig. 1) were carried out with mature gilthead seabream males (n = 106), with a body weight of 453 ± 7 g, in 2 m^3 aquaria. Briefly, the EE_2 or Tmx (Sigma-Aldrich) were incorporated in the commercial food at 5 or 100 µg/g food, respectively, using the ethanol evaporation method (0.3 l ethanol/kg of food) as described elsewhere (Shved et al., 2007a). The specimens were fed *ad libitum* three times a day with the pellet diet supplemented with EE_2 or Tmx for 50 days. After that, the specimens were fed with the commercial food for a further 88 days (recovery period) (Fig. 1). In order to evaluate the effect of EE_2 or Tmx on any induced adaptive immune response, the specimens were intraperitoneally injected with phosphate buffered saline (PBS) (control fish) or hemocyanin (200 µg/fish; Sigma Aldrich) and imject alum adjuvant (4 mg/fish; Thermo Scientific) (vaccinated fish) after 15 (priming) and 29 (booster) days of EE_2 or Tmx dietary intake. The samplings were carried out on days 16 (1 day postpriming, dpp) and 30 and 50 (1 and

21 days postbooster, dpb, respectively). A similar immunization schedule was carried out after 55 days (priming) and 71 days (booster) of the recovery period with specimens that were not vaccinated during EE_2 or Tmx exposure. A group that was neither EE_2 or Tmx-treated nor vaccinated served as control. The samplings were carried out on days 56 (1 dpp) and 72 and 88 (1 and 17 dpb, respectively). Specimens (n = 4 fish/treatment/time of sampling) were fasted for 24 h before sampling. They were tranquilized by 8 µl/l of clove oil and, immediately, anesthetized using 40 µl/l of clove oil, weighed and decapitated before the head kidneys and livers were removed and processed for gene expression analysis, as described below. In addition, the head kidneys were processed for functional activity assays and the gonads were removed and weighed; serum samples from trunk blood were obtained by centrifugation and immediately frozen and stored at – 80° C until use. Cell suspensions from head kidneys were obtained as described elsewhere (Chaves-Pozo et al., 2003, 2005a).

As an index of the reproductive stage and to evaluate the effect of the in vivo EE_2 or Tmx treatments, the gonadosomatic index (GSI) was calculated as 100 (MG/MB) (%), where MG is gonad mass and MB is body mass (both in grams).

The experiments described comply with the guidelines of the European Union Council (2010/63/EU) and the Bioethical Committees of the University of Murcia and IEO for the use of laboratory animals.

2.2 *Analysis of gene expression*

Total RNA was extracted from the liver (control and EE_2 or Tmx-treated) and head kidney (control, both vaccinated + EE_2 or Tmx-treated groups and vaccinated control) at day 16 of the treatments, 1 dpp, and at day 56, 1dpp, of the recovery period with TRIzol Reagent (Invitrogen), following the manufacturer's instructions, and quantified with a spectrophotometer (NanoDrop, ND-1000). Liver has been chosen for the expression of the hepatic vitellogenin and head kidney for the expression of *era*, *gper*, and the pro-inflammatory cytokine, interleukin 1b (*il1b*), and the anti-inflammatory *il10*. The RNA was then treated with amplification grade DNase I (1 U/µg RNA; Invitrogen) to remove genomic DNA traces that might interfere with the PCR reactions, and the SuperScript III RNase H Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNA with oligo-dT18 primer from 0.5 to 1 µg of total RNA, at 50° C for 50 min. The β-actin (*actb*) gene was analyzed by PCR performed with a Mastercycle Gradient Instrument (Eppendorf). Reaction mixtures were incubated for 2 min at 95° C, followed by 35 cycles of 45 s at 95° C, 45 s at the specific annealing temperature, 1 min at 72° C, and finally 10 min at 72° C.

The expression of the genes coding for *vtg*, *era*, *gper*, *il11b* and *il10*, was analyzed by real-time PCR performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). 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, 1 min at 60° C, and 15 s at 95° C. For each mRNA, gene expression was corrected by the ribosomal protein S18 gene (*rps18*) content in each sample using the comparative cycle threshold method, Ct method ($2^{-\Delta\Delta Ct}$). The gilthead seabream specific primers used are shown in Table 1. In all cases, each PCR was performed with triplicate samples and repeated in at least three independent samples.

Table 1. Gene accession numbers and primer sequences used for expression analysis. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html). All primers were used for real-time PCR, except *actb* primers that were used for conventional PCR.

Gene	Accession No.	Name	Sequence (5' → 3')
actb	X89920	F3	ATCGTGGGGCGCCCCAGGCACC
		R3	CTCCTTAATGTCACGCACGATTTC
gper	HG004163	F1	GGCTGCCAGAGAATGTCTTC
		R1	GTGGCCTGTGAGTGGGTAGT
il1b	AJ277166	F2	GGGCTGAACAACAGCACTCTC
		R3	TTAACACTCTCCACCCTCCA
il10	FG261948	F	TGGAGGGCTTTCCTGTCAGA
		R	TGCTTCGTAGAAGTCTCGGATGT
vtg	AF210428	F1	CTGCTGAAGAGGGACCAGAC
		R1	TTGCCTGCAGGATGATGATA
era	AF136979	F1	GCTTGCCGTCTTAGGAAGTG
		R1	TGCTGCTGATGTGTTTCCTC
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC
		R	CTTCTGCCTGTTGAGGAACC

2.3 *ROS production assay*

The production of reactive oxygen intermediate (ROS) was measured as the luminol-dependent chemiluminescence produced by 0.5 x 10^6 leukocytes (control, both vaccinated + EE_2 or Tmx-treated groups and vaccinated control) at day 50 of the treatments (21 dpb) and at day 88 of the recovery period (17 dpb) in the presence (stimulated) or absence (naïve) of 50 µg/ml of phenolextracted genomic DNA from *Vibrio anguillarum* ATCC19264 cells (VaDNA) for 48 h, as described elsewhere (Mulero et al., 2001). This was achieved by adding 100 µM luminol (Sigma-

Aldrich) and 1 µg/ ml phorbol myristate acetate (PMA, Sigma-Aldrich), while the chemiluminescence was recorded every 127 s during 1 h in a FLUOstart luminometer (BGM; LabTechnologies). The values reported are the average of triple readings from 3 different samples, expressed as the maximum and slope of the reaction curve from 127 to 1016 s, from which the apparatus background was subtracted.

2.4 Determination of IgM specific titer

The hemocyanin specific IgM titer was determined by an ELISA kit (Aquatic Diagnostic) following the manufacturer's instructions. In short, 1/100 and 1/1000 dilution of serum (control, both vaccinated + EE_2 or Tmx-treated groups and vaccinated control) at day 50 of the treatments (21 dpb) and at day 88 of the recovery period (17 dpb), respectively, were added to hemocyanin precoated 96-well ELISA plates, followed by a monoclonal antibody specific to seabream IgM (Aquatic Diagnostics) and an anti-rabbit IgG (whole molecule)-peroxidase antibody produced in goat (Sigma-Aldrich). Finally, the chromogen tetramethylbenzidine (TMB) was added and the absorbance was read at 450 nm using a FLUOstart luminometer (BGM; LabTechnologies).

2.5 Determination of IgM positive cells

The percentage of IgM positive cells was measured in aliquots of 0.5 x 10^6 head kidney total leukocytes (control, both vaccinated + EE_2 or Tmx-treated groups and vaccinated control) at day 88 of the recovery period (17 dpb). The cells were also incubated in the presence (stimulated) or absence (naïve) of hemocyanin, for 48 h, after which they were washed in PBS containing 2% charcoal/dextran-treated hormone-free fetal serum (FCS) (Life technologies) and 0.05% sodium azide (FACS buffer). Cells were then incubated with 0 and 2 µg/ml (1:100) of the anti-IgM antibody in PBS containing 2% FCS for 30 min at 4° C (Sepulcre et al., 2011). After washing, cells were incubated with a 1:1000 dilution of an FITC-conjugated rabbit for 30 min at 4° C, washed again, and analyzed by flow cytometry using a flow cytometer (BD Biosciences).

2.6 Statistical analysis

T and Unpaired tests were applied to determine differences between two groups. The critical value for statistical significance was taken as $p \leq 0.05$. The asterisks *, ** and *** refer to $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively. All statistical analyses were carried out using the GraphPad Prism 5 program.

3. RESULTS

3.1 EE_2 and Tmx promote a transient alteration in the gonadosomatic index and in vitellogenin gene expression

The survival of the animals exposed to EE_2 or Tmx was 100% during the trial. The GSI was lower in fish after 16 and 30 days of EE_2 dietary intake (Fig. 2a), as we have previously described (Cabas et al., 2011), although no differences with the control were observed at day 50 of EE_2 dietary intake or during the recovery period (Fig. 2b). However, GSI increased in fish at day 50 of Tmx dietary intake (Fig. 2a) and persisted at day 56 of the recovery period (Fig. 2b), although this parameter returned to values similar to the control fish at day 72 of the recovery period (Fig. 2b).

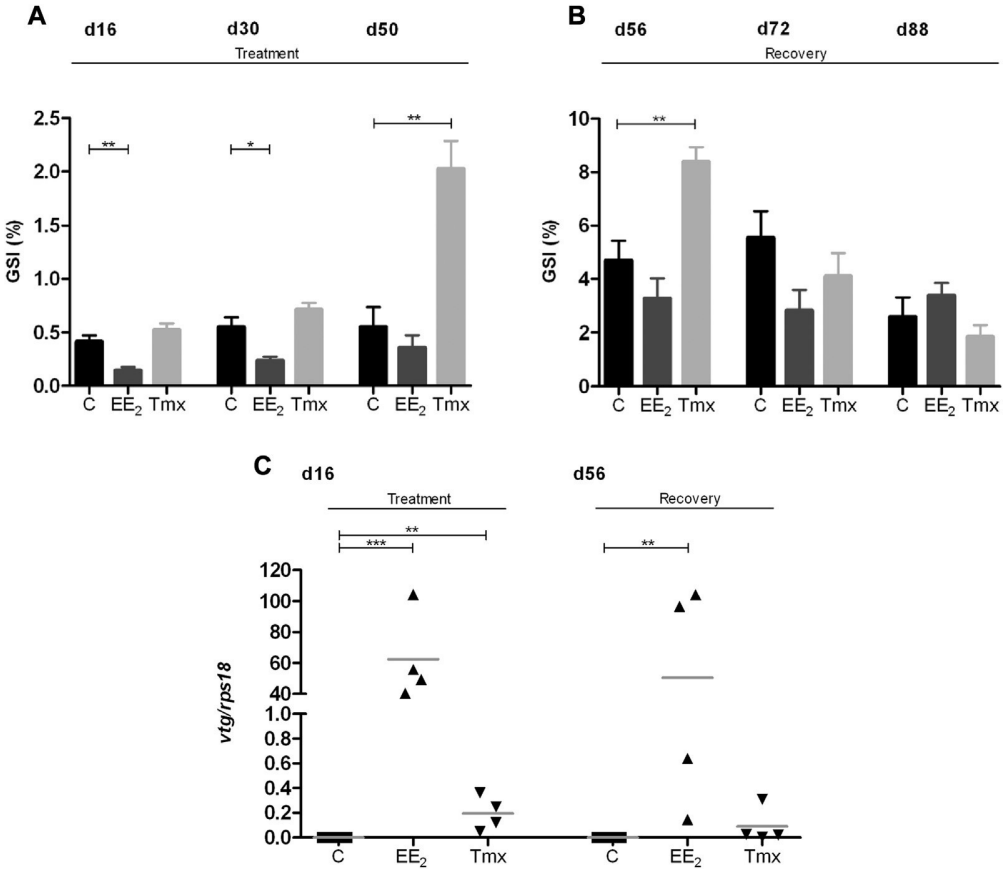

Figure. 2. EE_2 and Tmx promote a transient alteration in the gonadosomatic index (GSI) and in vitellogenin (vtg) gene expression in the gilthead seabream. The GSI was analyzed at days 16, 30 and 50 of the EE_2 and Tmx treatments (a), and at days 56, 72 and 88 of the recovery period (b). Each bar represents the mean ± SEM. The mRNA levels of vtg (c) were determined in the liver from control and EE_2 or Tmx-treated fish at day 16 of the treatments (1 dpp) and at day 56 of the recovery period (1 dpp) by real-time reverse transcription polymerase chain reaction (RT-PCR). The mean for each group of specimens is shown as a horizontal line. In all the analyses, the sample size was n ¼ 4 fish/group/time. Each bar represents the mean ± SEM. The asterisks denote statistically significant differences according to Student t test between the control and the EE_2 or Tmx-treated groups, at each time point. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$.

EE_2 promoted an estrogenic response in gilthead seabream after 16 days of exposure as it up-regulated hepatic *vtg* mRNA levels (Fig. 2c), as we have previously described in gilthead seabream (Cabas et al., 2011). Tmx also promoted an increase in hepatic *vtg* mRNA levels although this increase was much less pronounced (Fig. 2c). No significant changes in hepatic *vtg* expression were observed at day 56 of the recovery period of Tmx treated specimens, while the specimens treated with EE_2 showed strong individual variations in *vtg* expression at that time point (Fig. 2c).

3.2 Neither EE_2 nor Tmx alter the expression of genes encoding era and gper

The expression of *era* gene, which is constitutively expressed by gilthead seabream macrophages and lymphocytes (Liarte et al., 2011a, 2011b), was analyzed in head kidney after 16 days of EE_2 or Tmx dietary intake and at day 56 of the recovery period, in both cases 1 dpp (Fig. 3a). Immunization did not promote any modification of *era* mRNA levels (Fig. 3a). Moreover, neither EE_2 nor Tmx had modified the mRNA levels of *era* at these two times (Fig. 3a).

GPER is expressed in the head kidney of gilthead seabream although its expression is much lower than in liver and testis (Cabas et al., 2013a). GPER gene expression was not detected in the head kidney of the vaccinated fish, whether treated or not with EE_2 and Tmx, at day 16 (1 dpp) of treatment. Moreover, vaccination promoted a strong decrease in the *gper* gene expression at day 56 (1 dpp) of the recovery period (Fig. 3b) while EE_2 or Tmx did not affect *gper* expression.

3.3 EE_2 and Tmx transiently affect the innate immune response

The expression of genes encoding for two key cytokines, *il1b* and *il10*, was analyzed in head kidney after 16 days of EE_2 or Tmx dietary intake and at day 56 of the recovery period, i.e. 1 dpp (Fig. 3c, d). Vaccination resulted in an increased *il1b* expression, while dietary EE_2 and Tmx reversed this increase after 16 days of treatments (Fig. 3c), as previously observed in EE_2-bath exposed fish (Cabas et al., 2012). However, at day 56 of the recovery period, no differences in *il1b* expression were observed between the 4 groups analyzed (Fig. 3c). Neither the vaccination nor EE_2 or Tmx treatment affected *il10* gene expression after 16 days of treatment (Fig. 3d). Unexpectedly, at day 56 of the recovery period, an increase in *il10* gene expression was observed in the EE_2 treated fish compared with untreated, vaccinated fish (Fig. 3d).

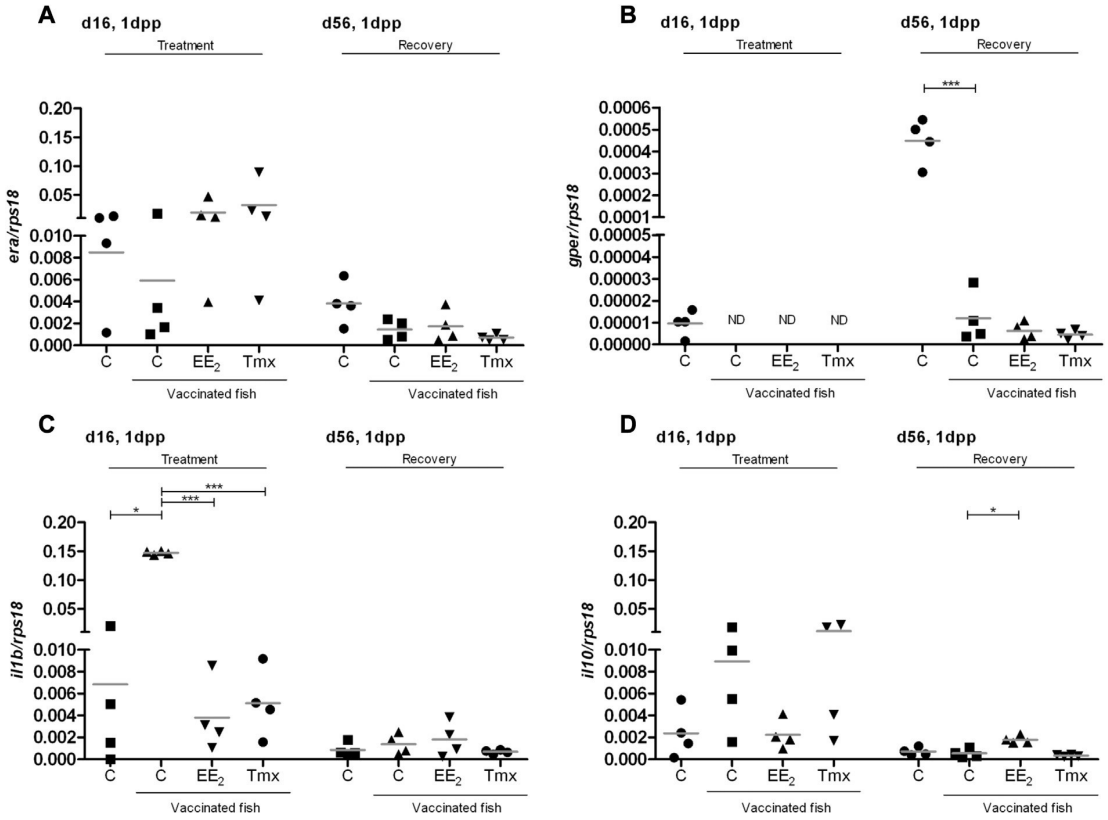

Figure. 3. EE_2 or Tmx exposure does not alter the expression of the genes encoding *era* and *gper* but regulates the expression of genes encoding for two key cytokines, *il1b* and *il10*, in head kidney. The mRNA levels of *era* (a), *gper* (b), *il1b* (c), and *il10* (d) were determined in the head kidney from control, and vaccinated + EE_2 or Tmx-treated groups and vaccinated control fish at day 16 of the treatments (1 dpp) and at day 56 of the recovery period (1 dpp) by real-time reverse transcription polymerase chain reaction (RT-PCR). Data represent means ± SEM of triplicates of the same sample. The mean for each group of specimens is shown as a horizontal line. The asterisks denote statistically significant differences according to Student t test between: (i) the control and the vaccinated control groups, at each time point, and (ii) the vaccinated control and both vaccinated plus EE_2 or Tmx-treated groups, at each time point. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$.

When ROS production was analyzed in both unstimulated and VaDNA-stimulated head kidney leukocytes from control and vaccinated fish (treated or not with EE_2 and Tmx) after 50 days (21 dpb) of dietary intake (Fig. 4a) and at day 88 (17 dpb) of the recovery period (Fig. 4b), immunization was seen to lower the ROS production in both unstimulated and VaDNA-stimulated cells (Fig. 4a, b). Interestingly, EE_2 and Tmx treatment resulted in increased ROS production in both unstimulated and VaDNA-stimulated head kidney leukocytes from vaccinated fish compared with untreated, vaccinated fish (Fig. 4a). However, this effect was not observed at day 88 of the recovery period (Fig. 4b). A similar effect of EE_2 and Tmx treatments was found in the number of head kidney acidophilic granulocytes (SSC^{high} cells), assayed by flow cytometry (Fig. 4c, d).

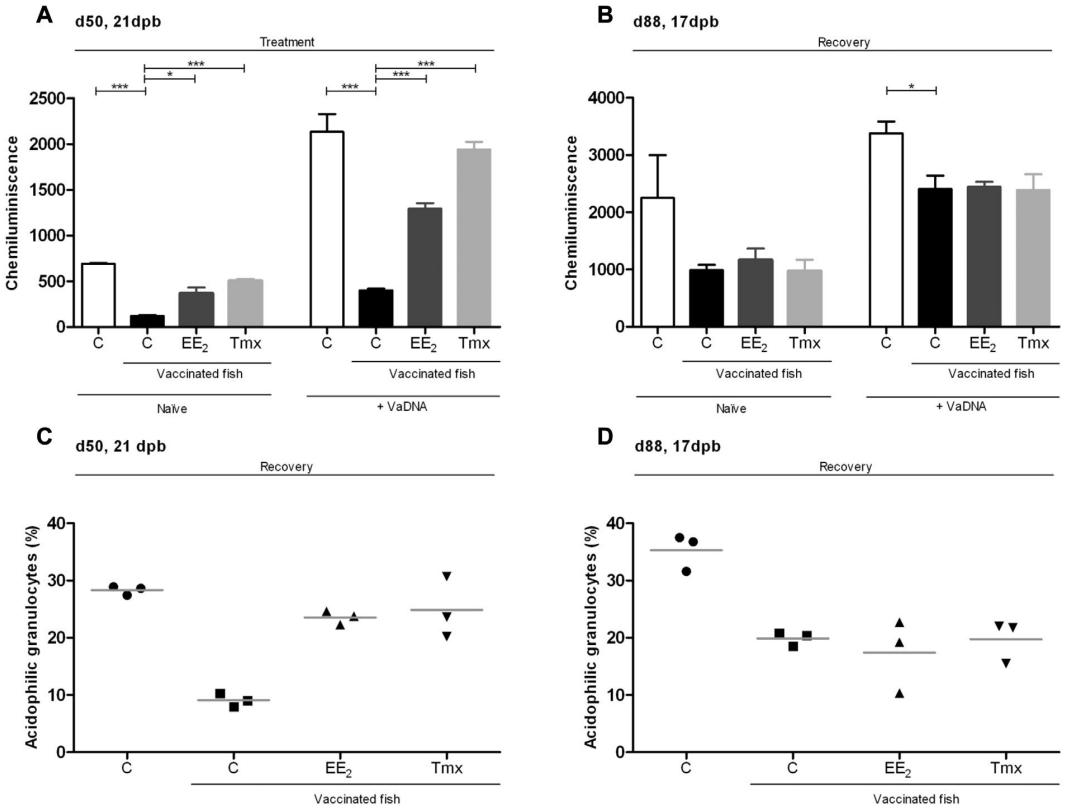

Figure 4. EE_2 and Tmx modulate the respiratory burst of leukocytes and the number of acidophilic granulocytes. (a, b)The respiratory burst was triggered by PMA (1 mg/ml) using a luminol-dependent chemiluminescence method of head kidney leukocytes from control, vaccinted + EE_2 or Tmx-treated groups and vaccinated control fish at day 50 of the treatments (21 dpb) (a) and at day 88 of the recovery period (17 dpb) (b) in the presence or absence of *Vibrio anguillarum* genomic DNA (VaDNA) during 48 h. Data represent means ± SEM of triplicates from three independent samples. (c, d) The percentage of acidophlic granulocytes (SSC^{high} cells) in the head kidney of control, vaccinated + EE_2 or Tmx-treated groups and vaccinated control fish at day 50 of the treatments (21 dpb) (c) and at day 88 of the recovery period (17 dpb) (d) was determined by flow cytometry. The mean for each group of specimens is shown as a horizontal line. The asterisks denote statistically significant differences according to Student t test between: (i) the control and the vaccinated control groups, at each time point, and (ii) the vaccinated control and both vaccinated plus EE_2 or Tmx-treated groups, at each time point. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$.

3.4 Tmx persistently affects the humoral adaptive immune response

The impact of EE_2 or Tmx exposure on the humoral adaptive immune response was evaluated as hemocyanin-specific IgM titer in the serum of vaccinated fish at day 50 (21 dpb) of EDC dietary intake and at day 88 (17 dpb) of the recovery period (Fig. 5a). As expected, all vaccinated fish showed very high specific IgM titers (Fig. 5a). Moreover, EE_2 and Tmx increased the IgM serum levels of immunized fish at day 50 of the treatments (Fig. 5a). Notably, however, at day 88 (17dpb) of the recovery period, vaccinated fish previously exposed to Tmx, but not to EE_2, showed a higher IgM titer than vaccinated, untreated fish (Fig. 5a). Concomitantly, the

percentage of head kidney IgM$^+$ B lymphocytes significantly increased at day 88 (17 dpb) of the recovery period in vaccinated fish that had previously been exposed to Tmx when compared with untreated, vaccinated fish (Fig. 5b). In addition, a similar increased number of IgM$^+$ B lymphocytes was observed in head kidney leukocyte from fish that had previously been exposed to EE_2 after 48 h of culture in the presence or absence of hemocyanin (Fig. 5c). Moreover, an increased number of head kidney IgM$^+$ B cells was also observed at day 88 of the recovery period (17 dpb) in vaccinated fish that had previously been exposed to EE_2 compared with untreated, vaccinated fish after 48 h of *in vitro* stimulation with the antigen (Fig. 5c).

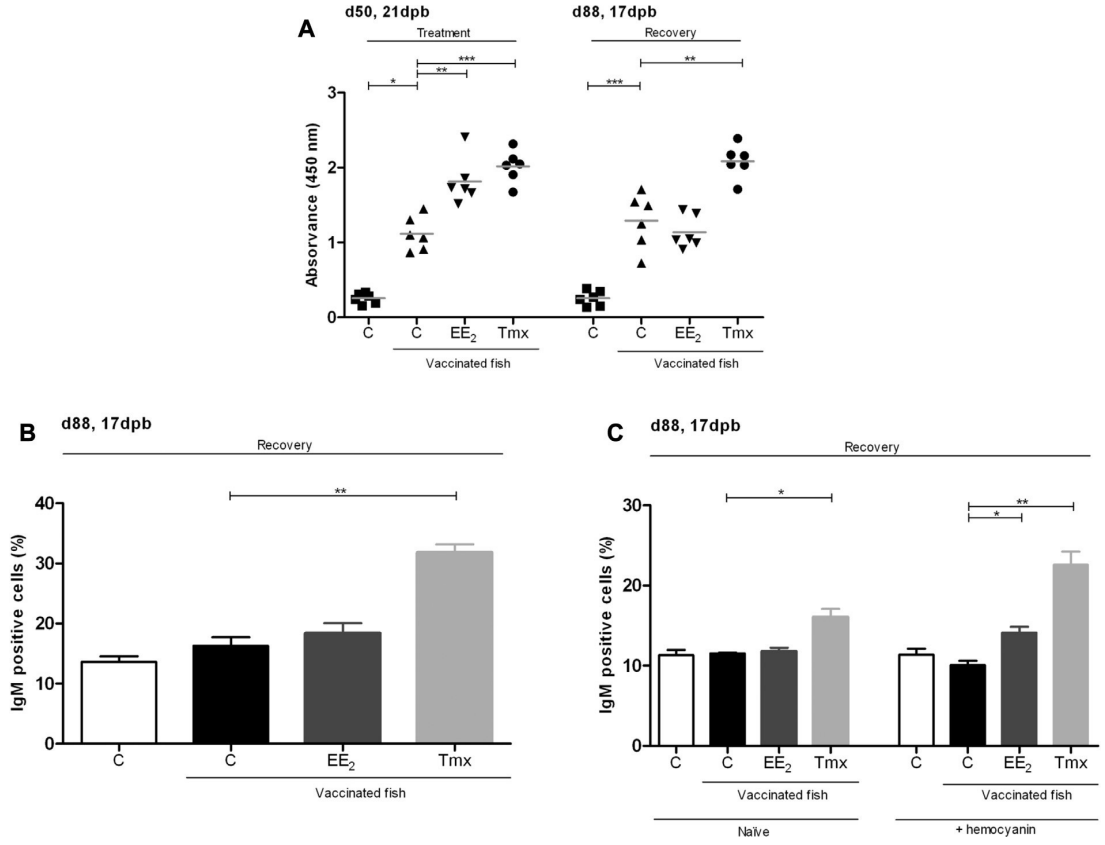

Figure 5. Tmx alters the antibody response and modulates the number of IgM$^+$ cells. (a) Hemocyanin-induced specific IgM levels were determined by ELISA on day 50 of the treatments (21 dpb) and on day 88 of the recovery period (17 dpb) (a). The absorbance was read at 450 nm using a FLUOstart luminometer. The sample size was n = 6 fish/group. The mean for each group of specimens is shown as a horizontal line (a). Head kidney leukocytes from control, vaccinated + EE_2 or Tmx- treated groups and vaccinated control fish at day 88 of the recovery period (17 dpb) were obtained to determine the percentage of IgM$^+$cells by flow cytometry. The sample size was n = 3 fish/group. Data represent means ± SEM of triplicates from three independent samples (b, c). The asterisks denote statistically significant differences according to Student *t test* between: (i) the control and the vaccinated control groups, at each time point, and (ii) the vaccinated control and both vaccinated plus EE_2 or Tmx-treated groups, at each time point. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$.

4. DISCUSSION

It has been demonstrated that estrogens affect the immune system in fish (Chaves-Pozo et al., 2009; Iwanowicz et al., 2009; Liarte et al., 2011a, 2011b) and that leukocyte function is modulated by E_2 (Chaves-Pozo et al., 2003; Law et al., 2001; Liarte et al., 2011b; Shelley et al., 2013; Wang and Belosevic, 1994; Watanuki et al., 2002; Yamaguchi et al., 2001) and by EE_2 (Cabas et al., 2012). In the light of the known presence of estrogenic EDCs in the environment and the increased use of immune status as an indicator of wild fish health, it is essential that mechanistic and physiological effects of estrogen on the immune function be elucidated in fish (Iwanowicz et al., 2014). The environmental impact of EE_2 on wildlife and humans has been intensively discussed in recent past years (Owen and Jobling, 2012). We have previously demonstrated that EE_2 acts as an EDC in gilthead seabream, since increased *vtg* mRNA levels, a yolk precursor protein produced by hepatocytes and generally accepted as a biomarker of estrogenic effects (Sumpter and Jobling, 1995), were observed after EE_2 exposure (Cabas et al., 2011; Cabas et al., 2012). Although, Tmx also promoted increased *vtg* expression in gilthead seabream, as occurred in Japanese medaka (Sun et al., 2007), such increase was much lower (300-fold increase compared with control group) than that promoted by EE_2 (96,000-fold increase compared with control group). These results would suggest an estrogenic and an anti-estrogenic role for Tmx in gilthead seabream as it has been described by clinical (Love et al., 1992; O'Regan and Gradishar, 2001) and preclinical (Jordan and Robinson, 1987) in some vertebrates. These differences could be due to the different ER subtypes involved in the modulation of Vtg production by EE_2 and Tmx, as was previously been suggested (Leanos-Castaneda and Van Der Kraak, 2007). In addition, it has been reported that the ligand binding site in the ER was critical to the compound's agonist or antagonist activity (Komm and Mirkin, 2014). Moreover, it has been described that high concentrations of Tmx caused plasma Vtg increases in males and decreases in females in zebrafish or in zebrafish and medaka (Sun et al., 2010; Sun et al., 2007). In our study, these observed effects disappeared after the suspension of the EE_2 and Tmx treatments, as has been described for EE_2 in zebrafish (Baumann et al., 2014). Moreover, while EE_2 promoted a decrease in the GSI during the first 30 days of exposure, Tmx promoted an increase but only after 50 days of exposure.

Recently, it has been demonstrated that E2 is capable of down regulating the immune function directly via leukocyte associated ERα in Channel catfish (Iwanowicz et al., 2014). Head kidney macrophages and lymphocytes, but not acidophilic granulocytes, of gilthead seabream constitutively express *era* gene (Liarte et al., 2011a). Exposure to EE_2 or Tmx promotes no changes in *era* mRNA levels in the head kidney of vaccinated gilthead seabream, as previously observed in EE_2-bath exposed specimens (Cabas et al., 2012). Unfortunately, we were not able to

analyze the mRNA expression of the other two known ERs in gilthead seabream, ERβ1 and ERβ2 (Pinto et al., 2006). Moreover, the head kidney of gilthead seabream expresses *gper* (Cabas et al., 2013a). Vaccination promoted a strong decrease in *gper* mRNA levels, which may be related to the mobilization of acidophilic granulocytes from the head kidney to the vaccination/infection site (Chaves-Pozo et al., 2005b), since these cells expressed very high levels of *gper* (Cabas et al., 2013a). Notably, despite the fact that EE_2 and Tmx bind to GPER (Albini et al., 2014; Filardo et al., 2000; Prossnitz et al., 2008; Revankar et al., 2005), its expression was not further modulated by the treatment with these two compounds. In agreement with this is the previous observation that both *in vitro* and *in vivo* GPER activation by G1, a GPER selective agonist, failed to modulate the gene expression of *gper* (Cabas et al., 2013a).

To the best of our knowledge, no studies have evaluated the effect of Tmx on the immune response in fish and the reversibility of the effects of EE_2 or Tmx on the immune response in gilthead seabream. Since the innate immune response is the first line of defence against infections, any effect of EE_2 or Tmx on this response could alter the susceptibility of fish to infection. EE_2 and Tmx are able to inhibit the vaccination-induced *il1b* gene expression as was previously described in EE_2-bath exposed fish (Cabas et al., 2012). Unexpectedly, we did not observe an increase in the *il1b* gene expression after vaccination during the recovery period probably due to the different reproduction stage of specimens at these time point. However, neither EE_2 or Tmx exposures nor immunization affected the gene expression profile of the anti-inflammatory cytokine, *il10*, during treatments or during the recovery period, in contrast that previously observed following G1 exposure (Cabas et al., 2013a).

ROS production has been proposed as a useful marker of endocrine disruption in fish, since this activity is altered by many EDCs (Bols et al., 2001) . As we have previously described (Cabas et al., 2013b), the ability to produce ROS was higher in head kidney leukocytes from unvaccinated fish than in those from vaccinated ones, i.e., ROS production decreased after vaccination. Interestingly, EE_2 and Tmx exposures prevent, at least in part, this effect, both in naïve and VaDNA-stimulated leukocytes. These results could be related to the reduced number of acidophilic granulocytes present in the head kidney of vaccinated fish and its full recovery by EE_2 or Tmx exposure, as these cells show a strong phagocytic and ROS production capacities (Sepulcre et al., 2002, 2007). Interestingly, no differences in the ROS-production ability between leukocytes from control and both EE_2 and Tmx-treated plus vaccinated fish were observed when the EE_2 and Tmx exposure ceased, suggesting a transient effect of EE_2 and Tmx in innate immunity. Studies are in progress to clarify whether the effect of EE_2 and Tmx in acidophilic granulocyte

numbers is mediated through the induction of myelopoiesis or the inhibition of mobilization.

The adaptive immune response in fish showed the expected characteristics of specificity and memory. *In vitro* studies have demonstrated the presence of two types of lymphocytes in fish, which are equivalent to B and T cells in mammals, the B lymphocytes and the antibodies (Igs) being key players in the adaptive immune response, although they are also implicated in the innate immune response (Zhang et al., 2010). In mammals five antibody isotypes have been identified (IgA, IgD, IgE, IgG and IgM), while in fish IgM has been identified as the most common Ig in the serum of teleosts, although IgD (Hordvik et al., 1999; Stenvik and Jorgensen, 2000; Wilson et al., 1997), IgT/IgZ (Hansen et al., 2005; Ryo et al., 2010) have also been identified. It has been previously demonstrated that natural and synthetic estrogens, including SERMs, have major effects on the immune system, particularly on B cells (Bernardi et al., 2014b). In gilthead seabream it has been previously demonstrated that total IgM levels decreased at 3 and 7 days after E2 post-injection (Cuesta et al., 2007). In the present work, gilthead seabream specimens were immunized with the widely used antigen hemocyanin in the presence of alum adjuvant in order to precisely evaluate the impact of EE_2 and Tmx exposure on the humoral adaptive immune response of this species using a monoclonal antibody to seabream IgM. The dietary intake of EE_2 and Tmx increased the specific IgM in vaccinated gilthead seabream although only Tmx increased the IgM titer, accompanied by an increase in the IgM^+ B cells, during the recovery period. The higher number of IgM^+ cells observed in naïve and hemocyanin-stimulated head kidney leukocytes from Tmx-treated fish during the recovery period would suggest a pivotal role for this compound in the homeostasis of the immune responses. However, further studies will be necessary to gain a deeper understanding of the mechanism underlying the immunotoxicology effects caused by this compound in this cell type in fish taking into account that E_2, but not SERMs, increased the Ig production and the number of Ig-producing cells in the bone marrow and spleen (Bernardi et al., 2014b; Erlandsson et al., 2002b).

In conclusion, our results indicate that the dietary intake of Tmx has estrogenic and anti-estrogenic effects. Both, EE_2 and Tmx alter *in vivo* the innate immune response of immunized fish, while only Tmx modulate the humoral adaptive immune response induced by an antigenic challenge. To our knowledge, the present study is the first to provide evidence for the ability of the innate immune response of fish to recover from the disruptive effects of EE_2 and Tmx, although no reversibility is observed in the effect of Tmx on the humoral adaptive immune response, at least after 88 days of recovery. The results obtained will be used to hopefully minimize the adverse impact of EE_2 and/or Tmx on the

sustainability of this species and can also be extrapolated to other species of interest in aquaculture.

Chapter 2

Selective estrogen receptor modulators differentially alter the immune response of gilthead seabream juveniles

EE_2, a synthetic estrogen used in oral contraceptives and hormone replacement therapy, Tmx, a selective estrogen-receptor modulator used in hormone replacement therapy, and G1, a GPER selective agonist, differentially increased the hepatic *vtg* gene expression and altered the immune response in adult gilthead seabream (*Sparus aurata* L.) males. However, no information exists on the effects of these compounds on the immune response of juveniles. This study aims, for the first time, to investigate the effects of the dietary intake of EE_2, Tmx or G1 on the immune response of gilthead seabream juveniles and the capacity of the immune system of the specimens to recover its functionality after ceasing exposures (recovery period). The specimens were immunized with hemocyanin in the presence of aluminium adjuvant 1 (group A) or 120 (group B) days after the treatments ceased (dpt). The results indicate that EE_2 and Tmx, but not G1, differentially promoted a transient alteration in hepatic *vtg* gene expression. Although all three compounds did not affect the production of reactive oxygen intermediates, they inhibited the induction *il1b* gene expression after priming. Interestingly, although Tmx increased the percentage of IgM-positive cells in both head kidney and spleen during the recovery period, the antibody response of vaccinated fish varied depending on the compound used and when the immunization was administered. Taken together, our results suggest that these compounds differentially alter the capacity of fish to respond to infection during ontogeny and, more interestingly, that the adaptive immune response remained altered to an extent that depends on the compound.

Key words: 17α-ethynylestradiol, tamoxifen, G1, immune response, juveniles, fish.

1. INTRODUCTION

EDCs is a term that covers both naturally produced compounds, such as estrogens and androgens, and a wide range of industrial chemicals released from many sources into the environment (Benotti and Brownawell, 2007; Desbrow et al., 1988; Kolpin et al., 2004; Sumpter and Jobling, 1995). Many of these chemicals are derived from pharmaceutical products and end up in the aquatic environment where they primarily affect aquatic organisms including fish because they interfere with the body's ability to regulate growth, its development, metabolism or other functions (Baumann et al., 2014; Bhandari et al., 2015; García-Reyero et al., 2011; Kloas et al., 2009; Scholz and Kluver, 2009). However, the effects of these compounds on the early life stages of fish have received little attention and, to the best of our knowledge, no studies exist on the effect of these these compounds on the immune system of juvenile specimens.

EE_2 is used in most oral contraceptive pills and in hormone replacement therapy, since it has 10 fold higher potency than E_2 to bind ER (Thorpe et al., 2003). EE_2 has a widespread presence in the aquatic environment (García-Reyero et al., 2011; Hinteman et al., 2006). The impact of EE_2 on wildlife and humans has been widely discussed over the past years (review by Owen and Jobling, 2012). It has been shown to be bioactive at concentrations below 2 ng/L and it disturbs several crucial processes in fish, such as gonad development, egg production, embryo mortality or courtship behavior (Kidd et al., 2007; Santos et al., 2014; Sarria et al., 2011; Zha et al., 2008). In addition, EE_2 is bio-accumulated due to its resistance to degradation and transfer throughout the food chain (Lai et al., 2002). The reversibility of some reproductive parameters in zebrafish exposed to EE_2 40 days after ceasing exposure have been recently analyzed by (Baumann et al., 2014).

Tmx is a nonsteroidal first-generation SERM, which was initially evaluated for the treatment of breast cancer in the early 1970s. Tmx is metabolized in the liver into a variety of metabolites which are mainly estrogenic (Clarke et al., 2001). Tmx acts as an antagonist competitor in the a-estrogen receptor (ERa) and as an agonist competitor in cholesterol metabolism (Coezy et al., 1982; Jordan and Koerner, 1975). The pharmacological targets of Tmx include ER, the multi-drug resistance gene product, permeability-glycoprotein (Callaghan and Higgins, 1995) and GPER (Revankar et al., 2005; Vivacqua et al., 2006b). Tmx can be found in wastewater in the range of 10 ng/L to 10 µg/L (Mater et al., 2014). In fish, dose-dependent effects have been reported on the rate of vitellogenin (*vtg*) synthesized in males (Sun et al., 2007). Moreover, an increase in the proportion of F1 males whose parents were exposed to concentrations of Tmx higher than 5 µg/L was observed (Sun et al., 2007) . Previous studies in fish have analyzed the impact of dietary Tmx on reproduction (Mandiki et al., 2005; Singh et al., 2015). Moreover, Tmx has been

proposed for use as a growth-promoting agent in aquaculture (Park et al., 2003) and in this context would pose an additional risk to aquatic organisms.

Selye noted in the early 1950s that some steroids induced biological effects only minutes after their application (Arukwe, 2008), mediated by specific receptors localized most frequently in the plasma membrane (Campbell et al., 2006; Casanova-Nakayama et al., 2011). The identification of a G protein-coupled estrogen receptor, a membrane estrogen receptor that binds E_2 and other estrogens, has opened up the possibility of exploring additional estrogen-mediated effects. However, information on the importance of GPER in immunity is scant. The first GPER-selective ligand identified was G1 (Bologa et al., 2006; Dennis et al., 2011), which has been extensively used to explore the biological significance of GPER in different tissues and organs (Prossnitz and Barton, 2011). The expression of GPER in multiple immune cells, including B and T cells, monocytes/macrophages, suggested that some estrogenic effects on the immune system could be mediated by GPER (Blasko et al., 2009; Rettew et al., 2010; Wang et al., 2008a).

The gilthead seabream (*Sparus aurata* L.) is a seasonal marine teleost hermaphrodite species of great commercial value, meaning that any impact of EDCs on its immune system is an important concern. We have previously demonstrated that immune cells of adult gilthead seabream express ERs (Liarte et al., 2011a) and GPER (Cabas et al., 2013a) and that the dietary intake of EE_2 (5 µg/g food), Tmx (100 µg/g food) (Rodenas et al., 2015a) and G1 (20 µg/g food) (Cabas et al., 2013a) promoted a differential increase in hepatic *vtg* gene expression. EE_2 and Tmx might alter the capacity of adult gilthead seabream to appropriately respond to infection although these compounds did not behave as immunosuppressors (Cabas et al., 2012) and only Tmx has a long-lasting effect on humoral adaptive immunity (Rodenas et al., 2015a). Moreover, G1 promotes an anti-inflammatory effect both *in vitro* and *in vivo* and fine-tunes adaptive immunity (Cabas et al., 2013a). In addition, we have illustrated the strong dependence of young gilthead seabream larvae on innate immunity and the lack of correlation between the achievement of immunocompetence and the formation of the lymphomyeloid organs and the detection of the adaptive immunity markers (Mulero et al., 2008).

The aim of this work was to demonstrate the effect of EE_2, Tmx and G1 exposure for approximately four months on the immune response of gilthead seabream juveniles and the capacity of the immune system to recover its functionality when the treatments with these compounds ceased.

2. MATERIALS AND METHODS

2.1 Animals, in vivo treatments and sample collection

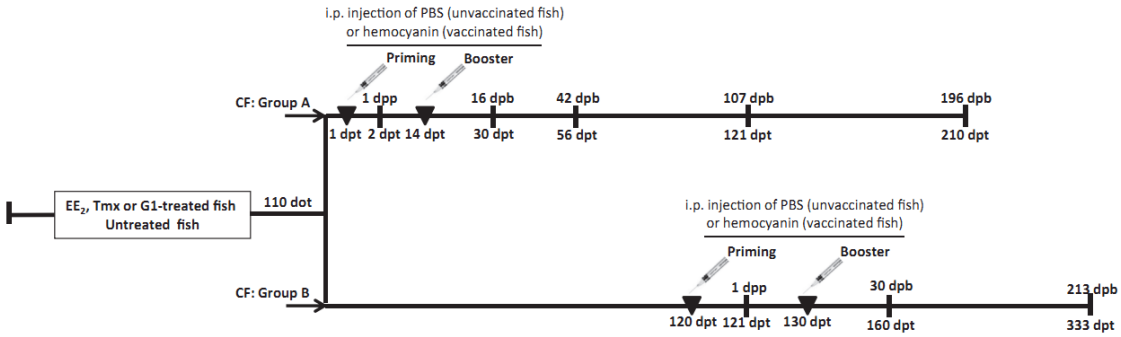

Figure 1. Schematic drawing of the experimental design: EE_2, Tmx or G1 dietary exposure and vaccination protocol of gilthead seabream juveniles (not to scale). Fish were exposed to a supplemented diet (treated fish) with 0.5 and 5 mg EE_2/g food, 10 and 100 mg Tmx/g food, 0.5 and 5 mg g G1/g food or to an unsupplemented diet (untreated fish) for 110 days (days of treatment, dot). Then, the specimens were fed with commercial food (CF) for 210 (group A) or 333 (group B) days (days post-treatment, dpt). Fish were intraperitoneally (i.p.) injected with phosphate buffered saline (unvaccinated fish) or hemocyanin plus imject alum adjuvant (vaccinated fish) at 1 (priming) and 14 (booster) dpt (group A) and at 120 (priming) and 130 (booster) dpt in specimens that were not previously immunized (group B). The samplings were carried out 110 dot and 1 day after priming (dpp) (2 dpt) and 16, 42, 107 and 196 days after booster (dpb) (30, 56 121 and 210 dpt) (group A) and on days 1 dpp (121 dpt) and 30 and 213 dpb (160 and 333 dpt) (group B).

Healthy specimens of gilthead seabream were maintained at the Oceanographic Centre of 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 comply with the guidelines of the European Union Council (2010/63/EU) and the Bioethical Committees of the University of Murcia and the Instituto Espanol de Oceanografía for the use of laboratory animals.

Treatments involving *in vivo* EE_2, Tmx or G1 exposure (Fig. 1) were carried out with two-month-old gilthead seabream specimens (n = 250/treatment), with a body weight (BW) which varied from 0.5 g (at the beginning of the experiment) to 71.2 ± 0.5 g (at the end of the experiments treatments), in 170 l aquaria. Briefly, EE_2 (0.5 and 5 µg/g food, 98% purity; Sigma), Tmx (10 and 100 µg/g food; Sigma-Aldrich) or G1 (0.5 and 5 µg/g food; Sigma-Aldrich) were incorporated in the commercial food using the ethanol evaporation method (0.3l ethanol/kg of food), as described elsewhere (Shved et al., 2007a). The specimens were fed three times a day *ad libitum* with the pellet diet supplemented (treated fish) with EE_2, Tmx or G1 or unsupplemented (untreated fish) for 110 days (days of treatment, dot). After that,

the specimens were fed with the commercial food for a further 210 (group A) or 333 (group B) days (recovery period, days post-treatment, dpt) (Fig. 1). In order to evaluate the effect of these compounds on an induced adaptive immune response, the untreated and the EE_2, Tmx or G1-treated specimens were intraperitoneally injected with keyhole limpet hemocyanin (45 µg/fish; Sigma-Aldrich) and imject alum adjuvant (4 mg/fish; Thermo Scientific) (vaccinated fish) or PBS (unvaccinated fish) on days 1 (priming) and 14 (booster) of the recovery period of group A. A similar immunization protocol was carried out at 120 days (priming) and 130 days (booster) of the recovery period with specimens of group B. Samples from group A were taken 1 day post-priming (dpp) (2 dpt) and 16, 42, 107 and 196 days post-booster (dpb) (30, 56, 121 and 210 dpt), while group B samples were taken at 1 dpp (121 dpt) and 30 and 213 dpb (160 and 333 dpt). Specimens (n = 6 fish/treatment/time of sampling) were fasted for 24 h before sampling. They were tranquilized by 8 µl/l of clove oil and immediately anesthetized using 40 µl/l of clove oil, weighed and decapitated before the head kidneys were removed and then processed for gene expression analysis, ROS production, determination of IgM positive cells and proliferation assays, as described below. Livers and spleens were weighed after removal and processed for gene expression and the determination of IgM positive cells, respectively. Serum samples from trunk blood were obtained by centrifugation and immediately frozen and stored at -80° C until use. Cell suspensions from head kidneys were obtained as described elsewhere (Chaves-Pozo et al., 2003, 2005b).

To evaluate the effect of the *in vivo* EE_2, Tmx or G1 treatments, the BW, hepatosomatic index (HSI) and splenosomatic index (SSI) were calculated at the end of the treatments and at the end of the recovery period. HSI is the 100 x (ML/MB)(%) and SSI is 100 x (MS/ MB)(%), where ML is liver mass, MS is spleen mass and MB is body mass (all in grams).

2.2 Analysis of gene expression

Total RNA was extracted from liver and head kidney from untreated and EE_2, Tmx or G1-treated (vaccinated or not) fish (n = 5 fish/treatment/time of sampling) with TRIzol Reagent (Invitrogen), following the manufacturer's instructions, and quantified with a spectrophotometer (NanoDrop, ND-1000). The RNA was then treated with amplification grade DNase I (1 U/µg RNA; Invitrogen) to remove genomic DNA traces that might interfere with the PCR reactions, and the SuperScript III RNase H Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNA with oligodT18 primer from 0.5 to 1 µg of total RNA, at 50° C for 50 min. The β-actin (*actb*) gene was analyzed by PCR performed with an Eppendorf Mastercycle Gradient Instrument (Eppendorf) to check cDNA quality. Reaction

mixtures were incubated for 2 min at 95° C, followed by 35 cycles of 45 s at 95° C, 45 s at the specific annealing temperature, 1 min at 72° C, and finally 10 min at 72° C.

The expression of the genes coding for hepatic *vtg* after treatments (at 1, 30, 56 and 121 dpt) and for head kidney *il1b* (a pro-inflammatory cytokine) and *il10* (an anti-inflammatory cytokine) one day after priming (1 dpp, at both 2, group A, and 121 days, group B, dpt) was analyzed by real-time PCR performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). 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, 1 min at 60° C, and 15 s at 95° C. For each mRNA, gene expression was corrected by the ribosomal protein S18 gene (*rps18*) content in each sample using the comparative cycle threshold method, Ct method ($2^{-\Delta\Delta Ct}$). The gilthead seabream specific primers used are shown in Table 1. In all cases, each gene was analyzed in triplicate.

Table 1. Gene accession numbers and primer sequences used for expression analysis. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html). All primers were used for real-time PCR, except *actb* primers that were used for conventional PCR.

Gene	Accession No.	Name	Sequence (5'/3')
actb	X89920	F3	ATCGTGGGGCGCCCCAGGCACC
		R3	CTCCTTAATGTCACGCACGATTTC
vtg	AF210428	F1	CTGCTGAAGAGGGACCAGAC
		R1	TTGCCTGCAGGATGATGATA
il1b	AJ277166	F2	GGGCTGAACAACAGCACTCTC
		R3	TTAACACTCTCCACCCTCCA
il10	FG261948	F	TGGAGGGCTTTCCTGTCAGA
		R	TGCTTCGTAGAAGTCTCGGATGT
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC
		R	CTTCTGCCTGTTGAGGAACC

2.3 ROS production assay

The production of reactive oxygen intermediate (ROS) was measured as the luminol-dependent chemiluminescence produced by 0.5 x 10^6 head kidney leukocytes from untreated and EE_2, Tmx or G1-treated fish (vaccinated or not) (n = 5 fish/treatment/time of sampling) after booster (at 16 dpb, 30 dpt; group A) in the presence or absence of 50 µg/ml of phenol-extracted genomic DNA from the bacterium *Vibrio anguillarum* ATCC19264 cells (VaDNA) for 16 h, as described elsewhere (Mulero et al., 2001). This was achieved by adding 100 µM luminol (Sigma-Aldrich) and 1 µg/ml phorbol myristate acetate (PMA; Sigma-Aldrich), while the

chemiluminescence was recorded every 127 s for 1 h in a FLUOstart luminometer (BGM; LabTechnologies). The values reported are the average of triple readings from three different samples, expressed as the maximum and slope of the reaction curve from 127 to 1016 s, from which the apparatus background was subtracted.

2.4 Determination of IgM positive cells

The percentage of IgM positive cells was measured in aliquots of 0.5 x 10^6 leukocytes from head kidney and spleen after booster (at 16 and 42 dpb, 30 and 56 dpt, respectively) (n = 3 fish/treatment/ time of sampling). In the proliferation assay, the leukocytes were incubated in the presence or absence of hemocyanin and/or 50 µg/ ml VaDNA in sRPMI containing 10% charcoal/dextran-treated hormone-free fetal bovine serum (hf-FBS, Hyclone) for 48 h. After that, the cells were washed in PBS containing 2% fetal calf serum (FCS, Life Technologies) and 0.05% sodium azide (FACS buffer). Cells were then incubated with 0 and 2 µg/ml (0, 1:100) immunoglobulin (Ig) M antibody (Ab) (Aquatic Diagnostic) (Sepulcre et al., 20011) in PBS containing 2% FCS for 30 min at 4° C. After washing, cells were incubated with a 1:1000 dilution of a fluorescein isothiocyanate (FITC)-conjugated rabbit for 30 min at 4° C, washed again, and analyzed by flow cytometry using a flow cytometer (BD Biosciences).

2.5 Proliferation assay

To assess the proliferative activity of head kidney IgM positive cells after booster (at 42 dpb, 56 dpt) (n = 3 fish/treatment/time of sampling), 5-ethynyl-2'-deoxyuridine (EdU) (Life Technologies) was added to aliquots of head kidney cell suspensions (0.5 x 10^6), incubated in the presence or absence of hemocyanin and/or 50 µg/ ml VaDNA, as previously described, for 2 h. To determine the percentage of double positive cells (EdU^+ and IgM^+ cells), EdU^+ cells were detected by fluorescent-azide coupling reaction with EdU according to the manufacturer's protocol (Click-iT; Life Technologies) and IgM^+ cells by immunofluorescence, and the cells were then analyzed by flow cytometry.

2.6 Determination of IgM specific titer

The hemocyanin specific IgM titer was determined by an ELISA kit (Aquatic Diagnostic), following the manufacturer's instructions. In short, serial serum dilution from untreated and EE_2, Tmx or G1-treated (vaccinated or not) fish (n = 5 fish/treatment/time of sampling) were added to hemocyanin-precoated 96-well ELISA plates, followed by a monoclonal Ab specific to seabream IgM and an anti-rabbit IgG (whole molecule)-peroxidase Ab produced in goat (Sigma-Aldrich).

Finally, the chromogen tetramethylbenzidine (TMB) was added, and absorbance was read at 450 nm using a FLUOstart luminometer (BGM; LabTechnologies).

2.7 *Statistical analysis*

ANOVA and Tukey tests were applied in a Bonferroni multiple range test to determine differences among groups. A Student t-test and an Unpaired tests were applied to determine differences between two groups. The critical value for statistical significance was taken as $p \leq 0.05$. The asterisks *, ** and *** refer to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. All statistical analyses were carried out using the GraphPad Prism 5 program.

3. RESULTS

3.1 *EE_2 and Tmx but not G1 increase hepatic vitellogenin gene expression*

The lowest concentration of EE_2 (0.5 µg/g food), Tmx (10 µg/g food) and G1 (0.5 µg/g food) used in this study did not promote any change in hepatic *vtg* transcript levels (data not shown). However, the highest concentration of EE_2 (5 µg/g food) and Tmx (100 µg/g food) increased hepatic *vtg* mRNA levels while no effect was detected at the highest concentration of G1 (5 µg/g food) at the end of the treatments, 110 days (Fig. 2a). For that reason, all the following analyses were carried out with the specimens exposed to the highest concentration of each compound. Notably, *vtg* gene expression returned to basal levels at 121 dpt in all treated groups (Fig. 2b).

3.2 *EE_2 and Tmx alter body weight*

The survival of young gilthead seabream specimens exposed to EE_2, Tmx or G1 was 100% during the trial (data not shown). EE_2 promoted a decrease and Tmx an increase in the BW of treated young gilthead seabream, which lasted during the recovery period (333 dpt) (Fig. 2c). All groups show similar food intake (data not shown).

3.3 *EE_2, Tmx and G1 exposures differentially affect the hepatosomatic and the splenosomatic index*

The liver and spleen from gilthead seabream juveniles were differentially altered by EE_2, Tmx or G1 treatments. Thus, at 110 dot, EE_2 exposure significantly increased both HSI (Fig. 2d) and SSI (Fig. 2e), while G1 only increased SSI (Fig. 2e). However, Tmx did not show any effect on these indexes (Fig. 2d,e). By 333 dpt, the

three compounds promoted an increase in the HSI (Fig. 2d) while the SSI increase promoted by EE_2 and G1 disappeared (Fig. 2e).

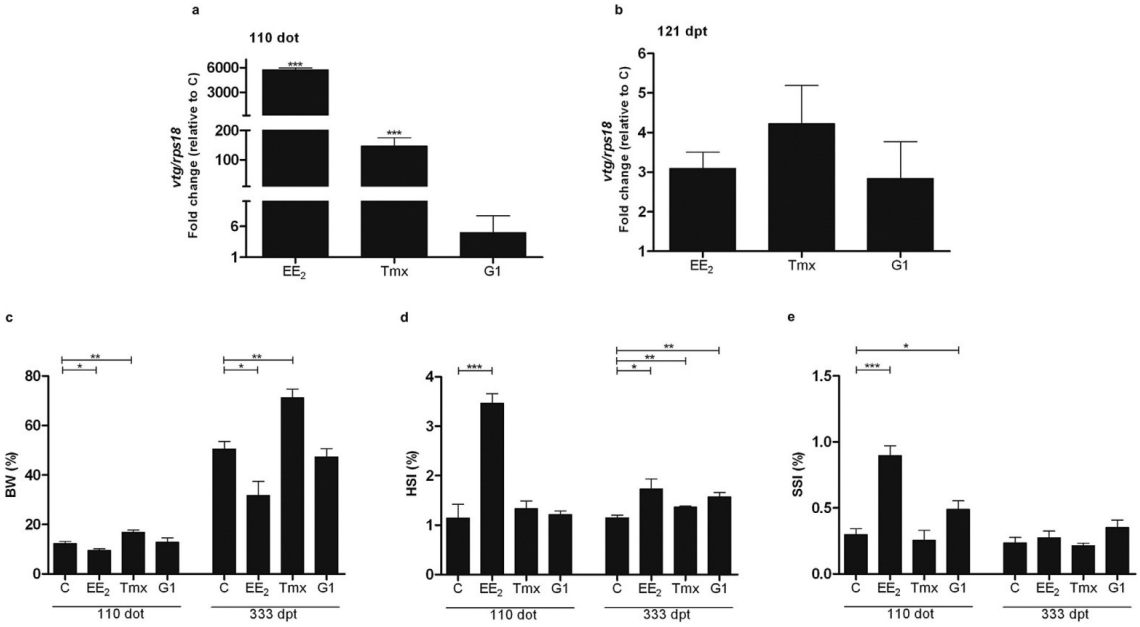

Figure 2. EE_2 and Tmx increase hepatic *vitellogenin* (*vtg*) transcript levels. The levels of *vtg* mRNA were determined in the liver of untreated and EE_2 (5 µg/g food), Tmx (100 µg/g food) or G1 (5 µg/g food)-treated fish at day 110 of treatments (dot) (a) and at day 121 after ceasing the treatments (dpt) (b) by real-time reverse transcription polymerase chain reaction (RT-PCR). Gene expression levels were normalized to *rps18* mRNA levels and were shown as relative to the mean of untreated fish (value 1). Each bar represents the mean ± SEM of triplicates of five independent fish. The sample size was n = 5 fish/group/time of sampling. Body weight (BW, c), hepatosomatic index (HSI, d) and splenosomatic index (SSI, e) were measured in specimens at 110 dot and at 333 dpt. Each bar represents the mean ± SEM of six independent fish. The sample size was n = 6 fish/group/time of sampling. In all cases, the asterisks denote statistically significant differences after Student *t-test* between the untreated and the EE_2, Tmx or G1-treated groups, at each time point. *p < 0.05; **p < 0.01 and ***p < 0.0 01.

3.4 EE_2, Tmx and G1 differentially modulates the innate immune response

The expression of genes coding for two key cytokines, *il1b* and *il10*, was analyzed in head kidney 1 dpp (2 and 121 dpt, group A and B, respectively). As expected, the immunization resulted in increased *il1b* transcript levels in specimens from group A, which was impaired by the dietary intake of EE_2, Tmx and G1 (Fig. 3a). In contrast, in specimens from group B, immunization promoted decreased *il1b* mRNA levels and only G1 significantly prevented this effect (Fig. 3b). Immunization did not alter the *il10* transcript levels in specimens from group A and only G1 significantly inhibited them in vaccinated fish (Fig. 3c). However, in specimens from group B, priming promoted slightly increased *il10* mRNA levels but the treatments show negligible effects (Fig. 3d).When we analyzed the production of ROS triggered by PMA in naïve and VaDNA-stimulated head kidney leukocytes from

untreated and EE_2, Tmx and G1-treated (vaccinated or not) gilthead seabream juveniles 16 dpb (30 dpt, group A), no significant differences were found among the groups (data not shown).

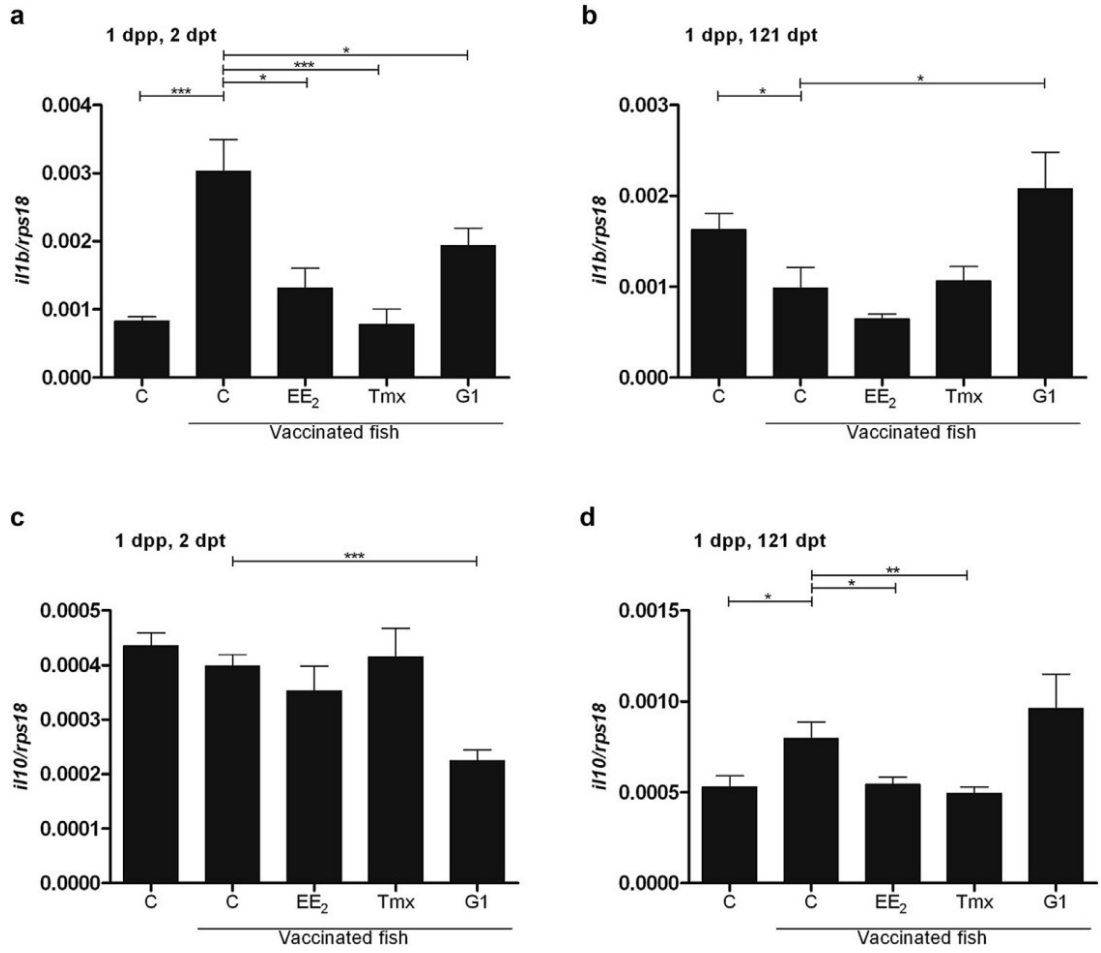

Figure. 3. EE_2, Tmx and G1 differentially modulate the innate immune response. The mRNA levels of *il1b* (a, b) and *il10* (c, d) were determined 1 day after priming (dpp) in the head kidney from untreated (vaccinated or not) and EE_2 (5 µg/g food), Tmx (100 µg/g food) or G1 (5 µg/g food)-treated (vaccinated) fish at 2 (group A; a, c) and 121 days after treatments (dpt) (group B; b, d) by real-time RT-PCR. Gene expression levels were normalized to *rps18* mRNA levels and data represent means ± SEM of triplicates of five independent fish. The sample size was n = 5 fish/group/time of sampling. The asterisks denote statistically significant differences after Student *t-test* between: (i) the unvaccinated or vaccinated untreated fish, within the same sampling time, and (ii) the untreated or EE_2, Tmx or G1-treated vaccinated fish, at each time point. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$.

3.5 Tmx increased the number of IgM positive cells in vaccinated fish

The percentage of IgM positive cells measured by flow cytometry increased in head kidney (Fig. 4a) and in spleen (Fig. 4b) of vaccinated and Tmx treated gilthead seabream at 16 dpb (30 dpt, group A).

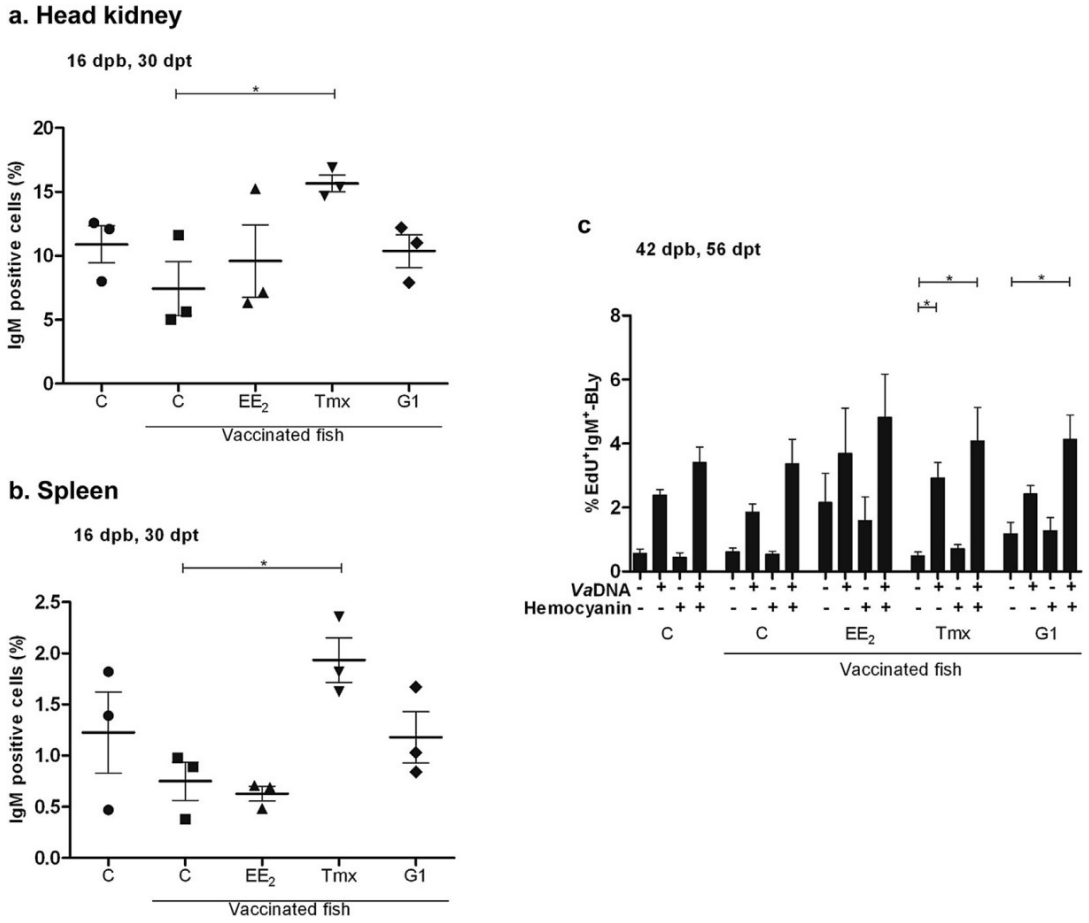

Figure 4. Tmx modulates the number of IgM positive cells in head kidney (a) and in spleen (b). The percentage of IgM-positive cells were determined in head kidney and spleen leukocytes from untreated (vaccinated or not) and EE_2 (5 mg/g food), Tmx (10 0 mg/g food) or G1 (5 mg/g food)-treated (vaccinated) groups at 16 dpb (30 dpt) by flow cytometry. The sample size was n = 3 fish/group. The mean for each group of specimens is shown as a horizontal line. The asterisks denote statistically significant differences after Student *t–test* between: i) the unvaccinated or vaccinated untreated fish, within the same sampling time, and (ii) the untreated or EE_2, Tmx or G1-treated vaccinated fish, at each time point. *p < 0.05; **p < 0.01 and ***p < 0.0 01. Tmx and G1 increased the number of the proliferative IgM positive cells in stimulated leukocytes (c). Head kidney leukocytes from untreated (vaccinated or not) and EE_2 (5 µg/g food), Tmx (100 µg/g food) or G1 (5 µg/g food)-treated (vaccinated) fish at 42 dpb (56 dpt) were obtained to determine the percentage of proliferating IgM-positive cells by flow cytometry in the presence or absence of hemocyanin and/or VaDNA after 48 h in culture. The data represent the mean ± SEM of three individual fish. The asterisks denote statistically significant differences among groups according to two-way ANOVA and Bonferroni post hoc test. *p < 0.05; **p < 0.01 and ***p < 0.0 01.

No differences were observed between untreated and EE_2, Tmx and G1-treated (vaccinated or not) groups in leukocytes of head kidney and spleen at 42 dpb (56 dpt, group A) (data not shown). Nevertheless, at this time point, vaccinated and Tmx or G1 treated fish were seen to have a significantly increased number of proliferating IgM positive cells following *in vitro* stimulation for 48 h with hemocyanin and VaDNA according to a two-way ANOVA (Fig. 4c).

3.6 EE_2, Tmx and G1 alter the antibody response

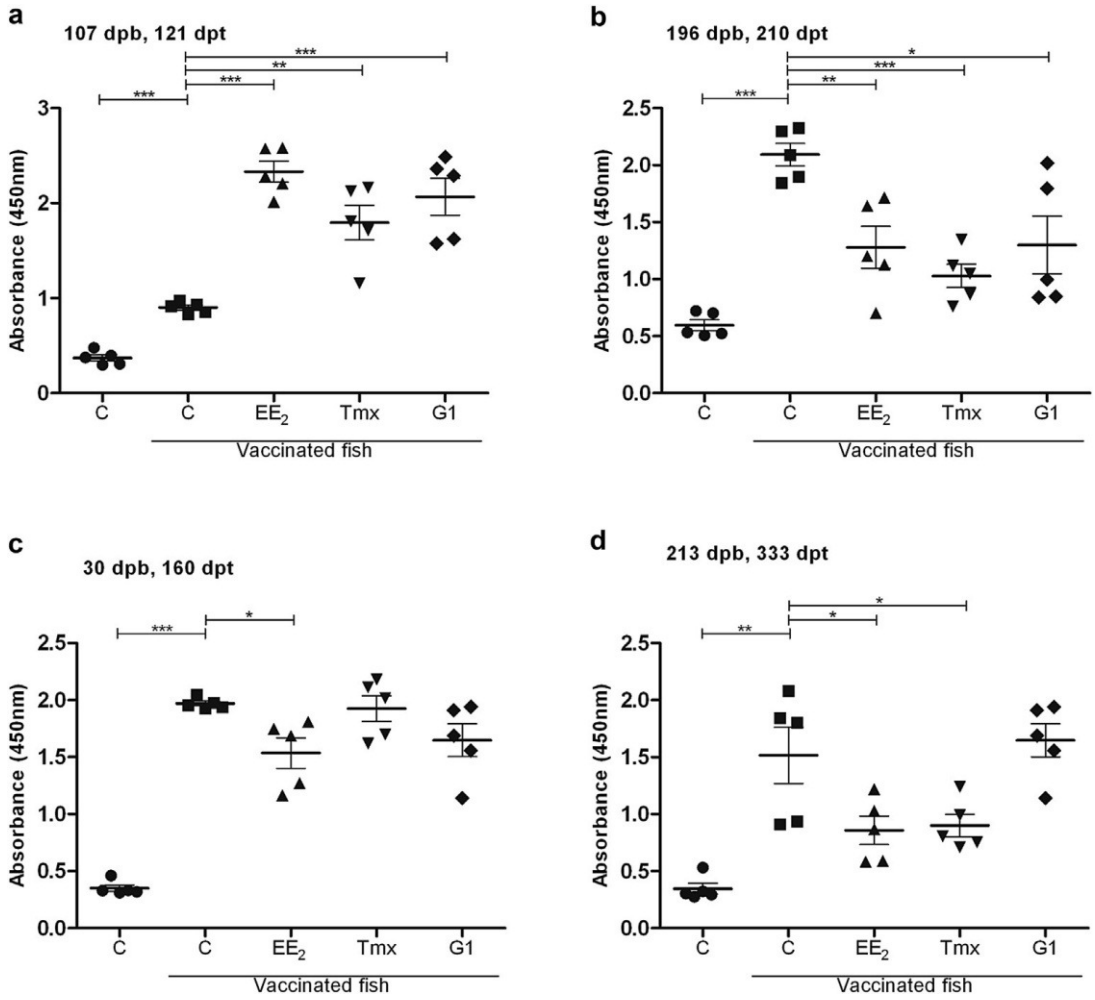

Figure 5. EE_2, Tmx and G1 differentially alter the antibody response. Hemocyanin-induced specific IgM levels were determined by ELISA after booster at 107 (a) and 196 dpb (b) (121 and 210 days after treatment, dpt, respectively, group A) and on 30 (c) and 213 (d) dpb (160 and 333 dpt, respectively, group B) from untreated (vaccinated or not) and EE_2 (5 mg/g food), Tmx (100 mg/g food) or G1 (5 mg/g food)-treated (vaccinated) fish. The data represent the mean ± SEM of absorbance value of five individual fish using a 1:236 serum dilution. The sample size was n ¼ 5 fish/group/time of sampling. The asterisks denote statistically significant differences after Student t-test between: i) the unvaccinated or vaccinated untreated fish, within the same sampling time, and (ii) the untreated or EE_2, Tmx or G1-treated vaccinated fish, at each time point. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$.

The impact of EE_2, Tmx and G1 exposure on the humoral adaptive immune response was evaluated as hemocyanin-specific IgM titers in the serum of gilthead seabream juveniles. As expected, vaccinated animals showed a stronger antibody response to the antigen than non-vaccinated fish, although the hemocyanin-specific IgM titers were different at all time points, perhaps as a result of the temperature of the water (ranging from 23 to 14 degrees). More importantly, EE_2, Tmx and G1 significantly increased the antibody titer of vaccinated fish 107 dpb (Fig. 5a) but decreased it at 196 dpb (Fig. 5b), respectively, when priming was performed 1 dpt

(group A). However, when priming was performed 120 dpt (group B), EE_2 diminished the antibody titer at 30 and 213 dpb (Fig. 5c,d) while Tmx diminished it only at 213 dpb (Fig. 5d). No effect was observed in G1-treated specimens at 30 and 213 dpb (Fig. 5c,d).

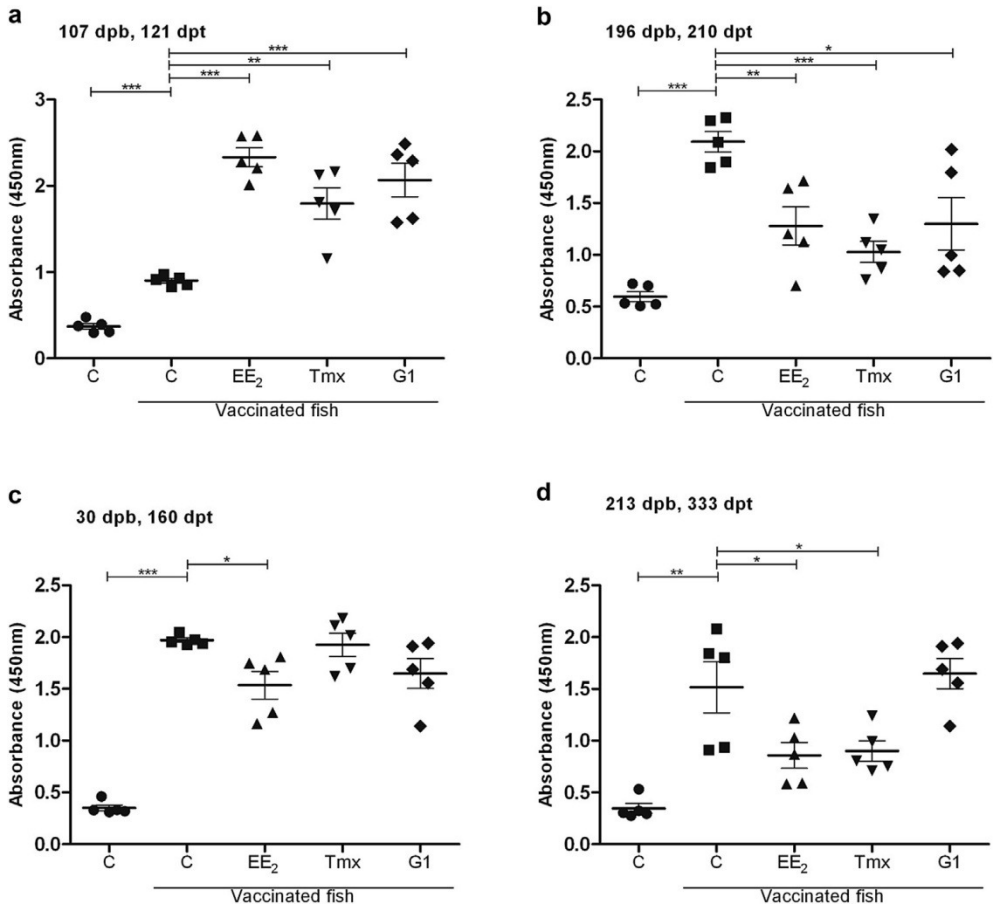

Figure 5. EE_2, Tmx and G1 differentially alter the antibody response. Hemocyanin-induced specific IgM levels were determined by ELISA after booster at 107 (a) and 196 dpb (b) (121 and 210 days after treatment, dpt, respectively, group A) and on 30 (c) and 213 (d) dpb (160 and 333 dpt, respectively, group B) from untreated (vaccinated or not) and EE_2 (5 µg/g food), Tmx (100 µg/g food) or G1 (5 µg/g food)-treated (vaccinated) fish. The data represent the mean ± SEM of absorbance value of five individual fish using a 1:236 serum dilution. The sample size was n = 5 fish/group/time of sampling. The asterisks denote statistically significant differences after Student *t-test* between: i) the unvaccinated or vaccinated untreated fish, within the same sampling time, and (ii) the untreated or EE_2, Tmx or G1-treated vaccinated fish, at each time point. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$.

4. DISCUSSION

Estrogens and androgens are regulators of fish immunity. A wide variety of compounds, acting as EDCs, seem to exhibit immunotoxicological actions (Milla et al., 2011). The impact of estrogenic EDCs on fish immune system is an important concern. In this respect, elucidation of the mechanistic and physiological effects of estrogen on the immune function in fish is essential (Iwanowicz et al., 2014) particularly in hermaphrodite species, such as the gilthead seabream. To the best of

our knowledge, no studies have evaluated the effect of EDCs on the immune response during fish ontogeny and, more interestingly, on the possible reversibility of their effects (Baumann et al., 2014), demonstrated that exposure to EE_2 results in very strong but reversible feminization of zebrafish during development.

Vtg, a yolk precursor protein produced by hepatocytes, is generally accepted as a biomarker of estrogenic effects (Leet et al., 2011; Sumpter and Jobling, 1995; Sun et al., 2007). We have previously demonstrated that EE_2 and Tmx differentially increased the level of *vtg* mRNA in adult specimens of gilthead seabream (Cabas et al., 2011; Cabas et al., 2013b; Garcia-Hernandez et al., 2016; Rodenas et al., 2015). In gilthead seabream juveniles, treated with the same concentrations of EE_2 and Tmx as were used in adult specimens, the effect of Tmx was much lower than that promoted by EE_2 (147 and 5728-fold increase, respectively, compared with control group). These differences in both adult and juvenile specimens could be due to the different ER subtypes involved in the modulation of Vtg production by EE_2 and Tmx, as it was previously been suggested in fish (Leanos-Castaneda and Van Der Kraak, 2007). However, G1 at 5 µg/g food did not promote any modification in the *vtg* mRNA levels (the weak increase promote in adult fish was observed with 20 µg G1/g food), confirming that G1 did not promote an evident estrogenic response and the specificity of the G1 agonist over GPER in gilthead seabream (Cabas et al., 2013a).

The length, body mass and growth rate of sticklebacks are influenced by municipal wastewater effluent (Pottinger et al., 2013). Similarly, the BW of Japanese medaka was reduced when exposed to water containing 25% effluent from a local water treatment plant (Zha and Wang, 2006). Although different experimental designs have been used, EE_2 promoted body growth inhibition in tilapia (Shved et al., 2008) and in zebrafish (Baumann et al., 2014), as we observed in gilthead seabream juveniles. However, Tmx promoted an increase in the BW of gilthead seabream juveniles while no effect was observed after G1 exposure. Both, the effect of EE_2 and Tmx in gilthead seabream juveniles were maintained 333 days after ceasing of the treatments, while zebrafish was able to compensate the delay in growth 40 days after the depuration of EE_2 in clean water (Baumann et al., 2014).

EDC-related hepatotoxicity has been reported in several fish species. Thus, it has been demonstrated that estrogenic EDCs, such as 4-nonylphenol, disrupted the liver architecture of tilapia with lyses, loss of nuclei, necrosis and fat infiltration (Abdulla Bin-Dohaish el, 2012); necrosis and a decrease in cell numbers were observed in rainbow trout (Uguz et al., 2003) and hepatocyte hypertrophy and damage in adult rare minnow (Zha et al., 2007) . Moreover, several studies have reported that they induced apoptosis in fish liver (Kaptaner and Unal, 2011; Piechotta et al., 1999; Weber et al., 2004) although treatment time, manner, and interspecies sensitivity are factors that may affect any responsiveness to toxicants.

Although we did not analyze the histopathological effects of any of the compounds used in this study, an increase in the HSI was observed after EE_2 treatment, while EE_2, Tmx and G1 promoted an increase in the HSI 333 dpt. Fewer studies exist on the effects of EDCs on fish spleen, and most of those that exist were focusing on the effects on T cell subsets, B cell functions, and dendritic cell and macrophage biology (Rogers et al., 2013). An increase in the SSI was promoted by EE_2 and G1 in our juvenile gilthead seabream specimens, which disappeared during the recovery period, while no effect was observed with Tmx. The impact of estrogenic compounds in liver and spleen deserve further investigation.

To determine the effect of EE_2, Tmx and G1 on the innate immune response as the first line of defense against infections, we analyzed the gene expression of two key cytokines, the pro-inflammatory, *il1b*, and the anti-inflammatory, *il10*, after priming as well as ROS production after booster. To the best of our knowledge, no studies have evaluated the effect of any of these compounds on the innate immune system of juvenile specimens and the reversibility of their effects. First, we observed that vaccination induces an increase in *il1b* gene expression, as previously described in adult specimens (Cabas et al., 2013b; Rodenas et al., 2015). However, this increase is not always observed probably due to the maturation stage of specimens and specific environmental conditions, such as water temperature (Rodenas et al., 2015a). A similar situation occurred with the *il10* transcript levels (Cabas et al., 2013a; Rodenas et al., 2015), suggesting that new studies are necessary to clarify the effects of vaccination on innate immune system in hermaphrodite fish during their life and reproductive cycles. As regards to *il1b* mRNA levels, EE_2, Tmx and G1 were able to inhibit vaccination-induced *il1b* mRNA levels in gilthead seabream juveniles, as previously described in adult specimens (Cabas et al., 2012; Cabas et al., 2013b; Rodenas et al., 2015) when priming was applied 1 dpt. However, when priming was applied 120 dpt, the decrease in *il1b* gene expression observed in vaccinated young fish was only inhibited by G1. Moreover, neither EE_2 nor Tmx exposure affected the gene expression profile of *il10*, while G1 inhibited *il10* mRNA levels, as occurred in adult specimens (Rodenas et al., 2015), only when priming was applied 1 dpt.

ROS production has been proposed as a useful marker of endocrine disruption in fish, since it is altered by many EDCs (Bols et al., 2001). Although EE_2 and Tmx prevent the ROS production after immunization in adult specimens (Cabas et al., 2013b; Rodenas et al., 2015), EE_2, Tmx and G1 had no effect on the ROS production in juveniles. In addition, G1 inhibited ROS production of leukocytes in non-vaccinated fish, but had no effect on vaccinated animals, further supporting the idea of a prominent role for GPER signaling in the homeostasis of the immune response, which is bypassed during immune challenge (Cabas et al., 2013a). All these results suggest that these compounds could alter the susceptibility of fish to

infection, depending on the compound, the maturational stage of the specimens and when the immunization is applied.

To the best of our knowledge, not studies have examined the influence of EE_2, Tmx or G1 on B cell function in fish although few studies have established the influence of E_2 on B cell function in fish, with contradictory results depending on the concentration, stimulation and period of exposure (Shelley et al., 2013). To precisely evaluate the impact of EE_2, Tmx and G1 exposure on the humoral adaptive immune response during ontogeny, gilthead seabream juveniles were immunized when the adaptive immune system is functional (Mulero et al., 2008) with the widely used antigen hemocyanin in the presence of imject alum adjuvant, as we previously used in adult gilthead seabream specimens (Cabas et al., 2012; Cabas et al., 2013a; Rodenas et al., 2015). More specifically, we focused on the effects on the percentage of the IgM positive B cell population and on antibody production, as we previously did in adult specimens (Cabas et al., 2012b; Cabas et al., 2013d; Rodenas et al., 2015a). Lymphocytes express ERs (Liarte et al., 2011a) and, more interestingly, B lymphocytes express GPER in gilthead seabream adult specimens (Cabas et al., 2015), which indicated that both natural and synthetics estrogens may modulate directly their function during an immune response and may also be a vulnerable target for estrogenic EDCs (Milla et al., 2011). Tmx increased the percentage of IgM positive cells in both head kidney and spleen of juveniles during the recovery period, as described in the head kidney of adult gilthead seabream specimens (Rodenas et al., 2015a). The higher number of proliferating IgM positive cells observed in the presence of the antigen hemocyanin in combination with VaDNA stimulated head kidney leukocytes from vaccinated and Tmx-treated juveniles during the recovery period would suggest a pivotal role for this compound in the homeostasis of the immune responses. Moreover, Tmx promoted an increase in the IgM titer in vaccinated juveniles 107 dpb when priming was applied 1 dpt, as previously shown in adult gilthead seabream (Rodenas et al., 2015a). However, the IgM titer decreased in vaccinated and Tmx-treated juveniles 196 dpb or 213 dpb when priming was applied 1 or 120 dpt, respectively. Moreover, neither EE_2 nor G1 promoted any modification in the percentage of IgM positive cells during the recovery period as Tmx did, although both had the same effect as Tmx on the IgM titer when priming was applied 1 dpt and only EE_2 when priming was applied 120 dpt. In mammals, E_2 but not SERMs increased Ig production and the number of Ig-producing cells in the bone marrow and spleen (Bernardi et al., 2014b; Erlandsson et al., 2002b). All of these results suggest that the effect of selective estrogen receptor modulators on the humoral adaptive immune response differs between adult and juveniles gilthead seabream, depending on the compound used and the time when the immunization was performed. Further studies will be necessary to gain a deeper understanding of the mechanism orchestrating B cell functions by natural and synthetic estrogens in fish.

Overall, our study enabled us to conclude that EE_2 and Tmx, but not G1, have disruptor endocrine effects in juveniles specimens of the hermaphrodite species, gilthead seabream. All of them altered the immune response of juveniles, in particular IgM positive lymphocyte responses, but none of them behave as immunosuppresor at the doses tested. The long lasting effect of EE_2 and Tmx on humoral adaptive immunity, when their endocrine disruptor effects were no longer apparent, deserves further investigation.

Chapter 3

Estrogens promote the production of antibodies through G protein-coupled estrogen receptor signaling pathway in fish

It is known that EE_2, the active ingredient in most formulations of contraceptive pills and one of the most potent synthetic estrogen, altered the immune response of gilthead seabream (*Sparus aurata* L.), a hermaphrodite protandric teleost fish. Moreover, EE_2 stimulated the antibody response of vaccinated gilthead seabream and this response depends on when the immunization is administered. IgM is the most evolutionary conserved isotyope, present in all vertebrates. This study aims to investigate the effects of dietary administration of EE_2 (5 µg/g food) on the immune lymphocyte populations and antibody production. For that, specimens were intraperitoneally injected with keyhole limpet hemocyanin and imject alum adjuvant at the end of the EE_2 treatment period, 76 dt. In this study, we report that EE_2 affects the percentage and proliferation of lymphocytes T and the differentiation of IgM positive cells as well as it induces the production of antibodies. These results correlate which those observed in G1 (a GPER specific agonist)-treated fish. It highlights that GPER signaling could be relevant in the estrogens-induced antibodies production.

Keywords: 17α-ethynylestradiol, immune response, lymphocytes, antibodies, fish, gilthead seabream

1. INTRODUCTION

The influence of estrogens on immune responses is, although controversial, well documented (Bouman et al., 2005; Cutolo et al., 2004; Karpuzoglu and Zouali, 2011; Straub, 2007). For many years, the immune system has been considered as a natural target for estrogen action (Khan and Ansar Ahmed, 2015) being corroborated by the clear sex-differences observed in autoimmune and inflammatory disorders.

Thus, estrogens modulate different aspects of immune response including those of adaptive immune response (Khan and Ansar Ahmed, 2015), which is mediated by lymphocytes, B and T, and antibodies, although in fish they also play a role in innate immue response, of which fish depend to a greater extent. In fact, estrogens affect different stages of B-cell development and modify the humoral response (Karpuzoglu and Zouali, 2011) and also a third generation of selective estrogen receptor modulators have been found to regulate B development and function (Bernardi et al., 2014). Moreover, it has recently described that the activation of ER direct influence antibody expression by binding to switch sites and regulatory elements in the immunoglobulin heavy chain locus of activated B cells (Jones et al., 2016). All these effects are possible due to the recognized presence of ER in immune cells (Straub, 2007), including lymphocytes (Schneider et al., 2014).

Although clasically the action of estrogens are mediated by the nuclar ER, ERα and ERβ, which function as hormone-inducible transcription factors, binding to the ERE located within the promoter region of target genes (Deroo and Korach, 2006), it has relatively recently identified that they are also able to rapidly activate transduction pathways via non-genomic mechanisms. These "additional" effects are mediated by a membrane anchored receptor called GPER which was identified by independent laboratories in the 2000s (Filardo et al., 2000; Thomas et al., 2005), and later shown that it can be activated by E_2 (Filardo et al., 2007; Filardo et al., 2002; Funakoshi et al., 2006). GPER-activation downstream mechanisms includes several siginaling pathways involving MAPKs, ERK, PI3K, cAMP and intracellular calcium, review in (Prossnitz and Barton, 2009; Prossnitz and Maggiolini, 2009). Nevertheless, there is much evidence that these categorized physiological responses to estrogens: genomic or rapid/nongenomic, are connected (Moriarty et al., 2006) (Ma and Pei 2007). Morever, both the identification of G1, a highly selective agonist of GPER (Bologa et al., 2006), and the study of several GPER KO mice models (Liu et al., 2009; Mizukami, 2010; Pang and Thomas, 2009) has allowed to define the role of GPER in human physiology, linking it with nervous, reproductive, cardiovascular, and immune systems; metabolism and obesity; cancer and cell growth; and inflammatory vascular diseases, reviewed in (Prossnitz and Barton, 2009) and opened the door to the generation of diagnostics and

therapeutics directed at individual estrogen receptors. However, the relevance of GPER for the immune system is understudied until now.

Xeno-estrogens or enviromental estrogens, known as EDCs, so causing endocrine disruption, pointed out as an important field to be considered in disease susceptibility (Schug et al., 2011), are synthetic or natural substances of high stability that exert toxicity by mimicking the effects of estrogens. As a marker of this estrogenic disruption, they induce the hepatic *vtg* expression (Sumpter and Jobling, 1995). Moreover, these substances that have been described to be involved in autoimmunity (Chighizola and Meroni, 2012; Inadera, 2006), reduces B cell precursors in mice (Erlandsson et al., 2002) and have been also found to activate GPER, review in (Prossnitz and Hathaway, 2015). EE_2, a synthetic analogue of E_2,is one of the most potent pharmaceutical estrogens which is intensively used in human therapeutic as a component of most of the oral contraceptives. Interestingly, approximately 16-68% of dose is excreted in the urine or feces (Johnson and Williams, 2004), arriving to the waste water treatment plants where it cannot be totally eliminated (Avar et al., 2016). Consequently, an unquantified load of estrogens are released into the aquatic environment, being a widespread problem in the environment due to its high resistance to degradation and where it can be absorbed by sediment and persists for long periods, taken up by aquatic biota (Aris et al., 2014; Matozzo and Marin, 2008) and being concentrated in animal tissues. It has been found in concentrations around 21.7 ng/l in a south-western European river (Mira, Portugal) (Rocha et al., 2016). In humans, the binding affinity of EE_2 to the ERs is one to two times higher than E_2, and EE_2 has been shown to be up to five times higher in some fish species, review by (reviewed by Aris et al., 2014). The effect of EE_2 on reproductive system has been extensively described (Fenske et al., 2005; Garcia-Hernandez et al., 2016; Nash et al., 2004; Pawlowski et al., 2004; Schafers et al., 2007; Tilton et al., 2005) but there are also several publications on its impact on immune response (Ahmed, 2000; Chalubinski and Kowalski, 2006).

The gilthead seabream (*Sparus aurata* L.) is a seasonal marine teleost of great commercial value in the Mediterranean area. Its hermaphrodite character made it an interesting animal model to analyze the role of estrogens, natural or synthetic, in the immune response. We have previously demonstrated that nuclear ERs and GPER are expressed in head kidney, bone marrow equivalent, leukocytes (Cabas et al., 2013a; Liarte et al., 2011a) and that estrogens modulate granulocyte functions in teleost fish through a GPER/cAMP/protein kinase A/CREB signaling pathway (Cabas et al., 2013a). We have also observed that EDCs altered the immune response of gilthead seabream by promoting some long-lasting effects even when their estrogenic disruptive effects were not present (Rodenas et al., 2015; Rodenas et al., 2016). Moreover, EE_2 bath-exposed specimens have altered their capacity to appropriately respond to infection although the compound does not behave as an

immunosuppressor (Cabas et al., 2012) and that EE_2 food intake stimulated the antibody response of vaccinated fish in adults (Rodenas et al., 2015) and modulated the IgM levels in juveniles (Rodenas et al., 2016). However, no information exits on the mechanisms involved on the effect of EE_2 on gilthead seabream B lymphocytes. In this context, the zeta-chain-associated protein kinase of 70 kDa, ZAP-70, and the paired box-5, Pax5, two gene lymphocyte markers have been previously characterized at least to some extent in teleost fish. ZAP-70, a tyrosine kinase that plays a critical role in initiating T-cell responses (Wang et al., 2010), is normally expressed in T cells and NK cells. Pax5, one of the major transcription factor in vertebrate B cell development (Morrison et al., 1998), is expressed from the pro-B cell through mature and activated B cell stages, and is down regulated during terminal differentiation being absent at the plasma cell stage (Cobaleda et al., 2007).

The aim of this study is to delve into how estrogens modulate lymphocyte populations and antibody production by analyzing some lymphocyte gene markers. Specifically, we have analyzed (i) the zeta-chain-associated protein kinase of 70 kDa, ZAP-70, (ii) the paired box-5, Pax5, and (iii) the antibody production. Finally, the involvement of GPER signaling in this context was assed and discussed.

2. MATERIALS AND METHODS

2.1 Animals, in vivo treatments and sample collection

Healthy specimens of gilthead seabream were maintained at the Oceanographic Centre of Murcia (Mazarrón, 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. They were 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. The environmental parameters, mortality and food intake, as well as behavior, were recorded daily.

In vivo EE_2 exposure (Fig. 1A) were carried out with two-month-old gilthead seabream specimens (n = 100/treatment), with a body weight (BW) which varied from 26.6 g to 63.2 ± 7.9 g (from the beginning to the end of the experiment), 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 elsewhere (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 unsupplemented (untreated fish) for 76 days (days of treatment, dt). After that, all the specimens were fed with the commercial food for a further 23 days (days post-treatment, dpt) (Fig. 1A). In order to evaluate the effect of EE_2 on any induced

immune response, specimens were intraperitoneally injected with keyhole limpet hemocyanin (KLH) (45 µg/fish; Sigma-Aldrich) and imject alum adjuvant (4 mg/fish; Thermo Scientific) (vaccinated/immunized fish) or phosphate buffered saline (PBS) (control/unvaccinated fish) at the end of the treatment period, 76 dt. Samples were taken at 40 and 76 dt and 1, 9 and 23 days post-immunization (dpi) or dpt. All groups show similar food intake. Specimens (n = 6 fish/treatment/time of sampling) were fasted for 24 h before sampling. They were tranquilized by 8 µl/l of clove oil and immediately anesthetized using 40 µl/l of clove oil, weighed and decapitated before the head kidneys and spleens were removed and processed for gene expression and/or flow cytometry analysis, as described below. Serum samples from trunk blood were obtained by centrifugation and immediately frozen and stored at -80 °C until use. Cell suspensions from head kidney and spleen were obtained as described elsewhere (Chaves-Pozo et al., 2003; Chaves-Pozo et al., 2005a;). Moreover, EE_2 (5 µg/g food, 98% purity; Sigma) or G1 (5 µg/g food; Sigma-Aldrich) were incorporated in the commercial food of two-month-old gilthead seabream specimens, as previously described, for 110 dt in order to obtain serum samples, for ELISA analysis, as describe below.

Other two *in vivo* experiences were carried out in gilthead seabream specimens in order to obtain serum samples, for ELISA analysis, as describe below: (1) specimens (650 g mean weight) were EE_2 bath exposure was administered at 0 5 and 50 ng/L as described elsewhere (Brown et al., 2007). Briefly, EE_2 was dissolved in a methanol:water solution (60% v/v) and added at a rate of 300 ml per aquaria, for 2 months. (2) Specimens with a body weight of 225 g were exposed to G1 in the commercial food to give a concentration of 0, 2, and 20 µg/fish/day treatment, as previously described, for up to 50 days, and (3) two-month-old specimens with a body weight varied from 0.5 to 71.2 g were exposed to EE_2 or G1 in the commercial food for 110 days.

The experiments described were approved by the Consejería de Agua, Agricultura y Medio Ambiente of the Región de Murcia, Spain (approval number A13160507).

2.2 *Determination of serum vitellogenin levels*

The serum *vtg* levels were determined by the enzyme-linked immunosorbent assay using acomercial kit (Aquatic Diagnostic), following the manufacturer's instructions. In short, 1:500 serum dilution from untreated and EE_2-treated fish (n = 6 fish/treatment/time of sampling) were added to flat-bottomed 96-well plates, followed by a commercial polyclonal antibody (Ab) against to gilthead seabream Vtg (1:100) and an anti-rabbit IgG (whole molecule)-peroxidase Ab (1:1000) (Sigma-Aldrich). Finally, the chromogen tetramethylbenzidine (TMB) was added, and the

absorbance was read at 450 nm using a FLUOstart luminometer (BGM; LabTechnologies).

2.3 *Analysis of gene expression*

Total RNA was extracted from head kidney from untreated and EE_2-treated (vaccinated or not) fish (n = 6 fish/treatment/time of sampling) at1, 9 and 23 dpi, dpt with TRIzol Reagent (Invitrogen), following the manufacturer's instructions, and quantified with a spectrophotometer (NanoDrop, ND-1000). The RNA was then treated with amplification grade DNase I (1 U/µg RNA; Invitrogen) to remove genomic DNA traces that might interfere with the PCR reactions, and the SuperScript III RNase H Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNA with oligo-dT18 primer from 1 mg of total RNA for 50 min at 50 °C. The b-actin (*actb*) gene was analyzed by PCR performed with an Eppendorf Mastercycle Gradient Instrument (Eppendorf) to check cDNA quality. Reaction mixtures were incubated for 2 min at 94 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at the specific annealing temperature (55 °C), 45 s at 72 °C, and finally 10 min at 72 °C.

On these samples, the expression levels of the gen coding the head kidney interleukin-1β (*il1b*; a key pro-inflammatory cytokine) were analyzed by real-time PCR performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). 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, 1 min at 60 °C, and 15 s at 95 °C. The gene expression was corrected by the ribosomal protein S18 gene (*rps18*) content in each sample using the comparative cycle threshold method, Ct method ($2^{-\Delta\Delta Ct}$). The gilthead seabream specific primers used are shown in Table 1. In all cases, samples were analyzed in triplicate.

Table 1. Gene accession numbers and primer sequences used for expression analysis. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/ zf_info/nomen.html). All primers were used for real-time PCR, except *actb* primers that were used for conventional PCR.

Gene	Accession No.	Name	Sequence (5'/30)
actb	X89920	F3	ATCGTGGGGCGCCCCAGGCACC
		R3	CTCCTTAATGTCACGCACGATTTC
il1b	AJ277166	F2	GGGCTGAACAACAGCACTCTC
		R3	TTAACACTCTCCACCCTCCA
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC
		R	CTTCTGCCTGTTGAGGAACC

2.4 *Zap70, IgM and Pax5 immunofluorescence*

The percentage of Zap70 positive, IgM positive and IgM positive Pax5 positive cells was determined by flow cytometry. In brief, the cytoplasmic protein Zap70, the IgM, the main Ig of serum, and the Pax5 were detected in aliquots of 0.5 x 10^6 leukocytes from head kidney at 1, 9 and 23 dpi, dpt and from spleen at 23 dpi, dpt (n = 6 fish/treatment/time of sampling) of untreated and EE_2-treated fish (vaccinated or not). The leukocytes were washed in PBS containing 2% fetal calf serum (FCS, Life Technologies) and 0.05% sodium azide (FACS buffer). Cells were fixed with 4% paraformaldehyde for 15 min at room temperature. After three rinses, cells were incubated in ice-cold PBS containing 1% BSA and saponin (Invitrogen) at 4˚C to permeabilize the membrane. Cells were then stained with 0 and 2 µg/ml (0, 1:100) of the rabbit Zap70 monoclonal antibody (Cell Signaling) or mouse monoclonal antibody specific to immunoglobulin M (IgM, Aquatic Diagnostic; Sepulcre et al., 2011), in PBS containing 2% FCS for 30 min at 4 °C. After washing, cells were incubated with a 1:1000 dilution of a phycoerythrin (PI)-conjugated rabbit or mouse, respectively for 30 min at 4 °C, washed again. In other experiments, cells were also stained with 0 and 2 µg/ml (0, 1:100) of Pax5 monoclonal antibody (Alexa 488 conjugate; Cell Signaling) in PBS containing 2% FCS for 1 h at room temperature. After washing, cells were analyzed by flow cytometry using a flow cytometer (BD Biosciences).

2.5 *Proliferation assay*

To assess the proliferative activity of head kidney and spleen Zap70 or IgM positive cells at 1, 9 and 23 dpi, dpt (n = 6 fish/treatment/time of sampling), 5-ethynyl-2'-deoxyuridine (EdU) (Life Technologies) was intraperitoneally injected for 2 h before sampling at each time point. Then, 0.5 x 10^6 aliquots of head kidney and spleen cell suspensions were used to determine the percentage of double positive cells (EdU^+ Zap70 positive or EdU positive IgM positive cells). EdU positive cells were detected by fluorescent-azide coupling reaction with EdU according to the manufacturer's protocol (Click-iT; Life Technologies) and Zap70 positive or IgM positive cells by immunofluorescence (as described above), and the cells were then analyzed by flow cytometry. The percentage of positive cells is given on head kidney R2 region: macrophages, lymphocytes and precursor cells, excluding acidophilic granulocytes (Esteban et al., 1998).

2.6 *Determination of serum IgM titer*

The total IgM levels and the IgM titer against hemocyanin (specific antigen to immunization program), lysozyme (unrelated antigen) were determined by ELISA (Aquatic Diagnostic), following the manufacturer's instructions. In short, serial

dilutions of pooled serum samples from the fish of the different *in vivo* experiences (described above) (n = 6 fish/treatment/time of sampling), were added to hemocyanin, lysozyme-precoated, flat-bottomed 96-well plates, followed by a monoclonal Ab (mAb) specific to IgM seabream (1:100) and an anti-rabbit IgG (whole molecule)-peroxidase Ab produced in goat (1:1000) (Sigma-Aldrich). Finally, the chromogen tetramethylbenzidine (TMB) was added, and the absorbance was read at 450 nm using a FLUOstart luminometer (BGM; LabTechnologies).

2.7 Statistical analysis

A Student *t*-test and an unpaired test were applied to determine differences between two groups. The critical value for statistical significance was taken as $p < 0.05$. The asterisks *, ** and *** refer to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. All statistical analyses were carried out using the GraphPad Prism 5 program.

3. RESULTS

3.1 EE_2 increases the hepatic vitellogenin serum levels but hardly modulates those of il1b in head kidney

The survival of gilthead seabream specimens was 100% during the trial (data no shown).

As a control for estrogenic endocrine disruption, we show that EE_2 significantly increased the hepatic Vtg serum level as we have previously described for hepatic *vtg* transcript levels in adult and juvenile gilthead seabream fish (Cabas et al., 2012, 2013b Garcia-Hernandez et al., 2016; Rodenas et al., 2015, 2016). So, EE_2-induced levels reached the maximum at the end of the treatment but decreased progressively with the cessation of the treatment, although they remain altered at 23 dpt (Fig. 1B). On the other hand, as a control for the immunization program, the expression of gene coding for a key pro-inflammatory cytokine, *il1b*, was analyzed in head kidney at 1, 9 and 23 dpi. As expected, the immunization resulted in increased *il1b* transcripts levels only at 1 and 9 dpi (Fig. 1C). However, the immunization-induced *il1b* gene expression levels were not significantly impaired by EE_2 (Fig. 1C).

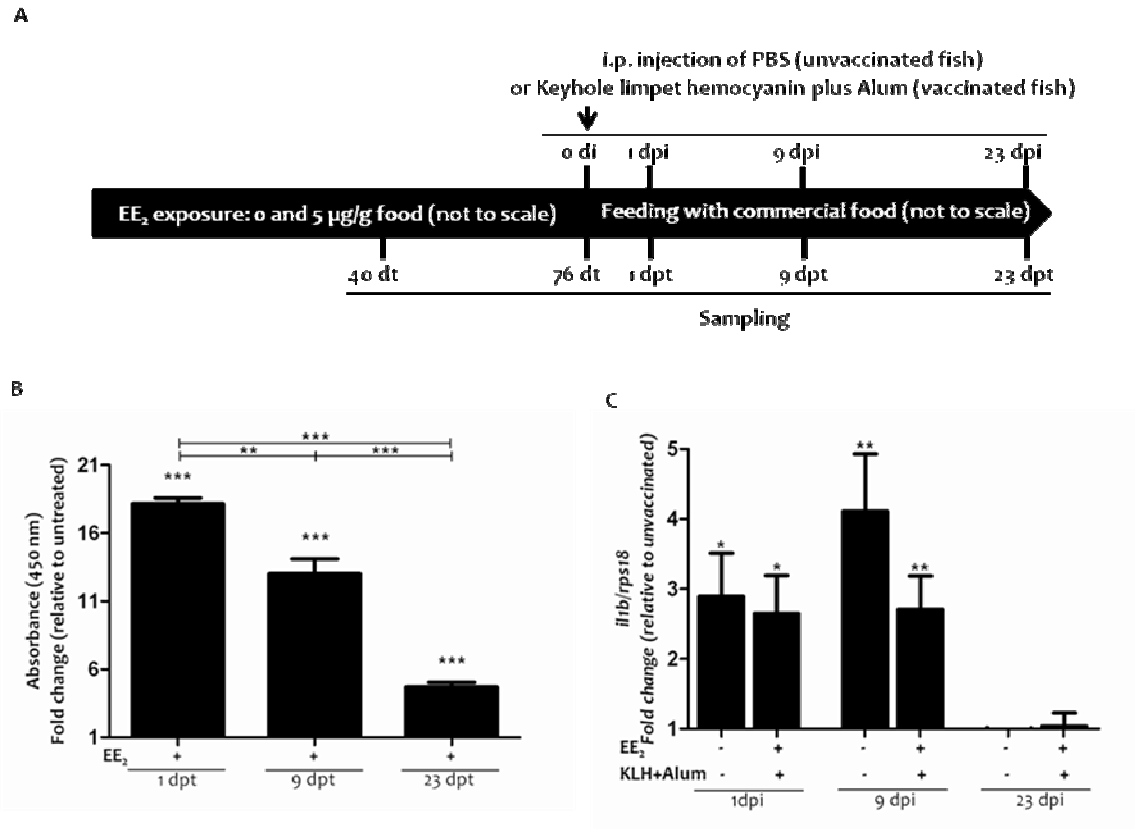

Figure 1. Schematic drawing of the experimental design: EE_2 dietary exposure and immunization protocol of gilthead seabream juveniles (not to scale) (A). Fish were exposed to an unsupplemented diet (untreated fish) or to a supplemented one with 5 µg EE_2/g food (treated fish) for 76 days (days of treatment, dt). Then, the specimens were fed with commercial food for additional 23 days (days post-treatment, dpt). Fish were intraperitoneally (i.p.) injected with phosphate buffered saline (unvaccinated or control fish) or keyhole limpet hemocyanin plus imject alum adjuvant (vaccinated fish) at 76dt. The samplings were carried out 40 and 76 dt and 1, 9 and 23 days post injection (dpi) (1, 9 and 23 dpt). **EE_2 exposure increases vitellogenin (Vtg) serum levels but hardly modulates *il1b* expression levels** (B-C). The levels of Vtg (B) were determined in the serum of untreated and EE_2 (5 µg/g food)-treated fish at day 1, 9 and 23 after ceasing the treatment (dpt) by Enzyme-Linked ImmunoSorbent Assay (ELISA). Levels were shown as relative to the mean of untreated fish (value 1). Each bar represents means ± SEM of duplicates of six independent fish. The sample size was n = 6 fish/group/time of sampling. The asterisks denote statistically significant differences after Student *t-test* between the untreated and the EE_2-treated group, at each time point and between the EE_2-treated groups at the different time points. *p < 0.05; **p < 0.01 and ***p < 0.001. The head-kidney mRNA levels of *il1b* (C) were determined in untreated and EE_2 (5 µg/g food)-treated fish (vaccinated or not) at 1, 9 and 23 days after injection (dpi) by real-time RT-PCR. Gene expression levels were normalized to *rps18* mRNA levels and data represent means ± SEM of triplicates of six independent fish. Levels were shown as relative to the mean of unvaccinated fish (value 1). The sample size was n = 6 fish/group/time of sampling. The asterisks denote statistically significant differences after Student *t*-test between the: (i) unvaccinated and vaccinated untreated fish , (ii) unvaccinated and vaccinated EE_2-treated fish, and (iii) untreated and EE_2-treated vaccinated fish , at each time point. *p < 0.05; **p < 0.01 and ***p < 0.001.

3.2 *EE_2 affects the percentage and proliferation of Zap70 positive cells*

The T-cell activation marker, Zap70, and the proliferation of these activated cells, were analyzed by flow cytometry in head kidney and spleen total leukocytes from untreated and EE_2-treated fish, both vaccinated or not, at 1, 9 and 23 dpi.

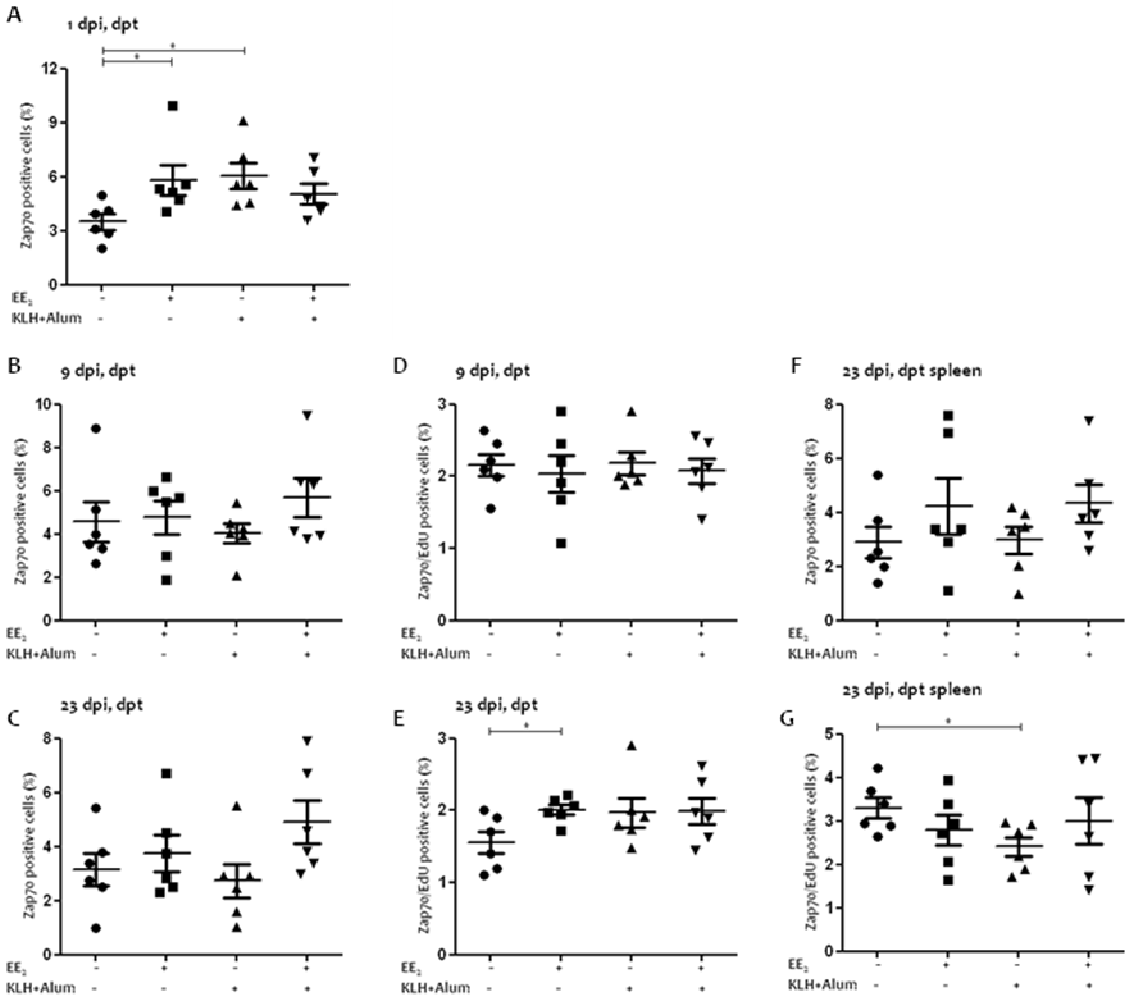

Figure 2. EE_2 exposure modulates the number and the proliferating Zap70 positive cells. The percentage of Zap70-positive cells (A, B, C, F) and that of proliferating Zap70-positive cells (shown as EdU^+cells) (D, E, G) were determined in head kidney (A-E) and spleen (F, G) leukocytes from untreated and EE_2 (5 µg/g food)-treated fish (vaccinated or not) at 1, 9 and 23 days after injection and post-treatment (dpi, dpt) by flow cytometry. The sample size was n = 6 fish/group/time of sampling. The mean for each group of specimens is shown as a horizontal line. The percentage is given on head kidney R2 region: macrophages, lymphocytes and precursor cells, excluding acidophilic granulocytes (Esteban et al., 1998). The asterisks denote statistically significant differences after Student *t*-test between the: (i) unvaccinated and vaccinated untreated fish, (ii) unvaccinated and vaccinated EE_2-treated fish, (iii) untreated and EE_2-treated unvaccinated fish and (iv) untreated and EE_2-treated vaccinated fish, within the same sampling time, at each time point. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$.

We firstly observed that immunization increase the percentage of Zap70 positive cells at 1 dpi in head kidney (Fig. 2A) and decrease that of proliferating Zap70 positive cells in spleen at 23 dpt (Fig. 2E). Interestingly, we also observed that EE_2 exposure increase the percentage of Zap70 positive cells in head kidney at 1dpt (Fig. 2A) and also increase that of proliferating Zap70 positive cells in the same tissue at 23 dpt (Fig. 2E) in unvaccinated fish.

3.3 EE_2 alters the differentiation and proliferation of head kidney IgM positive cells

The percentage of IgM positive cells determined by intracellular staining (total IgM), the co-expression of IgM with Pax5 as well as their proliferating rates, as EdU positive cells, were analyzed by flow cytometry in head kidney and spleen total leukocytes from untreated and EE_2-treated fish, both vaccinated or not, at 1, 9 and 23 dpi.

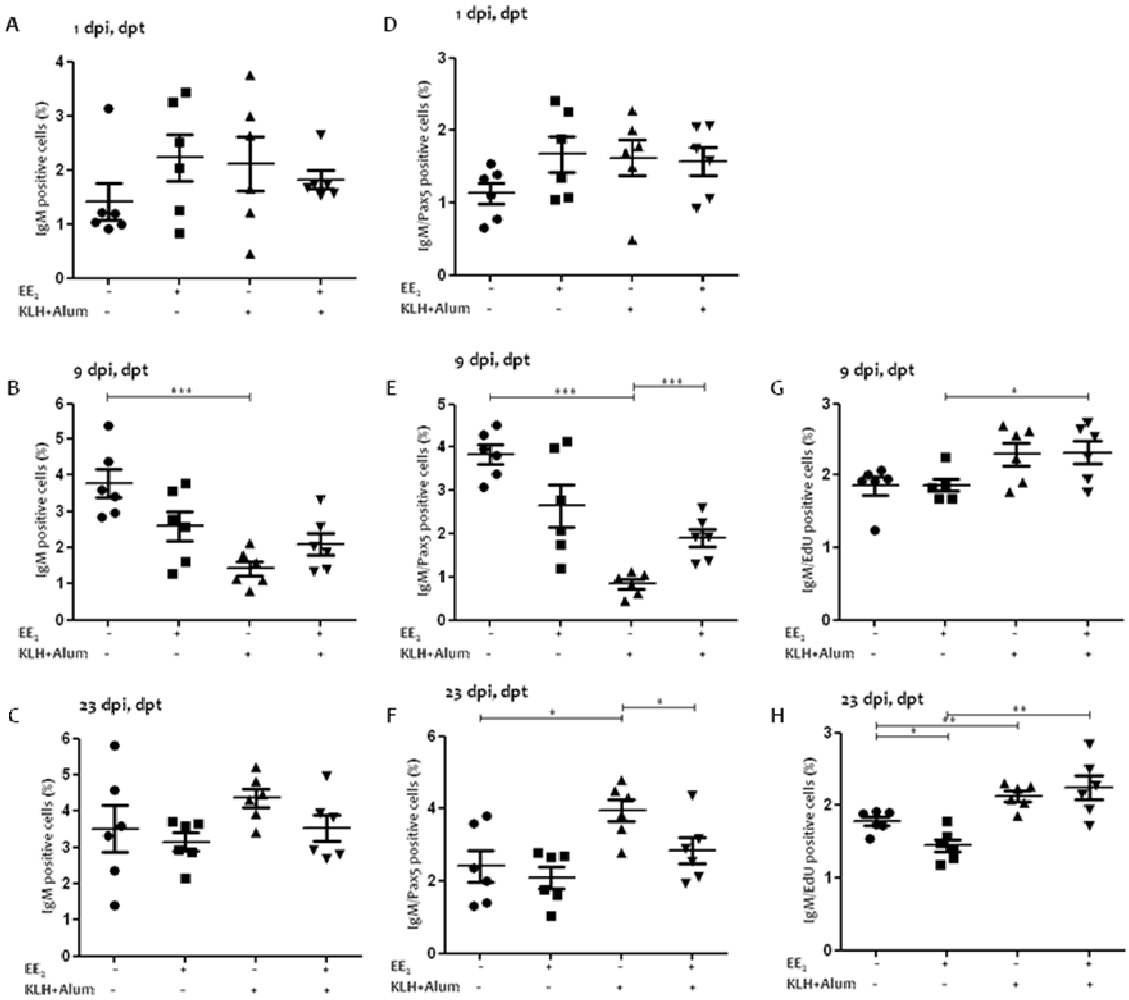

Figure 3. EE_2 exposure alters the IgM^+ cell population impaired the B cell lineage commitment. The percentage of IgM positive cells by intracellular (total IgM) staining (A, B, C), the percentage of IgM and Pax5 positive cells (D, E, F) and IgM and EdU positive (proliferating) cells (G, H) were determined in head kidney leukocytes from untreated and EE_2 (5 µg/g food)-treated fish (vaccinated or not) at 1, 9 and 23 dpi (1, 9 and 23 dpt) by flow cytometry. The sample size was n = 6 fish/group/time of sampling. The mean for each group of specimens is shown as a horizontal line. The percentage is given on head kidney R2 region: macrophages, lymphocytes and precursor cells, excluding acidophilic granulocytes (Esteban et al., 1998). The asterisks denote statistically significant differences after Student *t*-test between the: (i) unvaccinated and vaccinated untreated fish, (ii) unvaccinated and vaccinated EE_2-treated fish, (iii) untreated and EE_2-treated unvaccinated fish and (iv) untreated and EE_2-treated vaccinated fish, within the same sampling time, at each time point. *p < 0.05; **p < 0.01 and ***p < 0.001.

In head kidney, we firstly observed that immunization decrease the percentage of IgM positive cells at 9 dpt (Fig. 3B) and also decrease that of IgM and Pax5 positive cells at this time point (Fig. 3B), but not in the other time points studied. However, at 23 dpt, immunization increase the percentage of IgM and Pax5 positive cells (Fig. 3F) and also that of proliferating IgM positive cells (Fig. 3H). Moreover, in vaccinated fish, EE_2 exposure increase the percentage of IgM and Pax5 positive cells at 9 dpt (Fig. 3E) but decrease it at 23 dpt (Fig. 3F), when also decrease the proliferating IgM positive cells in unvaccinated fish (Fig. 3H). Nevertheless, neither immunization nor EE_2 exposure were observed to modulate these percentages in spleen leukocytes at 23 dpt (Fig. 4).

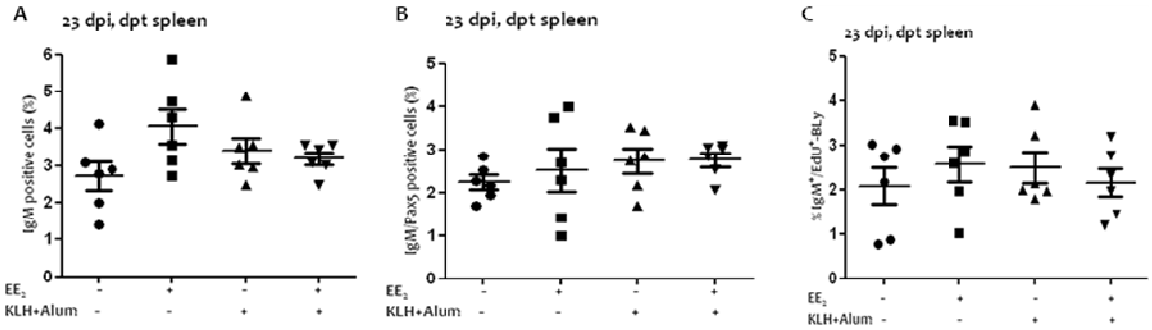

Figure 4. EE_2 in vivo treatment does not alter the IgM^+ cell population from spleen. The percentage of IgM positive cells by intracellular (total IgM) staining (A), the percentage of IgM and Pax5 positive cells (B) and IgM and EdU positive (proliferating) cells were determined in spleen leukocytes from untreated and EE_2 (5 µg/g food)-treated fish (vaccinated or not) at 1, 9 and 23 dpi (1, 9 and 23 dpt) by flow cytometry. The sample size was n = 6 fish/group/time of sampling. The mean for each group of specimens is shown as a horizontal line. The percentage is given on spleen R2 region: macrophages, lymphocytes and precursor cells, excluding acidophilic granulocytes (Esteban et al., 1998). The asterisks denote statistically significant differences after Student *t*-test between the: (i) unvaccinated and vaccinated untreated fish, (ii) unvaccinated and vaccinated EE_2-treated fish, (iii) untreated and EE_2-treated unvaccinated fish and (iv) untreated and EE_2-treated vaccinated fish, within the same sampling time, at each time point. *p < 0.05; **p < 0.01 and ***p < 0.001.

3.4 EE_2 significantly induces the production antibodies

Firstly, the hemocyanin-specific IgM titer was analyzed by ELISA in serum from untreated and EE_2-treated fish, both vaccinated or not, at 1, 9 and 23 dpi, dpt. Curiously, we observed that the unvaccinated EE_2-treated fish has a higher hemocyanin-specific IgM titer at 1dpi, dpt (Fig. 5A).

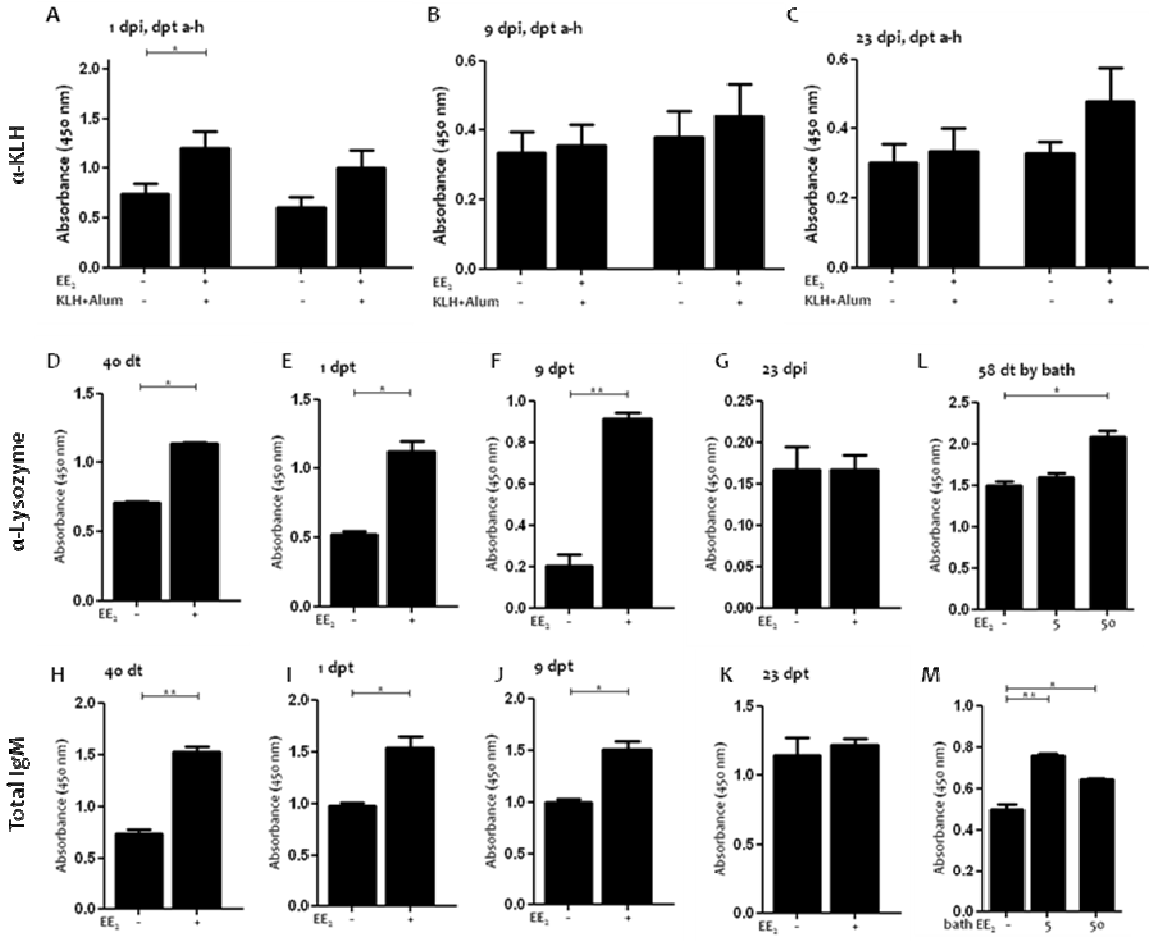

Figure 5. EE_2 exposure induces the antibody production. Hemocyanin-specific IgM titer was determined by ELISA after injection at 1 (A), 9 (B) and 23 (C) dpi (1, 9 and 23 dpt, respectively) from untreated and EE_2 (5 µg/g food)-treated fish (vaccinated or not). Lisozime-specific IgM titer (D-G, L) as well as total IgM levels (H-L, M) were determined by ELISA after 40 dt (D, H) and at 1 (E, I), 9 (F, J) and 23 (G, K) dpt and after 58 days of bath exposure (0, 5 and 50 ng/L) (L, M) from untreated and EE_2-treated fish. The data represent the mean ± SEM of absorbance value of pool sera of six individual fish at 1:10 serum dilution at 1 dpi in hemocyanin and lisozime-induced IgM levels and at 1:100 serum dilution 9 and 23 dpi. The sample size was = 6 fish/group/time of sampling. The asterisks denote statistically significant differences after Student *t*-test between the untreated and EE_2-treated fish (vaccinated or not), at each time point. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$.

After that, the unrelated lysozyme-IgM titer and the total IgM titer were analyzed at 40 dt and 1, 9 and 23 dpt in serum from untreated and EE_2-treated juveniles fish and from EE_2-bath exposure adults specimens. Interestingly, we observed that EE_2 significantly increase the lysozyme (a non-related antigen) (Fig.

5D-F) and the total IgM titer (Fig. 5H-J) at 40 dt and 1 and 9 dpt, but the effect disappear at 23 dpt. Significant similar results were obtained in gilthead seabream adults exposed to EE_2 by bath for approximately two months (Fig. 5L, M).

3.5 GPER signaling is involved in the EE_2-induced antibody production

In order to evaluate the effect of GPER activation on antibodies production, the unrelated lysozyme-IgM titer and the total IgM titer were analyzed in serum from untreated and G1-treated adults fish at 30 dt and from EE_2 and G1-treated juveniles at 110 dt. Interestingly, we observed that G1-treated fish had a higher lysozyme (Fig. 6A) and total IgM (Fig. 6C) titers at 30 dt.

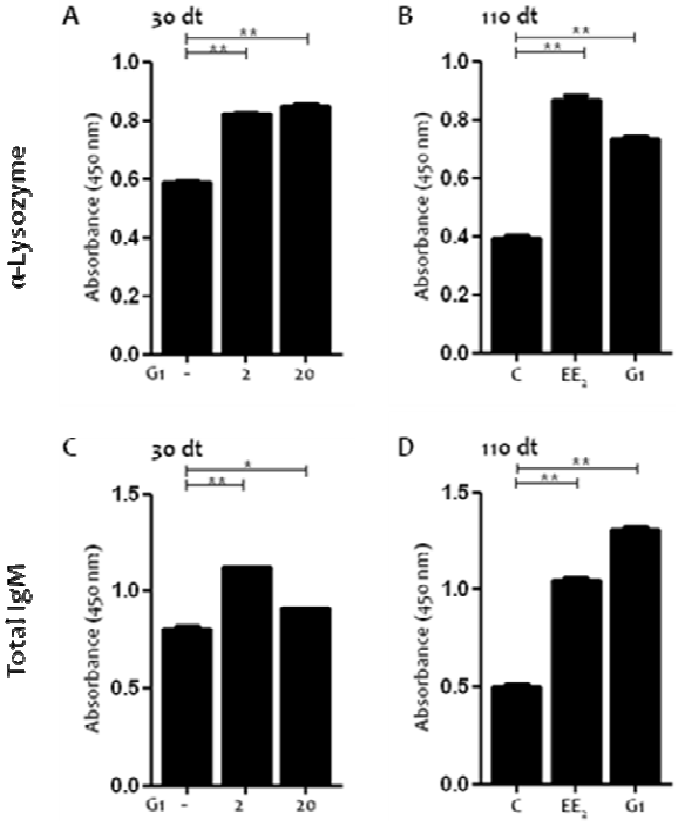

Figure 6. GPER activation *in vivo* modulates the production of antibody. Lisozime-specific IgM (A, B) titer as well as total IgM levels (C, D) were determined by ELISA after 30 days of G1 treatment (0, 2 and 20 µg/fish/day) or after 110 dt of EE_2 (5 µg/g food) and G1(5 µg/g food)- treatment in juveniles fish. The data represent the mean ± SEM of absorbance value of pool sera of six individual fish at 1:10 serum dilution. The sample size was = 6 fish/group/time of sampling. The asterisks denote statistically significant differences after Student *t*-test between the untreated and EE_2-treated fish, at each time point. *$p < 0.05$; **$p < 0.01$ and ***$p < 0.001$.

4. DISCUSSION

The fact that estrogens have a key modulator role of immune system is well known (Straub, 2007). Thus, they have been linked with autoimmunity (Bynote et al., 2008), hematopoiesis, and others. On the other hand, the action of xeno-estrogens on this regard, as exogenous estrogens both natural and synthetic able to mimicking the function of estrogens, are being taken into account in an important way. However, significant knowledge gaps exist between exposures to EDCs and disorders in wildlife populations. More importantly, there are no studies about the effects of the component in many frequently used contraceptives, EE_2, in B cell population and in the production of antibodies.

The most used indicator of exposure to exogenous estrogens in aquatic species is hepatic Vtg and also choriogenins (Chgs), vitelline envelope proteins (Kurauchi et al., 2005). As we previously demonstrated, the *vtg mARN* levels increases after EE_2 exposure but returned to basal levels when the exposure cesed (Rodenas et al., 2015; Rodenas et al., 2016). Here we show the same results with induction at Vtg serum levels by EE_2 exposure.

To determine the effect of EE_2 on the innate immune response as provides a first line of defense against many common microorganisms and being essential for the control of common bacterial infections, we analyzed the gene expression of the key pro-inflammatory cytokine, *il1b*. We observed that vaccination induces an increase in *il1b* gene expression, as previously described in adult and juvenile specimens (Cabas et al., 2013a; Rodenas et al., 2015, 2016). Nevertheless, the EE_2-treatment did not impair the immunization-induced *il1b* expression levels, in contrast as we have previously seen after 110 dt (Rodenas et al., 2016).

The capacity of EE_2 to modulate lymphocyte population and their antibody production function was then evaluated. To the best of our knowledge, no information exits on the influence of EE_2 on the T and B cell subsets. A reduction in the number of circulating lymphocytes, indicative of immunosuppression, has been shown in fathead minnow exposed to potent estrogenic effluents (Filby et al., 2007) and after the treatment with SERMs and E_2 which decreased thymus weight and cellularity (Bernardi et al., 2014). In mammals, it is well known that T cells play a central role in adaptive immune response and several subsets of T cells have a distinct function involved in both humoral and cell-mediated immune. Here, we demonstrated that EE_2 promoted in unvaccinated fish an increase in the percentage and in the proliferation rate of T lymphocytes, showed as Zap70-positive cells, a cytoplasmic tyrosine kinase mainly expressed in T cells that plays a critical role in the events involved in initiating T-cell responses by the antigen receptor (Wang et al., 2010). Moreover, due to its participation in B cell receptor (BCR) signaling in chronic

lymphocytic leukemia (Wang et al., 2010), ZAP70 is considered as an attractive candidate for treatment. Besides, it has been reported that signaling defects in ZAP70 mutant mouse may result in autoimmune disease (Au-Yeung et al., 2009). In fish, similar functions of T cells known for mammals have been reported *in vivo* and *in vitro*, review in (Nakanishi et al., 2015), and also ZAP70 have been used to identify T cells for carp (Piazzon et al., 2015) and zebrafish (Yoon et al., 2015).

Regarding to IgM positive cell populations, no modulation was observed in the percentage of IgM positive cells, contrary that was previously described (Rodenas et al., 2015; Rodenas et al., 2016). Nevertheless, here we observed that EE_2 exposure modulate the percentage of IgM and Pax5 co-expressing cells and their proliferative rate, indicating that EE_2 exposure could modulate the B cell linage commitment as Pax5 is the major transcription factors that play role in vertebrate B cell development expressed at a pro-B stage and is maintained until plasma cell differentiation.

Antibody levels are potentially related to disease resistance and could be used as selective markers for genetic disease resistance (Kachamakova et al., 2006). In general, environmental conditions are considered important for the antibody levels in animals. (Gonzalez et al., 1988)

In this context, steroid hormones (including E_2) act directly on the B cells to suppress IgM production *in vitro* (Hou et al., 1999) or to stimulate antibody production. Moreover, diethylstilbestrol and bisphenol A, other estrogenic EDCs, enhance IgM antibody production *in vitro*. These results suggest that xenoestrogens are involved in antibody production and can be an etiologic or modulating factor in the development of autoimmune diseases (Inadera, 2006; Murakami et al., 1992). Besides, IgM is the most potent complement activator

On the other and, until the identification of GPER as an estrogen binding receptor in 2005, it was thought that estrogens only mediated their effect through their clasical action which is mediated by nuclear ER, ERα and ERβ. Nevertheless, there were some rapid effects that could not be explained by this clasical action, so the relevance of GPER in these additional estrogen-mediated effects is importat to considered, even more in the context of therapeutics applications directed at individual ERs. Thus, although information in very scarse, there are some studies that describe that GPER contributes and/or has been linked to estrogen-induced thymic atrophy (Wang et al., 2008a), the impairement of T cells production in the thymus (Isensee et al., 2009), a suppressive phenotype of CD4 regulatory T cells (Brunsing et al., 2013) and inducing IL-10 in Th17 effector populations (Brunsing and Prossnitz, 2011). Moreover, GPER knockout mice show impaired estrogen-mediated protection against an experimental autoimmune encephalomyelitis model (Wang et al., 2009), whereas G1 has a beneficial role in multiple sclerosis.

Therefore, taken together our results show that estrogens can modulate lymphocyte populations and clearly induce antibodies production in vertebrates through GPER signalling pathway.

Chapter 4

G protein-coupled estrogen receptor 1 regulates human neutrophil functions

The role of estrogens in immune functioning is relatively well known under both physiological and pathological conditions. Neutrophils are the most abundant circulating leukocytes in humans, and their abundance and function are regulated by estrogens since they express ERs. Traditionally, estrogens were thought to act via classical nuclear ERs, namely ERα and ERβ. However, it was observed that some estrogens induced biological effects only minutes after their application. This rapid, "non-genomic" effect of estrogens is mediated by a membrane anchored receptor called GPER1. Nevertheless, the expression and role of GPER1 in the immune system has not been exhaustively studied and its relevance in neutrophil functions remains unknown. In the present study, we show that human neutrophils express a functional GPER1 which regulates their functions through AMP/PKA/CREB, p38 MAPK and ERK signaling pathways. Thus, GPER1 activation in vitro increases respiratory burst of neutrophils, extends their life span and drastically alters their gene expression profile. Collectively, our results demonstrate that GPER1 activation promotes the polarization of human neutrophils towards a pro-inflammatory phenotype and point to GPER1 as a potential therapeutic target in immune diseases where neutrophils play a key role.

Key words: GPER1, neutrophils, human.

1. INTRODUCTION

Estrogens play an important role in many areas of human physiology, including reproduction, immunity, and vascular and nervous system biology, as well as in diseases, such as cancer, depression and reproductive disorders (Jia et al., 2015). Moreover, differences between the sexes have been demonstrated in the number, differentiation state and function of immune cells (Hughes and Choubey, 2014; Klein et al., 2015), and in the incidence of autoimmune and chronic inflammatory diseases, to which women are more susceptible (Gilliver, 2010; Gonzalez et al., 2010). This sex difference has been partially attributed to sex steroids, and estrogens are considered key modulators of the immune system (Straub, 2007). The biological effects of estrogens are classically mediated by the ERs, ERα and ERβ, which function as hormone-inducible transcription factors, binding to the ERE located within the promoter region of target genes (Edwards, 2005). Although estrogens mainly act by this classic genomic mechanism, it has relatively recently been confirmed that they are also able to rapidly activate transduction pathways via non-genomic mechanisms. GPER1, previously known as GPR30, an orphan receptor designation, was identified in the early 2000s by independent laboratories (Filardo et al., 2002; Revankar et al., 2005; Thomas et al., 2005). Thus, GPER1 was identified as the receptor mediating the non-genomic effects of estrogens through intracellular calcium mobilization and the synthesis of phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Revankar et al., 2005). Moreover, the GPER1-signaling mechanisms include the rapid activation of MAPKs, ERK-1, ERK-2 and PI3K, and increased cytosolic cAMP and calcium (Filardo et al., 2002; Prossnitz and Barton, 2009; Prossnitz and Maggiolini, 2009).

As GPER1 is known to bind many of the same ligands as classical ERs, the identification of a nonsteroidal, high-affinity, highly selective agonist of GPER1, G1 (Bologa et al., 2006), which can selectively bind GPER1 in the same cell where ERs are present, has enabled the role of GPER1 in human physiology to be defined and opened the door to the generation of diagnostic and therapeutic strategies directed at individual ERs (Prossnitz and Barton, 2014). In addition, GPER1 knockout mice models reveal no reproductive deficits but multiple physiological alterations and a lack of estrogen-mediated effects in numerous tissues, including those of the immune system (Prossnitz and Hathaway, 2015). In addition, GPER1 activation has been shown to mediate anti-inflammatory protective effects in rodent models of multiple sclerosis (Blasko et al., 2009; Hirahara et al., 2013; Yates et al., 2010) and ischemia-reperfusion injury (Bopassa et al., 2009; Deschamps and Murphy, 2010; Murata et al., 2013). These findings suggest that GPER1 could be a potential target for new therapies against a range of inflammatory or autoimmune diseases which display gender dimorphism as a result of the regulation of physiological and immunological processes by sex steroids (Klein et al., 2015). Despite all this evidence

pointing to the crucial role of GPER signaling in the regulation of the inflammatory response, the impact of GPER1 signaling in innate immune cells is largely unknown. We have previously shown that estrogens are able to modulate granulocyte functions in teleost fish through a GPER1/cAMP/protein kinase A (PKA)/cAMP response element-binding protein (CREB) signaling pathway (Cabas et al., 2013a). Whether this signaling pathway is evolutionary conserved is unknown. Although human neutrophils have been shown to express ERα and ERβ (Stygar et al., 2006), the direct impact of estrogens on neutrophil functions is largely unknown. Earlier studies showed that neither physiologic nor pharmacologic estrogen concentrations of estradiol affect human neutrophil apoptosis (Ottonello et al., 2002). However, there are some observations showing that estrogens modulate neutrophil function. Thus, it has been described that estrogens are able to reduce superoxide anion release by human neutrophils (Bekesi et al., 2000; Bekesi et al., 2007). More recently, it has been reported that terminally differentiated neutrophil-like HL-60 cells express functional ERα, ERβ and GPER1 (Blesson and Sahlin, 2012). Therefore, in this study we sought to address the question of whether human neutrophils express a functional GPER1, using its specific agonist G1. It was found that GPER1 is constitutively expressed by human neutrophils and that its engagement by G1 activates a cAMP/PKA/CREB signaling pathway that results in increased respiratory burst, extended life span and the induction of genes encoding pro-inflammatory mediators.

2. MATERIALS AND METHODS

2.1 Ethics statement

Human samples were collected after obtaining informed written consent by healthy donors. All experimental protocols were approved by the Ethic Committee of the Azienda Ospedaliera Universitaria Integrata di Verona (Italy). The methods were carried out in accordance with the approved guidelines.

3.2 Cell purification and culture

Neutrophils were isolated under endotoxin-free conditions from buffy coats of healthy donors to reach 99.7% (Cassatella et al., 1993; Zimmermann et al., 2015). Eosinophils were isolated from the granulocyte fraction by the Eosinophil Isolation Kit (Miltenyi Biotec). Human monocytes were isolated from PBMCs, after Ficoll-Paque gradient centrifugation of buffy coats, by anti-CD14 microbeads (Miltenyi Biotec). Dendritic cells were obtained from monocytes by a 5-day culture with 20 ng/ml interleukin-4 (IL-4) and 20 ng/ml granulocyte-macrophage colony-stimulating factor (G-CSF) (R&D Systems) (Sozzani et al., 1998). Immediately after purification,

cells were suspended in RPMI-1640 culture medium supplemented with 10% low-endotoxin fetal bovine serum (<0.5 EU/ml; from BioWhittaker-Lonza), treated or not with several stimuli, including 10-100 µM G1 (Tocris, 50 mM stock in DMSO), 1 µg/ml ultrapure *Escherichia coli* lipopolysaccharide (LPS) (0111:B4; Alexis), 10 ng/ml tumor necrosis factor α (TNFα) (Peprotech), 1000U/ml G-CSF (Myelostim, Italfarmaco Spa), 100U/ml interferon γ (IFNγ) (R&D Systems), and 200U/ml IL-10 (R&D Systems), and then plated on 12/24-well tissue culture plates (Greiner Bio-One) to be cultured at 37 ° C in a 5% CO_2 atmosphere. Control cells were incubated in the presence of 0.2 % dimethyl sulfoxide to achieve the same concentration of dimethyl sulfoxide as 100 µM G1-treated cells. After the desired incubation period, cells were collected and centrifuged at 300 *g* for 5 min. The resulting supernatants were immediately frozen and stored at -20 ° C, while the corresponding pellets were extracted for total RNA or lysed for protein analysis, as described below.

2.2 Reverse transcription quantitative real-time PCR (RT-qPCR)

Briefly, total RNA was extracted from 10^7 neutrophils using the RNeasy Mini Kit, according to the manufacturer's protocol (Qiagen) and reverse transcribed using SuperScript III (Life Technologies). Reverse transcription quantitative real-time (RT-qPCR) was performed in triplicate from 5 ng cDNA for each sample, using the Fast SYBR Green Master Mix (Life Technologies) and ViiA™ 7 real-time PCR system (Life Technologies). RT-qPCR analyses were performed using gene-specific primer pairs from Life Technologies (Table 1). The reaction conditions, identical for all primer sets, were as follows: 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Data were calculated using qGENE (Ramakers et al., 2003) and linRegPCR 7.0 (Simon, 2003) software (http://www.gene-quantification.de/download.html) and then expressed as relative mRNA levels ± SEM, after normalization with the control ribosomal protein L32 mRNA expression levels.

2.3 Flow cytometry analysis

Neutrophils (10^5) were suspended in 50 ml PBS containing 10% complement-inactivated human serum (for Fc receptor blocking). Cells were then stained for 15 min at room temperature with 1:25 FITC antihuman CD66b (clone G10F5), 1:25 PE antihuman CD11b, 1:50 PerCP-Cy5.5 antihuman CD16 (clone 3G8), 1:25 APC-Cy7 anti-human CD62L (clone 145/15) antibodies (all from Miltenyi Biotec). GPER1 expression was analyzed using 0, 0.2, 0.4, 0.8 and 2 µg/ml (0, 1:1000, 1:500, 1:250 and 1:100, respectively) of an affinity-purified rabbit polyclonal anti-GPER1 (sc-48525-R, Santa Cruz Biotechnology) for 30 min at 4 °C followed by a 1:500 dilution of a PE-conjugated goat anti-rabbit IgG (H+L, Life Technologies) for 30 min at 4 °C. For competition studies, a 10-fold molar excess of a commercial blocking peptide (sc-

48515 P, Santa Cruz Biotechnology) was used. Data analysis was performed using FlowJo software Version 8.8.6 from Tree Star (Ashland, OR, USA). Phenotypic cell analysis was performed in live cells, identified as singlet Vybrant DyeCycle Violet-negative cells (Life Technologies).

2.4 Western blotting

Whole-cell (Zimmermann et al., 2015) and cytosol and light membrane (secretory vesicles plus plasma membrane) fraction (Dusi et al., 1996) proteins were obtained, solubilized in Laemmli sample buffer and then subjected to immunoblot by standard procedures using the following antibodies: 1:1,000 anti-phospho-CREB (#9198), 1:1,000 anti-phospho-p38 MAPK (#9211) and 1:1,000 anti phospho-ERK (#9106) from Cell Signaling; 1:200 anti-GPER1 (sc-48525-R), 1:1,000 anti-PKA (sc-903) and , 1:1,000 anti-IκBα (sc-371), from Santa Cruz Biotechnology; 1: 2,000 mAb anti-β-tubulin (#T5293) from Sigma-Aldrich; and anti-GP91-phox from Abcam (#ab139371). Blotted proteins were detected and quantified using the Odyssey infrared imaging system (LI-COR Biosciences).

2.5 Detection of cytokine release

Cytokine concentrations in cell-free supernatants were measured by specific human ELISA kits for C-X-C motif chemokine ligand 8 (CXCL8) (Immunotools) and IL-1 receptor antagonist (IL-1RA) (R&D Systems), according to the manufacturers' instructions. Detection limits of these ELISAs were: 40 pg/ml for IL-1RA and 8 pg/ml for CXCL8.

2.6 Respiratory burst

Superoxide anion (O_2^-) release was estimated by the cytochrome C reduction assay, as previously described (Costantini et al., 2010).

2.7 Statistical analysis

Data are expressed as means ± SEM, unless otherwise indicated. Statistical evaluation was performed using 1-way ANOVA followed by Tukey's post hoc test. p values < 0.05 were considered statistically significant.

3. RESULTS

3.1 *Human neutrophils express GPER1*

Using Western blot, it was found that several human immune cell types, including monocytes, neutrophils, eosinophils and dendritic cells, all robustly expressed GPER1 protein (Fig. 1a).

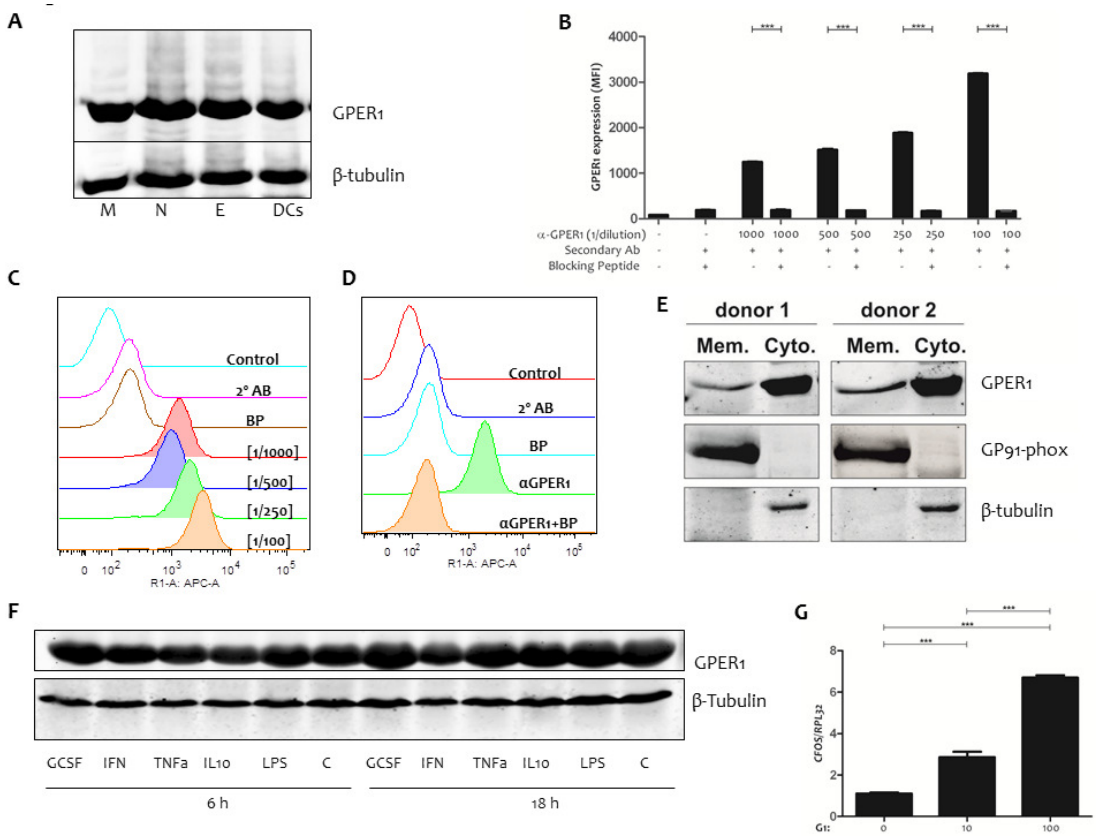

Figure 1. GPER1 protein levels on human neutrophils and other human immune cells. (A) Western blot analysis of GPER1 in monocytes (M), neutrophils (N), eosinophils (E) and dendritic cells (DCs). (B) Mean fluorescence intensity (MFI) of the GPER1 positive cells measured by flow cytometry in human neutrophils (>99 % purity) unstained (control, C), staining with secondary antibody (AB) and blocking peptide (BP) (as additional control), or with 1:1000, 1:500, 1:250 and 1:100 dilution of αGPER1 AB, preincubated or not with the BP. Values represent means ± SEM in two donors. The asterisks denote statistically significant differences among groups according to one-way ANOVA and Tukey post hoc test. *** $p < 0.001$. (C-D) Representative fluorescence histograms of these same stainings. (E) Western blot analysis of GPER1 in cytosol and light membrane (secretory vesicles plus plasma membrane) fractions. β-tubulin and GP91-phox were used as markers of the cytosol and light membrane fractions, respectively. The results of neutrophil fractions obtained from two donors are shown. (F) Western blot analysis of neutrophils treated with G-CSF, IFNγ, TNFα , IL-10 or LPS for 6 and 18h. β-tubulin was used as loading control. The results are representative of three different experiments. (G) Neutrophils were stimulated with 0 (control), 10 or 100 μM G1 for 1h. Afterward, the mRNA levels of *CFOS* were determined by real-time RT-PCR. Gene expression is normalized against *RPL32* mRNA levels. Each bar represents the mean ± SEM of three donors.

The expression of GPER1 by neutrophils was further confirmed by flow cytometry, by means of which neutrophils were found to be immunostained with GPER1 Ab in a dose-dependent manner, while staining was fully blocked by pre-adsorption of the GPER1 Ab with a specific blocking peptide (Figs. 1b-d). Notably, neutrophils expressed GPER1 in the plasma membrane, since the immunostaining was performed with live neutrophils. This result was further confirmed by subcellular fractionation studies, in which GPER1 was found to be present in the light membrane fraction (Fig. 1e). In addition, stimulation of neutrophils for 6 and 24 h with G-CSF, IFNγ, TNFα, IL10 or LPS failed to alter GPER1 protein levels, as assayed by western blot (Fig. 1f). We next investigated whether GPER1 was functional in human neutrophils using its specific agonist G1. Stimulation of neutrophils with 10 and 100 μM G1 resulted in a dose-dependent increased transcript level of *CFOS*, a marker of GPER1 activation (Albanito et al., 2007), in these cells (Fig. 1g). These G1 concentrations were selected for further experiments.

3.2 GPER1 regulates human neutrophil activation and life span

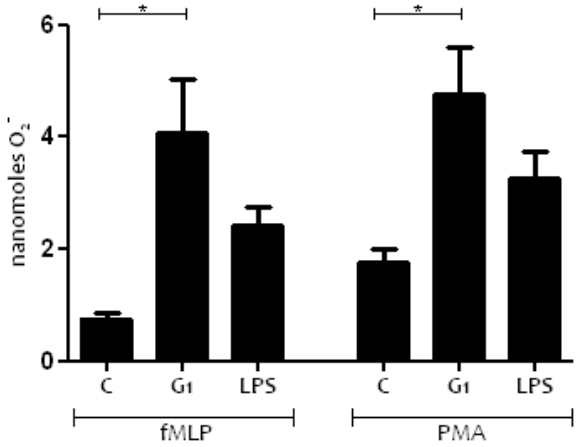

Figure 2. GPER1 signaling modulates the respiratory burst of neutrophils. Neutrophils were incubated with 0 (control), 100 μM G1 or LPS for 2h. Afterward, the superoxide anion (O_2^-) release (nmol) by these cells triggered by fMLP or by PMA was measured using a cytochrome C reduction assay. Values represent means ± SEM in triplicate and are representative of multiple independent experiments. The asterisks denote statistically significant differences among groups according to one-way ANOVA and Tukey post hoc test. * $p < 0.05$. No symbol means not significant.

G1 was able to significantly prime the production of O_2^- triggered by N-formylmethionine-leucyl-phenylalanine (fMLP) or phorbol myristate acetate (PMA) in human neutrophils, and even more sharply than LPS (Fig. 2). In addition, the transcript levels of *IL1B*, *CXCL8*, *PTGS2* (*COX2*), *SOCS3, GCSF,* and to some extent of *IL1RA* also increased upon 10 and 100 μM G1 stimulation, while those of *TNFA* were unaffected (Fig. 3a and data not shown). Consistent with these results, GPER1 engagement with 10 and 100 μM G1 increased in a dose-dependent manner the

release of pro-inflammatory CXCL8 by human neutrophils (Fig. 3b), but had no effect on the release of anti-inflammatory IL-1RA (Fig. 3c).

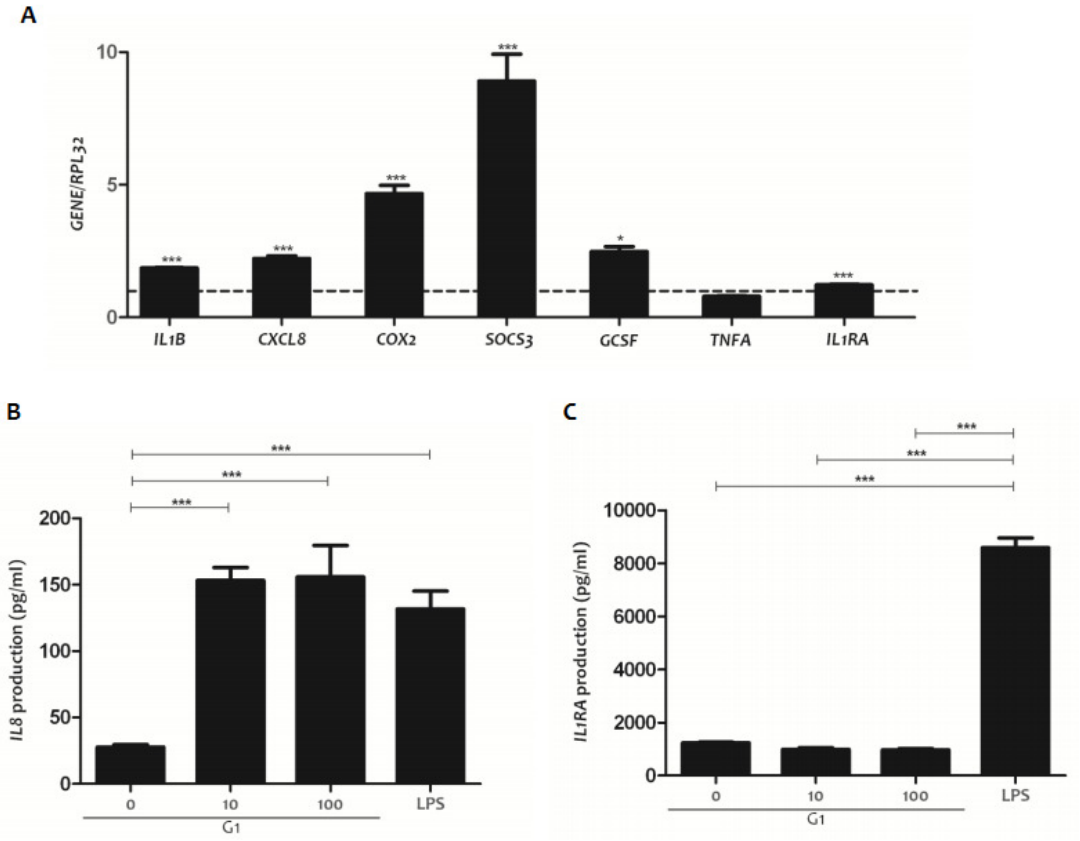

Figure 3. GPER1 activation regulates the gene expression profile of human neutrophils. (A) Neutrophils were stimulated with 0 (control) and 100 µM G1 for 1h. Afterward, the mRNA levels of *CFOS* , *IL1B*, *CXCL8*, *COX2*, *SOCS3* and *GCSF*, and *TNFA* and *IL1RA* were determined by real-time RT-PCR. Gene expression is normalized against *RPL32* mRNA levels and then against non-stimulated cells. Each bar represents the mean ± SEM of three donors. (B, C) Neutrophils were stimulated with 0 (control), 10 or 100 µM G1 or LPS for 16 h and then CXCL8 (B) and IL-1RA (C) concentrations were measured by ELISA. The data represent the mean ± SEM of three donors. The asterisks denote statistically significant differences compared with control group according to one-way ANOVA and Tukey post hoc test. **p* <0.05, ** *p* < 0.01, *** *p* < 0.001. No symbol means not significant.

The above results prompted us to analyze the effect of GPER1 activation on neutrophil viability and the surface expression of CD11b and CD62L, two widely used activation markers (García-Vicuna et al., 1997). Both LPS and G1 treatments were equally efficient in increasing neutrophil viability (Fig. 4a). In addition, a significant upregulation of total CD11b surface expression (Fig. 4b) and CD62L shedding (Fig. 4c) was observed in neutrophils stimulated with either LPS or G1. Collectively, these results suggest that GPER1 engagement promotes human neutrophil activation, which is characterized by the usual co-upregulation of CD11b and shedding of CD62L.

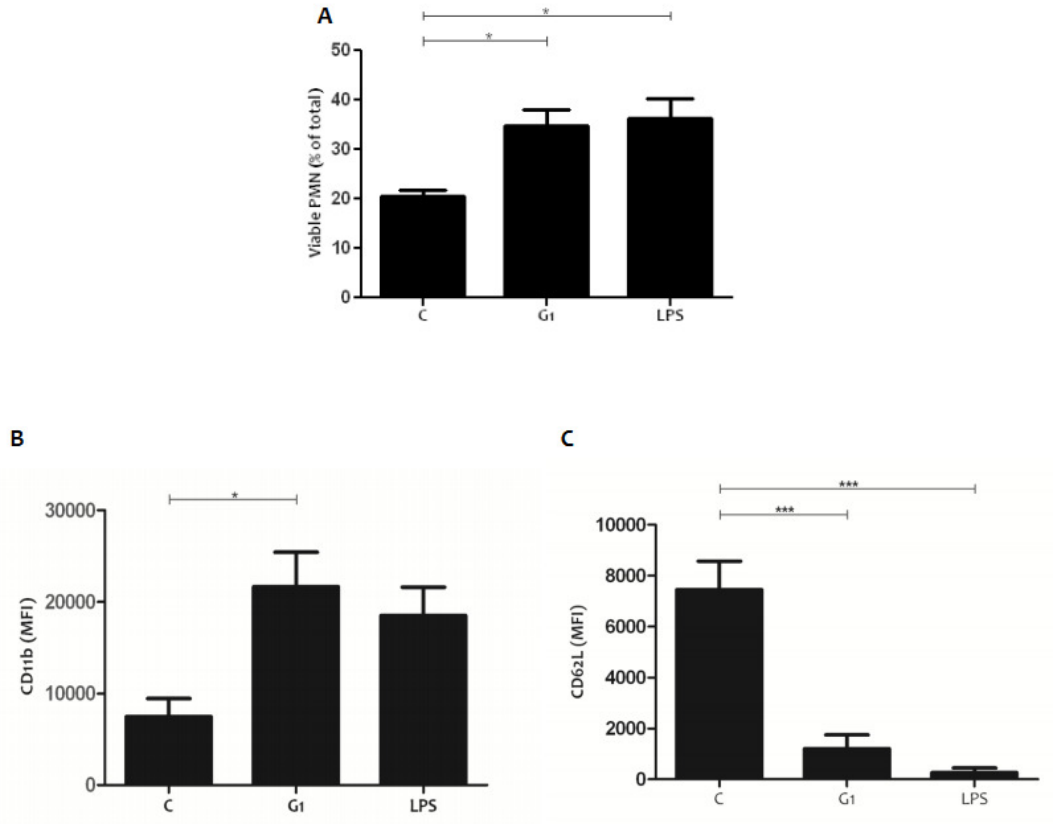

Figure 4. GPER1 regulates human neutrophil activation and life span. Neutrophils were stimulated with 0 (control), 100 μM G1 or LPS over night and afterward, the percentage of vibrant-positive neutrophils (A) and the mean fluorescence intensity (MFI) of CD11b (B) and CD62L (C) positive cells were determined by flow cytometry. The data represent the mean ± SEM of three donors. The asterisks denote statistically significant differences compared with control group according to one-way ANOVA and Tukey post hoc test. * $p < 0.05$, * $p < 0.01$, * $p < 0.001$. No symbol means not significant.

3.3 GPER1 signals via cAMP/PKA/CREB, ERK and p38 MAPK pathways in human neutrophils

As GPER1 activation promotes adenylate cyclase, p38 MAPK and ERK (Filardo et al., 2002; Prossnitz and Barton, 2009; Prossnitz and Maggiolini, 2009) activation in human cancer cells, we analyzed the activation of these signaling pathways in neutrophils stimulated with G1 for 30, 60 and 90 min or with PMA, as a positive control. CREB phosphorylation was seen to increase in neutrophils treated with G1 compared with the control at the three time points tested, although the increase was more pronounced after 60 and 90 min (Fig. 5a). After 30 min of exposure, G1 treatment weakly promoted p38 MAPK phosphorylation but did not alter PKA levels, while modestly increased ERK phosphorylation at the three time tested (Fig. 5B), and failed to promote IκBα degradation (data not shown). As expected, PMA stimulation resulted in very strong phosphorylation of CREB, ERK and p38 MAPK (Figs. 5A and 5B).

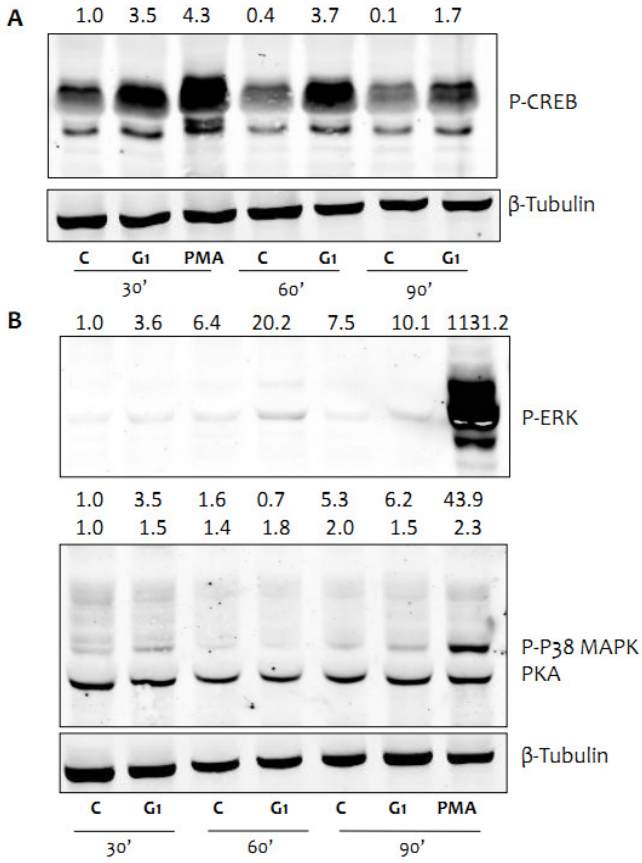

Figure 5. GPER signaling via the cAMP/PKA/CREB pathway but it also signals through p38 MAPK and ERK. Western blot analysis of P-CREB (A) and P-P38 MAPK, PKA and P-ERK (C) in neutrophils stimulated with 0 (control) or 100 µM G1 for 30, 60 or 90 minutes, or PMA as a positive control. The quantification of the band intensity is shown over each lane. The blots are representative of three independent experiments.

4. DISCUSSION

As estrogens have been shown to affect multiple cell components of the immune system, they have attracted significant interest as potential modulators of autoimmune diseases (Jia et al., 2015). Although many important estrogenic responses are mediated by the two nuclear ERs ERα and ERβ (Edwards, 2005), increasing evidence suggests that the non-genomic effects mediated by GPER1 signaling play crucial roles in mouse models of various human inflammatory disorders (Bopassa et al., 2009; Deschamps and Murphy, 2010; Hirahara et al., 2013; Murata et al., 2013; Yates et al., 2010), where the receptor mainly shows an anti-inflammatory role. However, although there have been almost 900 publications on GPER1 since its discovery in the early 2000s and its expression in immune cells has been described (Barton, 2016), the *in vivo* role of GPER1 in the regulation of neutrophil function still remains enigmatic. The present study shows that GPER1 is functionally expressed in human neutrophils, extending a previous study reporting the functionality of this receptor in terminally differentiated neutrophil-like HL-60 cells (Blesson and Sahlin, 2012). Curiously, GPER1 expression was unaffected by

neutrophil stimulation with different cytokines and LPS. Although GPER1 was originally reported to localize in the endoplasmic reticulum in different cancer cell lines (Revankar et al., 2005), we found GPER1 in the plasma membrane of human neutrophils, as has been shown for human eosinophils (Tamaki et al., 2014). Since its discovery, the localization of GPER1 has been a controversial aspect. Thus, although most studies reported an internal membrane localization, other studies showed it localized in plasma membrane (Filardo et al., 2002) and even the nucleus (Madeo and Maggiolini, 2010). Thus, the functional impact of this observation in human neutrophils is worthy of study.

GPER1 seems to be functional in human neutrophils and its engagement with G1 promotes their activation, as evidenced by: (i) the induction of *cFOS* expression, a known marker of the activation of the GPER signaling pathway (Albanito et al., 2007), (ii) the increased respiratory burst activity of the cells primed by different agents, e.g. LPS and fMLP, (iii) the increase in CD11b expression and CD62L shedding, the two classical markers associated with neutrophil activation *in vitro* (Garcia-Vicuna et al., 1997), and (iv) the induction of several genes encoding key proinflammatory mediators, such as *IL1B*, *PTGS2*, *GCSF* and *CXCL8*. However, the activation induced by GPER1 signaling is not identical to the classical activation observed with LPS, since while both G1 and LPS stimulate CXCL8 release, G1 fails to induce the release of *IL1RA*. These observations contrast with the inability of GPER1 to consistently enhance the respiratory burst of bony fish granulocytes and its anti-inflammatory effect on these cells in which the sustained induction of genes encoding Ptgs2, prostaglandin D2 synthase (PTGDS) and IL-10 was observed upon G1 stimulation (Cabas et al., 2013a). Moreover, G1 promotes a differential effect on neutrophil-respiratory burst compared to estrogens, which have been found to reduce superoxide anion release by human neutrophils (Bekesi et al., 2000; Bekesi et al., 2007). However, it has enhancing effect on ROS production by human neutrophils similar to that seen for para-nonyphenol, also described as a GPER1 agonist (Prossnitz and Barton, 2014). Taken together, these results suggest certain differences in the effect of GPER1 signaling in neutrophils across different vertebrate groups.

Another interesting observation made possible by the present study is that the stimulation of human neutrophils with the GPER1 specific agonist G1 resulted in an increased neutrophil life span, since estrogens delay human neutrophil apoptosis (Molloy et al., 2003). However, GPER stimulation has been found to inhibit spontaneous human eosinophil apoptosis through the inhibition of caspase-3, while promoting caspase-3-dependent apoptosis in IL-5-stimulated eosinophils (Tamaki et al., 2014). Therefore, the regulation of human granulocyte life span by GPER1 seems to be complex and requires further research.

The signaling pathways downstream of GPER1 have been found to be very complex in human cancer cell lines and although at least it is known that they involve adenylate cyclase/cAMP/PKA/CREB, p38 MAPK, and ERK (Albanito et al., 2007; Filardo et al., 2002; Prossnitz and Barton, 2009; Prossnitz and Maggiolini, 2009), no studies have so far examined GPER1 signaling in mammalian immune cells. We have previously reported that GPER1 signals via an adenylate cyclase/cAMP/PKA/CREB in bony fish granulocytes (Cabas et al., 2013a). Similarly, it was found here that GPER1 not only uses a similar signaling pathway in human neutrophils but also signals through p38 MAPK and ERK, as has been demonstrated in cancer cell lines (Albanito et al., 2007; Filardo et al., 2002; Prossnitz and Barton, 2009; Prossnitz and Maggiolini, 2009). However, GPER1 failed to signal via NF-κB, in contrast to ER signaling which is known to inhibit NF-κB pathway by different mechanisms, including enhancing IKBα levels (Xing et al., 2012).

Therefore, our results point to the relevance of the estrogen-mediated effects through GPER1 in human neutrophils in both physiologic and pathologic conditions. Furthermore, neutrophil regulation by GPER1 signaling must be considered in the context of endocrine disruption, since xeno-estrogens, synthetic or natural substances of high stability that exert toxicity by mimicking the effects of estrogens, such as bisphenol A, some pesticides and ethynylestradiol, are involved in autoimmunity (Chighizola and Meroni, 2012) and have been found to activate GPER1 (Prossnitz and Hathaway, 2015).

In summary, our results demonstrate that human neutrophils constitutively express a plasma membrane-anchored GPER1, which regulates their life span and promotes their activation. These results suggest a crucial role for GPER1 signaling in autoimmune and chronic inflammatory diseases in which estrogens and neutrophils are involved. They also point to GPER1 as a promising therapeutic target.

Conclusions

I. The dietary intake of EE_2 and Tmx could be considered as an endocrine disruptor mechanism in giltead seabream as they increase the expression levels of the gene encoding for hepatic vitellogenin. This increase is independent on the developmental stage of the specimens but is higher in the case of EE_2. However, the dietary intake of G1 does not modify *vtg* expression levels.

II. The endocrine disrupting effect of EE_2 and Tmx dissapears when the treatment ceases.

III. Dietary administration of EE_2, Tmx or G1 at the doses tested does not result in immunosuppression in gilthead seabream.

IV. EE_2, Tmx or G1 decrease the ability of specimens to respond to an immune stimulus *in vivo* by inhibiting the production of the gene that encodes the pro-inflammatory cytokine IL-1β. Moreover, they barely affect ROS production by phagocytes.

V. The effect of EE_2, Tmx and G1 on the humoral adaptive immune response differs between adult and juvenile gilthead seabream.

VI. EE_2 and Tmx increase antibody production in vaccinated fish during dietary treatment. However, only Tmx increases antibody production and the numbers of IgM^+ B lymphocytes during the recovery phase.

VII. Dietary intake of EE_2 increases the abundance and proliferation of T and IgM^+ B lymphocytes during a primary immune response. In addition, it also increases the production of antibodies via the GPER1 signaling pathway.

VIII. Human neutrophils express a functional GPER1 which regulates their functions through cAMP/PKA/CREB, p38 MAPK and ERK signaling pathways.

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Resumen en español

Abreviaturas

ADN	Ácido desoxirribonucleico
AMPc	Adenosín monofosfato cíclico
ARNm	ARN mensajero
cFOS	Proto-oncogene *cfos*
Cox2	Ciclooxigenasa 2
CREB	Potreína de unión al elemento de respuesta a AMPc
CXCL8	Interleuquina 8
DEs	Disruptores endocrinos
Dot	Días de tratamiento
Dpb	Días post-booster
Dpp	Días post-priming
Dpt	Días post-tratamiento
E_2	17β-estradiol
EE_2	17α-etinilestradiol
ERK	Quinasas reguladoras de señales extracelulares
FAO	Organización de Naciones Unidas para la Agricultura y la Alimentación
G-CSF	Factor estimulante de colonias de granulocitos
GPCR	Receptores asociados a proteína G
G1	Agonista específico de GPER
GAs	Granulocitos acidófilos
GPER1	Receptor de estrógenos asociado a proteína G 1
IFN	Interferon
Ig	Inmunoglobulina

IGS	Indice gonadosomático
IHS	Indice hepatosomático
IL	Interleuquina
IL1RA	Antagonista del receptor de la Interleuquina
IPCS	Programa Internacional de Seguridad de las Substancias Químicas
fMPL	N-Formilmeteonina-leucil-fenilalanina
ISS	Indice esplenosomático
LPS	Lipopolisacárido
Ly	Linfocitos
MAPK	Proteína quinasa activada por mitógenos
MSRE	Modulador selectivo de los receptores de estrógenos
PBS	Solución fosfato salina
ppt	Partes por trillón
PTGDS	prostaglandina D2 sintetasa
RC	Riñón cefálico
REs	Receptores de estrógenos
ROIs	Intermediarios de oxígeno reactivos
Tmx	Tamoxifeno
TNF	Factor de Necrosis Tumoral
TLR	Receptores similares a Toll
Vtg	Vitelogenina

Resumen

En el transcurso de esta Tesis Doctoral se ha explorado el efecto del 17α-etinilestradiol (EE_2) y del tamoxifeno (Tmx) sobre el sistema inmunitario de la dorada (*Sparus aurata* L.) durante y después de la exposición a estos compuestos. El primero es un estrógeno sintético siendo el principal ingrediente activo de la píldora anticonceptiva, mientras que el Tmx es un modulador selectivo de los receptores de estrógenos con efecto anti-proliferativo en el tejido mamario. Debido al amplio uso y a la incompleta degración, son contaminantes habituales de los medios acuáticos. La dorada es un pez teleósteo marino y una de las especies por excelencia de la acuicultura marina, con un gran valor en la acuicultura mediterránea. Además se ha determinado el papel del receptor de estrógenos asociado a proteína G, GPER1 en la biología de neutrófilos humanos.

En primer lugar, hemos evaluado el efecto del EE_2 y del Tmx administrados mediante dieta en determinados marcadores de disrupción estrogénica en doradas adultas. Hemos confirmado que el EE_2 provoca una respuesta estrogénica y que el Tmx también altera estos marcadores, aunque el efecto fue menos pronunciado. También hemos investigado la capacidad de ambos compuestos de modular la respuesta inmunitaria inducida tras una vacunación así como su capacidad de recuperación tras el cese de la exposición. Los resultados indican que el EE_2 y el Tmx inhiben de manera transitoria la inducción de la expresión del gen inteleuquina 1β (IL)-1β e incrementan la producción de intermediarios de oxígeno reactivos (ROIs) en leucocitos de riñón cefálico (RC) de peces vacunados. Por otro lado, el EE_2 y el Tmx estimulan la producción de anticuerpos en peces vacunados aunque sólo en los expuestos a Tmx se produjo una alteración del porcentaje de linfocitos (Ly) B inmunoglubulina M positivos (IgM^+) en peces durante el periodo de recuperación.

Tras observar que el EE_2 y el Tmx alteran la respuesta inmunitaria de ejemplares adultos, nos planteamos determinar si estas consecuencias se producen también en juveniles. Además introdujimos el compuesto G1, agonista específico de GPER ya que también altera la respuesta inmunitaria de adultos de dorada. El EE_2 y el Tmx, pero no el G1, promueven una respuesta diferente y transitoria en la expresión del gen hepático de la *vtg*. Aunque estos tres compuestos no afectan la producción de ROIs, provocan la inhibición de la inducción de la expresión del gen *il1b*, al igual que ocurría en adultos. Destacamos, que a pesar de que el Tmx incrementa el porcentaje de Ly B-IgM^+ RC y en bazo durante el periodo de recuperación, la producción de anticuerpos en peces vacunados varía dependiendo del compuesto utilizado y de cuando se produjo la vacunación.

Los resultados previos nos llevaron a investigar la influencia del EE_2 en la respuesta inmunitaria centrándonos en los Ly y en la actividad humoral tras el cese de la exposición. En concordancia con lo anteriormente observado, el EE_2 incrementa la expresión del gen hepático de la *vtg*. De manera significativa, afecta al porcentaje y a la capacidad de proliferación de los Ly T y B-IgM^+. Además, describimos por primera vez que el EE_2 induce la producción de anticuerpos y que este efecto está mediado a través de la señalización por GPER1.

Finalmente, dado que los estrógenos son capaces de modular funciones de granulocitos en dorada a través de la señalización por GPER, hemos realizado la caracterización funcional de GPER1 en neutrófilos humanos. Estas células expresan GPER1 de manera funcional en la membrana plasmática, ya que tras la activación *in vitro* con el agonista específico G1, produjo el incremento de los niveles de transcripción de CFOS, marcador de activación de GPER1. Además incrementa la producción ROIs y altera de manera drástica el perfil de expresión de genes, además de incrementar la viabilidad de neutrófilos humanos a través de diferentes mecanismos de señalización. En definitiva, nuestros resultados muestran por primera vez que la activación de GPER1 produce la polarización de neutrófilos humanos hacia un fenotipo pro-inflamatorio y pone de manifiento la potencia de GPER1 como diana terapéutica en enfermedades inmunitarias en los que los neutrófilos juegan un papel fundamental.

1. INTRODUCCIÓN

Actualmente, la población mundial se enfrenta a un gran número de retos que van de los impactos derivados de la crisis económica y financiera hasta los fuertes cambios que se están produciendo en el clima. Al mismo tiempo, hay que compaginar las necesidades nutricionales de una población que está creciendo muy activamente, teniendo en cuenta que los recursos naturales son limitados (FAO, 2016). La pesca es un recurso finito que en las últimas décadas ha sido sometido a tales niveles de explotación que en muchos casos ha puesto en peligro su sostenibilidad. El desarrollo de la acuicultura desde inicios de la década de los 80 ha permitido tener una fuente alternativa de productos pesqueros. En este contexto, la pesca y la acuicultura contribuyen de manera crucial al bienestar general y a su prosperidad ya que el sector produce sustento e ingresos, de manera directa e indirecta, a una gran parte de la población mundial. La acuicultura se perfila como uno de los sectores proveedores de producto animal con mayor crecimiento y, en las próximas décadas, la producción total de peces capturados más los producidos en acuicultura superará a la producción de vaca, cerdo y ave de corral. El control de la reproducción de los peces y su alimentación ha experimentado un crecimiento que permite proveer de manera continua y estable las necesidades de pescado que la población demanda (FAO, 2016).

Los peces poseen un sistema inmunitario tanto innato como adaptativo. Sin embargo, el sistema inmunitario innato está más desarrollado. Se cree que el sistema inmunitario adaptativo apareció en una etapa muy temprana en la evolución de los vertebrados, hace 450 millones de años, con los primeros vertebrados con mandíbulas (Gnathostomata). La posición filogenética basal que ocupan los peces en la filogenia de los vertebrados lo hace un grupo muy atractivo para los estudios de inmunidad comparada. Tenemos que considerar que la mayoría del conocimiento del sitema inmunitario y de los patógenos de peces proceden de especies de acuicultura (Rauta y col., 2012).

Nuestro modelo de estudio, la dorada (*Sparus aurata* L.), es un pez teleósteo marino hermafrodita, protándrico, y de puesta estacional. Tiene un gran valor comercial en la región mediterránea ya que es una de las principales especies de peces cultivada. Además, es una de las especies que más ha contribuido al conocimiento de la respuesta inmunitaria y a la eliminación de enfermedades. El RC, su principal órgano hematopoyético, está compuesto por granulocitos acidófilos (GAs), Ly, macrófagos y células precursoras, siendo los Ly los responsables de la respuesta inmunitaria adaptativa producida por anticuerpos.

La investigación del sistema inmunitario en vertebrados inferiores es indispensable para entender su evolución siendo los peces un excelente modelo para el estudio de la inmunología comparada. Por lo tanto, los peces, en general, y los teleósteos, en particular, son utilizados como modelos de investigación para estudiar alteraciones genéticas (Ota y Kawahara, 2014), enfermedades cerebrales (Stewart y col., 2012; Kalueff y col., 2014) o toxicolçogicas (Hill y col., 2005) e inmunológicas (Goody y col., 2014), entre otras (Lieschke y Currie, 2007; Löhr y Hammerschmidt, 2011). Los peces teleósteos comparten diferentes aspectos del desarrollo con los mamíferos y, también, comparten algunas de las características del sistema endocrino, incluyendo las hormonas, los receptores y las cascadas de señalización (Löhr y Hammerschmidt, 2011).

Los órganos linfoides presentes en mamíferos también lo están en peces, excepto los ganglios linfáticos y la médula ósea (Press y evensen, 1999). Por lo tanto, el riñón (anterior o cefálico, RC), el timo y el bazo son los principales órganos linfoides en teleósteos (Zapara y col., 2006), siendo el RC el equivalente a la médula ósea en vertebrados y el principal órgano hematopoiético hasta la etapa adulta (Zapata y col., 2006).

La inmunidad innata es un mecanismo de defensa esencial en peces. Está formada por barreras epitelial o mucosa y por la respuesta humoral y celular. Además juega un papel esencial en la respuesta adaptativa y en la homeostasis (Uribe y col., 2011).

La respuesta inmunitaria celular de los peces incluye una gran variedad de leucocitos, que incluye fagocitos (monocitos/macrófagos y granulocitos) así como células citotóxicas no específicas (Secombes, 1996). Los fagocitos son los más importantes en la respuesta inmunitaria innata por su capacidad para eliminar virus, bacterias y parásitos (Rowley y col., 1988; Secombes y Fletcher, 1992; Sepulcre y col., 2002) y, además, pueden iniciar la activación y regulación de la respuesta inmunitaria específica (Clem y col., 1985, 1991; Vallejo y col., 1992). Las células principales involucradas en la fagocitosis de peces son los neutrófilos y los macrófagos (Secombes y Fletcher, 1992). Eliminan bacterias principalmente a través de la producción de ROIs durante la explosión respiratoria. Por otro lado, en la dorada, los GAs del RC son las células fagocíticas más activas y abundantes en esta especie (Sepulcre y col., 2002; Chaves-Pozo y col., 2004a). Además, están consideradas funcionalmente equivalentes a los neutrófilos en mamíferos, ya que son los granulocitos circulantes más abundantes (Sepulcre y col., 2002), con gran capacidad fagocítica y con capacidad para producir ROIs (Sepulcre y col., 2002, 2007), producen citoquinas en respuesta a numerosos estímulos inmunológicos (Chaves-Pozo y col., 2004a; Sepulcre y col., 2007) y expresan varios receptores similares a Toll (TLR), con la excepción de TLR3 (Sepulcre y col., 2007).

La inmunidad adaptativa está mediada por Ly y anticuerpos. Estos últimos neutralizan virus y facilitan la fagocitosis de patógenos mediante opsonización y la activación clásica de la vía del complemento (Sakai, 1984). Centrándonos en los anticuerpos o Ig, hay que destacar que son factores esenciales en la respuesta inmunitaria adaptativa producidos por los Ly B. Durante mucho tiempo se pensó que en peces sólo existía la IgM. Sin embargo, en estudios recientes se han identificado en peces teleósteos otras Igs como la IgD, la IgZ (Danilova y col., 2005), y la IgT en trucha (*Oncorhynchus mykiss*) y pez globo (*Tetraodon biocellatus*) (Hansen y col., 2005; Savan y col., 2005b), similar a la IgZ en pez cebra. Esta Ig está presente en las mucosas con funciones anti-patogénicas sólo en el intestino, similar a la IgA en animales de sangre caliente y a la IgX en anfibios (Zhang y col., 2010; Zhu, 2012).

Tanto los macrófagos como los Ly expresan el receptor de estrógenos (RE)α, uno de los tipos de REs nucleares, así como el receptor de estrógenos asociado a proteína G (GPER1) mientras que los GAs, el equivalente a neutrófilos humanos, expresan sólo GPER1 (Liarte y col., 2011a; Cabas y col., 2013, 2015). La expresión de estos REs, hace vulnerables a la modulación por estrógenos, tanto naturales como sintéticos, a las células del sistema inmunitario.

La superfamilia de los REs nucleares se caracteriza por la gran diversidad funcional de sus miembros a pesar de su gran similitud estructural. Hay una gran variedad de ligandos que se unen de manera específica a ellos y, de esta manera, participan en la regulación de una gran variedad de actividades, como el crecimiento, el desarrollo y la homeostasis, entre otras. Los complejos ligando-receptor reconocen promotores de transcripción en las secuencias de ADN, elementos de respuesta a estrógenos, resultando en la inducción o represión de la actividad de los genes. La similitud entre estos elementos de respuesta a estrógenos así como entre los receptores indican una estrategia general conservada en el control hormonal de la transcripción por esteroides. (Wahli y Martínez, 1991).

Hasta la fecha, se considera que el ancestro común para vertebrados mandibulados (Gnathostomata) tiene dos formas distintas de RE nucleares, REα y REβ (Thornton, 2001). Estas dos formas de REs se han encontrado en mamíferos, peces, aves, reptiles y anfibios (Katsu y col., 2013). Para contextualizar, en peces, en 1989, se describió la secuencia completa para un RE nuclear en trucha (*Onchorhyncus mykiss*) (Pakdel y col., 1989). Los efectos del estrógeno endógeno natural, 17β-estradiol (E_2), están regulados, al menos, por tres subtipos de REs, REα, REβ1 y REγ (Katsu y col., 2013; Nelson y Habibi, 2013). El REγ en peces parece estar relacionado con el REβ1, sugiriendo una duplicación del gen en teleósteos. Como consecuencia, el REβ y el REγ han sido nombrados como REβ1 y REβ2, respectivamente (Hawkins y col., 2000). Además, una cuarta isoforma (ERα2) ha

sido descrito en trucha (Nagler y col., 2007). En general, los REs se expresan en diferentes tipos celulares incluyendo las células inmunitarias (Phiel y col., 2005; Ascenzi y col., 2006; Pierdominici y col., 2010) y la predominancia de un subtipo sobre otro podría cambiar los efectos de los estrógenos, promoviendo o amortiguando la inflamación (Straub, 2007). Centrándonos en nuestro modelo, la dorada, el REα, el REβ1 y el REβ2 se expresan en orgános reproductores y no reproductores como hígado, corazón, ovario, piel, testículo y RC (Pinto y col., 2006). Poblaciones celulares de RC, de macrófagos y de Ly expresan constitutivamente el gen *era*. Además, los macrófagos participan en el papel inmuno modulador del E2, como sugiere su capacidad para inducir la expresión del gen ERβ2 y el incremento en la expresión de genes que codifican para el ERα, el ERβ1, citoquinas pro-inflamatorias, quimioquinas y moléculas remodeladoras. Además, los factores solubles producidos por macrófagos tratados con E2 disminuyen la habilidad y la capacidad fagocítica en RC. Sin embargo, los GAs no expresan ninguno de los tres genes de REs (ERα, ERβ1 y ERβ2) (Liarte y col., 2011a). Como previamente hemos mencionado, estas células están consideradas como neutrófilos fagocíticos con algunas similitudes morfológicas con células humanas (Weinreb, 1963).

Desde hace más de 40 años se sabe que los estrógenos, además de su acción clásica mediada a través de la activación de los REs nucleares, pueden también producir respuestas de señalización rápida no genómica mediadas por receptores asociados a membrana (Szego y Davis, 1967). Por lo tanto, otros receptores deben estar implicados en la acción del E2 ya que se han visto efectos en células que no tienen REs nucleares (Gu y col., 1999; Nadal y col., 2000; Qiu y col., 2003). A pesar de que previamente se habían descrito efectos rápidos de los estrógenos, no fue hasta 2005 cuando se identificó un receptor huérfano GPER, como un receptor de membrana intracelular para estrógenos (Revankar y col., 2005; Thomas y col., 2005). En 2013, resultados previos en nuestro laboratorio mostraron que los GAs de dorada expresan GPER tanto a nivel de ARNm como de proteína (Cabas y col., 2013). Sin embargo, no hay estudios sobre la presencia de este receptor en los neutrófilos humanos y su acción en estas células inmunitarias. Los peces teleósteos y los mamíferos, divergieron de la línea de vertebrados hace 200 millones de años. Por lo tanto, una comparación de las características funcionales de GPER1 es estas dos especies debería de revelar si la unión de los estrógenos y la transducción de señales son funciones básicas de estas proteínas en vertebrados (Thomas, 2010). Dentro del grupo de receptores asociados a proteína G (GPCRs) sólo una pequeña parte de proteínas del receptor se expresan en la membrana plasmática mientras que la mayor parte permanecen en el retículo endoplasmático (Filardo y col., 2007; Petaja-Repo y col., 2000). El agonista específico de GPER, G1, tiene gran afinidad por GPER en corvina y se ha comprobado que mimetiza la acción del E_2 en un ensayo de maduración de oocitos, indicando que G1 es una herramienta farmacológica para investigar la acción específica de los estrógenos en vertebrados. Así, hay numerosas

evidencias que muestran que la vía de señalización de estos receptores está conservada en vertebrados. Tanto en teleósteos como en humanos, GPERs activan proteínas G, resultando en un aumento en la producción de AMPc (Thomas y col., 2005; Pang y col., 2008; Cabas y col., 2013).

Es evidente que el sistema inmunitario interacciona con el resto de sistemas de nuestro organismo, siendo esta influencia claramente evidente con el sistema reproductor (Tokarz y col., 2015). La interacción del sistema reproductor con el sistema inmunitario se atribuye a los esteroides sexuales y a los receptores de hormonas que están presentes en las células inmunitarias (Khan y Ansar-Ahmed, 2016). En efecto, la regulación de la respuesta inmunitaria es diferente en hombres y mujeres debido a la presencia de diferentes hormonas. Así se ha observado una mayor prevalencia de enfermedades autoinmunes en mujeres. Debido a la presencia de receptores específicos para los esteroides sexuales en células inmunitarias, las terapias basadas en estrógenos están consideradas como prometedoras estrategias en enfermedades autoinmunes como esclerosis múltiple o diabetes autoinmune, con una mayor prevalencia en mujeres (Gourdy y col., 2016).

A pesar de los considerables esfuerzos para reducir la contaminación ambiental, estamos siendo testigos de la aparición incontrolada de nuevos compuestos en el medio acuático. Las principales fuentes de contaminación del medio acuático son municipales (generadas por hogares) y los residuos de los hospitales así como los generados por una mala utilización de medicamentos caducados o cosméticos. Además, otra fuente de contaminación son los criaderos de animales en los que se suele utilizar compuestos esteroideos (ayudan al crecimiento) y antibióticos (prevención de posibles infecciones) (Hirsch y col., 1999). La mayoría de los fármacos no son eliminados durante los procesos de tratamiento de aguas, debido a sus propiedades físico-químicas. Las sustancias activas procedentes de medicamentos pueden acumularse en los tejidos y ser transferidos a través de la cadena trófica, convirtiéndolos en extremadamente peligrosos para los organismos vivos, incluyendo los humanos. Algunas de estas sustancias están consideradas como disruptores endocrinos (DEs) que pueden interaccionar directamente con los receptores hormonales mimetizando o antagonizando su acción. También se puede producir una interacción directa con un gran número de proteínas que controlan la liberación de hormonas hacia células o tejidos diana. Según la definición del Programa Internacional en Seguridad de las Substancias Químicas (IPCS) de 2002: "Un DE es una substancia exógena o una mezcla que altera la función o funciones del sistema endocrino y como consecuencia causa efectos adversos sobre la salud de un organismo, o su progenie, o incluso de (sub)poblaciones".

Los mayores efectos observados, tanto en animales salvajes o de experimentación como en humanos, ocurren cuando la exposición a los DEs tiene lugar en los momentos de mayor vulnerabilidad y en concentraciones que provocan alteraciones en la regulación endocrina. Los efectos que más preocupan son los que ocurren durante las primeras etapas del desarrollo tanto en humanos como en animales, ya que estos efectos son a menudos irreversibles y pueden no ser evidentes hasta etapas más tardías de la vida. Los DEs tienen la capacidad de interferir con los tejidos y órganos en desarrollo y alterar a su función por lo que pueden alterar la susceptibilidad a diferentes tipos de enfermedades a lo largo de la vida. Son necesarios esfuerzos interdisciplinares para identificar los químicos que son responsables del incremento de la incidencia de enfermedades relacionadas con el sistema endocrino y su disfunción. Los DEs no se limitan sólo a las vías estrogénica, androgénica y tiroidea ya que también interfieren con el metabolismo, el almacenamiento de grasas, el desarrollo óseo y el sistema inmunitario. Esto sugiere que todos los sistemas podrían verse afectados y sus efectos dependen del nivel y del tiempo de exposición, siendo especialmente críticos cuando se produce durante el desarrollo.

Respecto a las vías de actuación, los DEs actúan a través de los REs nucleares, receptores esteroideos no nucleares (ej. REs de membrana), receptores no esteroideos (ej. receptores de neurotransmisores como el receptor de serotonina, dopamina o nor-epinefrina), receptores huérfanos (ej. receptor de aril hidrocarburos, AhR), vías enzimáticas involucradas en la biosíntesis de esteroides y/o metabolismo y otros mecanismos que convergen bajo los sistemas endocrino y reproductor (Diamanti-Kandarakis y col., 2009).

El término xenoestrógeno significa literalmente "estrógeno extranjero". Estos compuestos proceden de compuestos químicos que muestran un cierto grado de afinidad con los estrógenos naturales y que se han ido acumulando en el medio ambiente. Pueden encontrarse en productos derivados de la industria, incluyendo recipientes para guardar comida, fitoestrógenos, productos de limpieza, electrónicos, plásticos, medicamentos como píldoras anticonceptivas u hormonas de reemplazamiento estrogénico y, muchos de ellos, son considerados DEs.

A pesar que los DEs son una clase muy heterogénea de sustancias, en la presente Tesis Doctoral nos hemos centrado en el EE2 y en el Tmx. Estas dos suntancias, procedentes de la industria framacéutica, se unen a los REs e interfieren con numerosos aspectos que controlan la homeostasis del organismo a través de estrógenos incluyendo el balance entre crecimiento celular/apoptosis (Diamanti-Kandarakis y col., 2009; Zoeller y col., 2010).

El EE_2 es un compuesto sintético con homología estructural a la hormona sexual femenina E_2. El EE_2 fue el principal estrógeno esteroideo sintético administrado de forma oral, sintetizado en 1983 por Hans Herloff Inhoffen y Walter Hohlweg. Se usa en la mayoría de formulaciones modernas de píldoras orales anticonceptivas combinadas. Aproximadamente del 16-68% del EE2 tomado por boca es excretado en la heces o en la orina (Johnson y Williams, 2004). Está presente en los ecosistemas acuáticos, en concentraciones elevadas, y se ha llegado a detectar más de 41 ng/l (Hua y col., 2016). El EE_2 y otros estrógenos provocan daños en el ecosistema acuático mediante disrupción del sistema endocrino en poblaciones salvajes. Estos daños incluyen la condición de intersexualidad, reduciendo por tanto la capacidad reproductora de las especies (Arnold y col., 2004). Estos daños son provocados incluso a muy bajas concentraciones, en partes por trillón (ppt). Además, la exposición durante el desarrollo induce de manera transgeneracional fenotipos de discapacidad reproductora (Bhandari y col., 2015).

En estudios previos de nuestro grupo de investigación se ha comprobado que el EE_2 produce la infiltración de GAs y Ly B en el testículo e interrumpe la espermatogénesis. Además, incrementa de manera dosis dependiente la expresión de genes que codifican para citoquinas, quimioquinas y moléculas correlacionadas con la infiltración de leucocitos. También se ha comprobado que altera el nivel de los esteroides sexuales en el suero, el perfil de expresión génica de algunas moléculas relevantes en la estereidogénesis y de los REs. Algunos de estos efectos varían según el estado de desarrollo de los ejemplares. El EE_2 *in vitro* inhibe la producción de ROIs y la actividad fagocítica y altera el perfil de expresión de genes inmunitarios en macrófagos.

Otro importante xenoestrógeno es el Tmx. Es un derivado sintético del trifeniletileno que es utilizado de manera satisfactoria para prevenir la formación de tumores mamarios en ratones. Desde su introducción en 1997, es utilizado como la primera línea de tratamiento para el cáncer de pecho positivo para REs en ambos sexos. Además, es usado como adyuvante (tras cirugía, radiación y/o quimioterapia) (Colleoni y col., 2006), en el tratamiento de la metástasis del cáncer de pecho y para reducir la incidencia en mujeres con riesgo alto de padecer cáncer de pecho (Fisher y col., 2015). El Tmx presenta baja afinidad por los REs. Sin embargo, sus metabolitos, endoxifen y 4-hidroxitamoxifen, poseen 100 veces más afinidad que el Tmx. Estos compuestos interaccionan con los REs en los tejidos diana, mamarios y no mamarios, para producir un fenotipo complejo, provocando efectos agonistas y antagonistas dependiendo del tejido. Actúa como antagonista de los estrógenos en tejido mamario pero como agonista parcial en el útero y en el hueso. Debido a esta doble acción, el Tmx se describe como un modulador selectivo de los receptores de estrógenos (MSRE). El Tmx tiene acciones pro- y anti-estrogénicas mediadas por su unión competitiva con los REs provocando un cambio conformacional. Además,

actúa como agonista de GPER1 (Revankar y col., 2005; Vivacqua y col., 2006). Debido a que es un MSRE, no sólo antagoniza la acción de los estrógenos mediante el boqueo de su unión, si no que mimetiza otras acciones a través de su unión por REs. Así produce formación de pólipos en el endometrio, cáncer endometrial invasivo, sarcoma uterino o tromboembolismo venoso, entre otros.

Este tipo de fármacos, de momento, no son eliminados por los sistemas de depuración de aguas (Zhang y col., 2013). Por lo tanto, hay una alta probabilidad de que lleguen al medio ambiente y su presencia en aguas residuales, aguas superficiales y su potencial presencia en agua potable está generando cierta preocupación (Booler y col., 2014). La mayoría de los estudios realizados han detectado niveles elevados de este compuesto en aguas residuales urbanas superiores a 42 ng L-1, e incluso ha sido detectado en cursos de agua en más de 200 ng L-1 (revisado en Ferrando-Climent y col., 2014). En el noreste de España se ha detectado en concentraciones entre 25 y 38 ng L-1.

El desarrollo del agonista selectivo de GPER1 que no activa los REs, G1, ha facilitado la investigación de este receptor. Al igual que en mamíferos, G1 tiene uan alta afinidad por la union a GPER1 de croaker (*Micropogonias undulate*) (Pang y col., 2008; Thomas y col., 2005) y mimetiza la acción de E_2 en un biensayo de maduración de ovocitos de peces, indicando que G1 es una herramienta muy útil para investigar los efectos derivados de la modulación de GPER1. Además, se ha confirmado en dorada la especifidad del agonista G1 sobre GPER1 (Cabas y col., 2013).

Con estos antecedentes, la presente Tesis Doctoral pretende determinar si el posible efecto derivado de la presencia de xenoestrógenos sobre el sistema inmunitario de un pez teleósteo marino como la dorada, de gran interés en acuicultura, desaparece cuando la exposición cesa. Además nos interesa conocer si estas posibles ocurren de igual manera según el estado de desarrollo de los ejemplares. Faltaría alguna frase del uso del G1. Finalmente hemos estudiado la importancia del GPER1 en la regulación de la biología de los neutrófilos humanos.

2. OBJETIVOS

Este trabajo tiene los siguientes objetivos específicos:

I. Determinar el efecto de la administración en dieta de EE_2 y de Tmx sobre la respuesta inmunitaria de ejemplares adultos de dorada así como su capacidad de recuperación tras el cese de los tratamientos.

II. Determinar el efecto de la administración en dieta de EE_2, de Tmx y de G1 sobre la respuesta inmunitaria de ejemplares juveniles de dorada así como su capacidad de recuperación tras el cese de los tratamientos.

III. Evaluar los efectos derivados de la administración de EE_2 en dieta sobre la actividad de los linfocitos de dorada.

IV. Realizar la caracterización funcional del receptor de estrógenos asociado a proteína G, GPER1, en neutrófilos humanos.

3. PRINCIPALES RESULTADOS Y DISCUSIÓN

3.1 Efecto del EE_2 y del Tmx sobre la respuesta inmunitaria de machos adultos de dorada y análisis de dicha respuesta tras el cese de los tratamientos.

Trabajos previos de nuestro grupo de investigación han demostrado que el EE_2 actúa como disruptor endocrino en dorada ya que provoca una respuesta estrogénica *in vivo* al inducir la expresión hepática del gen que codifica para la vitelogenina (*vtg*) (40.000 veces más comparada con la expresión en los ejemplares no tratados) (Cabas y col., 2012), aumento de expresión que es considerado marcador de disrupción estrogénica (Sumper y Jobling, 1995). El EE_2 modula *in vivo* e *in vitro* la respuesta inmunitaria de machos adultos de dorada (Cabas y col., 2011; Cabas y col., 2012) y, aunque, no actúa como sustancia inmunosupresora, modula la capacidad de los ejemplares de responder apropiadamente frente a una infección (Cabas y col., 2012). Sin embargo, al inicio de esta Tesis Doctoral, desconocíamos si los efectos adversos provocados por el EE_2 desaparecen una vez que cesa el tratamiento y cuanto tiempo hace falta para ello. Por otra parte, quisimos conocer si otro estrógeno sintético, el Tmx (MSREs) es también capaz de actuar como disruptor endocrino en machos adultos de dorada y si tiene algún efecto sobre la respuesta inmunitaria. Al igual que en el caso del EE_2, también quisimos conocer si los posibles efectos provocados por el Tmx desaparecen tras el cese del tratamiento. En la bibliografía no hemos encontrado referencias sobre la evaluación del efecto del Tmx en la respuesta inmunitaria en peces ni, por tanto, de su capacidad de recuperación tras el cese del tratamiento. Los resultados obtenidos están recogidos en el capítulo 1 (Rodenas y col., 2015).

Machos adultos de dorada con un peso de 453 ± 7 g fueron tratados con 0 (control del tratamiento) ó 5 µg EE_2/g de comida ó 100 µg Tmx/g de comida durante 50 días (dt). El tiempo máximo de tratamiento con EE_2 administrado en la dieta en dorada, hasta ese momento, había sido de 28 días (Cabas y col., 2011, 2013). Posteriormente, los ejemplares fueron alimentados con dieta comercial durante 88 días más (dpt). Para analizar el efecto del EE_2 sobre la respuesta inmunitaria, los ejemplares fueron inmunizados/vacunados con hemocianina en presencia de aluminio, como adyuvante, o tratados con PBS, como control de la inmunización, tanto durante el tratamiento (a los 15, *priming*, y 29, *booster*, dt) como tras el cese del tratamiento (a los 55, *priming*, y 71, *booster*, dpt) (en este último caso a animales que no habían sido inmunizados durante el tratamiento). Se tomaron muestras de hígado (tratados, con EE_2 o Tmx, o no tratados, sin inmunizar), riñón cefálico y sangre (tratados, con EE_2 o Tmx, o no tratados, sin inmunizar e inmunizados) los días 16 (1 día después del *priming*, dpp), 30 y 50 (1 y 21 días después del *booster*, dpb,

respectivamente) dt. Tras el cese del tratamiento, las muestras se tomaron los días 56 (1 dpp), 72 y 88 (1 y 17 dpb, respectivamente). En ambos casos, las muestras fueron procesadas para la extracción de ARN, la producción de ROIs y el análisis del título específico de IgM frente al antígeno usado y del porcentaje de células IgM+.

En nuestro trabajo, en el que hemos observado un aumento en la expresión del gen de la *vtg* de 96.000 veces en el grupo tratado con EE_2 respecto al grupo no tratado a los 16 dt, confirmamos el carácter disruptor del EE_2 en dorada. A los 56 dpt se mantiene el aumento en los niveles de expresión del gen de la *vtg* aunque se observa una fuerte variación individual. El Tmx también tiene un efecto disruptor en machos adultos de dorada ya que provoca un aumento en el nivel de expresión del gen que codifica para la *vtg*. Sin embargo, este aumento no es tan pronunciado si lo comparamos con el que provoca el EE_2 (incremento 300 veces superior comparado con el grupo no tratado) aunque sí que vuelve a valores similares a los del grupo no tratado a los 56 dpt. Estos resultados sugieren un papel estrogénico y anti-estrogénico del Tmx en dorada, como ha sido descrito previamente en estudios clínicos (Love y col., 1992; O'Regan y Gradishar, 2001) y preclínicos en algunos vertebrados (Jordan y Robinson, 1987). Estas diferencias pueden deberse a los diferentes subtipos de REs involucrados en la modulación de la producción de Vtg, como previamente se había sugerido (Leanos-Castaneda y Van Der Kraak, 2007). Además se ha descrito que concentraciones elevadas de Tmx provocan un incremento de los niveles plasmáticos de Vtg en machos mientras que disminuye en hembras de pez cebra o en pez cebra y medaka (Sun y col., 2010, 2007).

El EE_2, además, provoca una disminución del índice gonadosomático (IGS), tal y como ha sido previamente descrito por Cabas y col. (2011, 2012) durante los primeros días de tratamiento (hasta 28 días); sin embargo, el IGS de los animales tratados adquiere valores similares al de los animales control a tiempos más largos de tratamiento (50 días) y tras el cese del tratamiento. El Tmx, sin embargo, incrementa el IGS al final del tratamiento (50 días) y persiste con un nivel más alto que en los ejemplares control hasta 56 días después del cese del tratamiento volviendo, posteriormente, a niveles similares al de los ejemplares control (72 días).

Como primer paso para determinar el efecto del EE_2 y del Tmx sobre la respuesta inmunitaria analizamos los niveles de expresión génica de los REs, REα y GPER1, en RC ya que, como se ha descrito previamente, los macrófagos y los Ly los expresan constitutivamente (Cabas y col., 2015) mientras que los GAs, principales componentes celulares del riñón cefálico de dorada, sólo expresan *gper* (Cabas y col., 2013). Ni el EE_2 ni el Tmx modulan la expresión del ERα en RC como tampoco lo hace la inmunización. Debido a que *gper* se expresa en menor medida en RC que en otros tejidos como hígado o testículo (Cabas y col., 2013) y a que la inmunización provoca la movilización de GAs al sitio de vacunación/infección (Chaves-Pozo y col.,

2005b), no pudimos detectar su expresión en peces inmunizados a 16 dt (1dpp), tratados o no. Sin embargo, 56 dpt (1 dpp) se produce una fuerte disminución de los niveles de ARNm de *gper* en peces vacunados, tratados o no.

Para determinar el efecto del EE_2 y del Tmx sobre la respuesta inmunitaria innata analizamos la expresión de dos citoquinas clave, una citoquina pro-inflamatoria, la *il1b,* y una citoquina anti-inflamatoria, la *il10*, en un intento de poder utilizarlas como marcadores de disrupción endocrina de la respuesta inmunitaria de los machos adultos de dorada. El EE_2 inhibe la inducción de la expresión del gen que codifica para la *il1b* en respuesta a la inmunización, como previamente había sido descrito en doradas expuestas a EE_2 mediante baño (Cabas y col., 2012). De manera inesperada, este incremento no se produjo cuando la inmunización se realizó tras el cese del tratamiento, lo que pudo ser debido al diferente estado de desarrollo de los ejemplares. Respecto a la *il10*, sólo observamos un incremento 56 tras el cese del tratamiento en el grupo tratado con EE_2 y vacunado con respecto al grupo vacunado. El Tmx, al igual que el EE_2, es capaz de inhibir la inducción de la expresión de la *il1b* en peces vacunados. Sin embargo, no produce ninguna alteración en los niveles de expresión de la *il10*. También analizamos la producción de ROIs, al haber sido propuesta como un excelente marcador de disrupción endocrina en peces (Bols y col., 2001). Como se ha descrito previamente, la capacidad para producir ROIs es mayor en leucocitos de RC en peces no inmunizados que en aquellos que sí lo están (Cabas y col., 2013b). Nosotros observamos que la producción de ROIs disminuye con la inmunización pero tanto el EE_2 como el Tmx previenen este incremento en leucocitos, estimulados (con un patrón molecular asociado a patógenos como el ADN genómico de *Vibrio anguillarum*) o no, tras 16 horas de incubación. Observamos un efecto similar en el número de GAs en RC de los distintos grupos mediante citometría de flujo. Como hemos mencionado, tras la inmunización se produce la movilización de GAs al sitio de vacunación/infección (Chaves-Pozo y col., 2005b), con lo que podríamos inferir que el EE_2 y el Tmx producen el reclutamiento de GAs al RC. Tras el cese de los tratamientos, el EE_2 y el Tmx no previenen el incremento de la producción de ROIs, sugiriendo un efecto transitorio de estos disruptores endocrinos.

La respuesta inmunitaria adaptativa en peces muestra las esperadas características de especificidad y de memoria. Estudios *in vitro* han demostrado la presencia de dos tipos de Ly, equivalentes a las células B y T en mamíferos. Respecto a los anticuerpos, la IgM ha sido identificada como la más común en el suero de peces teleósteos (Zhang y col., 2010). Previamente se ha demostrado que los estrógenos, tanto los naturales como los sintéticos, alteran el sistema inmunitario, en particular a las células B (Benardi y col., 2014). El impacto del EE_2 y del Tmx en la respuesta inmunitaria adaptativa ha sido evaluado analizando la presencia de IgM específica en el suero de los peces vacunados tras dos

inmunizaciones (*priming* y *booster*). Como cabía esperar, los animales inmunizados mostraron unos niveles mayores de IgM específica frente al antígeno usado. Al final del tratamiento, el EE_2 y el Tmx incrementan el nivel de IgM específica en peces vacunados. Sin embargo, sólo el Tmx incrementa el título de IgM acompañado de un incremento en el porcentaje de células IgM+ tras el cese del tratamiento. Este aumento podría sugerir un papel esencial de este compuesto en la homeostasis de la respuesta inmunitaria. Sin embargo, se necesitan más estudios para poder entender el mecanismo por el que este compuesto produce estos efectos en este tipo celular en peces, teniendo en cuenta que el E2, pero no los MSREs, incrementan la producción de Ig y el número de células productoras de Ig en RC y en bazo (Benardi y col., 2014; Erlandsson y col., 2002).

En conclusión, nuestros resultados indican que la administración del Tmx en la dieta produce efectos estrogénicos y anti-estrogénicos. Tanto el EE_2 como el Tmx alteran *in vivo* la respuesta inmunitaria innata en peces inmunizados mientras que sólo el Tmx modula la respuesta adaptativa humoral inducida por un reto inmunitario. Hasta donde conocemos, nuestro estudio aporta las primeras evidencias de la capacidad de recuperación de la respuesta inmunitaria innata de peces tras los efectos de disrupción provocados por el EE_2 y el Tmx aunque los efectos producidos por el Tmx en la respuesta inmunitaria humoral adaptativa se mantienen al menos hasta los 88 días tras el cese del tratamiento. Los resultados obtenidos podrán ser usados para minimizar los efectos adversos provocados por el EE_2 o el Tmx en dorada e, incluso, pueden ser extrapolados a otras especies de interés en acuicultura.

3.2 *Efecto del EE_2, del Tmx y del G1 sobre la respuesta inmunitaria de juveniles de dorada y análisis de dicha respuesta tras el cese de los tratamientos.*

A continuación, quisimos evaluar si el efecto del EE_2 y del Tmx en juveniles de dorada, antes de la diferenciación sexual, es similar al que tienen en los machos adultos tanto durante el tratamiento como tras su cese. En este estudio, incluimos el tratamiento de los ejemplares con G1, un agonista selectivo de GPER1 (Bologa y col., 2006), ya que su uso *in vivo* e *in vitro* en machos de dorada nos permitió demostrar que los estrógenos son capaces de modular las actividades de los GAs, células que no expresan los REs nucleares pero si GPER, aunque a las concentraciones utilizadas en dicho estudio no actuaba como disruptor endocrino (Cabas y col., 2013b). Con el fin de profundizar en el conocimiento del efecto de estos compuestos, aumentamos tanto el tiempo de tratamiento (110 días) como el tiempo de estudio tras el cese de los tratamientos (333 días), con respecto a los

estudios previos realizados con machos adultos. Los resultados obtenidos están recogidos en el capítulo 2 (Rodenas y col., 2016).

Ejemplares de dorada (71.2 ± 0.5 g) fueron tratados con los tres compuestos mencionados a la concentración que previamente habíamos usado para adultos de dorada (5 µg EE_2/g comida, 100 µg Tmx/g comida y 5 µg G1/g comida) y otra concentración 10 veces inferior de cada compuesto (0.5 µg EE_2/g comida, 10 µg Tmx/g comida y 0.5 µg G1/g comida) durante 110 días. Posteriormente, los ejemplares fueron alimentados con dieta comercial durante 333 días más (dpt). En primer lugar, comprobamos que las dosis bajas (0.5 µg EE_2, 10 µg Tmx y 0.5 µg G1) no producen ninguna modificación en los niveles de transcripción de *vtg*, es decir no actúan como disruptores endocrinos, al menos en el tiempo de tratamiento analizado (110 días). Sin embargo, las dosis altas de EE_2 y Tmx (5 µg y 100 µg, respectivamente) tienen el mismo efecto que en los machos adultos siendo, también en los juveniles, el efecto del Tmx menor que el provocado por el EE_2 (147 y 5728 veces de incremento, respectivamente, comparado con el grupo control). Esta diferencia, tanto en adultos como en juveniles, podría ser debida a los diferentes subtipos de REs implicados en la modulación de la producción de la Vtg por EE_2 y Tmx, como previamente se había sugerido para peces (Leaños-Castañeda y col., 2007). La expresión del gen de la *vtg* volvió a valores basales a los 121 dpt en todos los grupos tratados. Sin embargo, el G1 no produce ninguna modificación en los niveles de expresión de ARNm de *vtg*, confirmando que no promueve una respuesta estrogénica, tal y como se ha descrito en machos adultos (Cabas y col., 2013b).

Teniendo en cuenta que se ha descrito que el EE_2 inhibe el crecimiento en tilapia (Shved y col., 2008) y en pez cebra (Baumann y col., 2014), en los juveniles de dorada analizamos el efecto del EE_2, del Tmx y del G1 sobre el peso corporal y sobre el índice hepatosomático (IHS) y el índice esplenosomático (ISS). El peso corporal disminuye con el tratamiento de EE_2 y aumenta con el de Tmx. Estos efectos se mantienen hasta los 333 dpt. Además, el EE_2 provoca el incremento del índice hepatosomático (IHS) y del índice esplenosomático (ISS). Mientras que el primero (IHS) permanece alterado hasta los 333 dpt, la alteración del ISS desaparece a ese tiempo. Debido al estado de desarrollo de los juveniles, no pudimos evaluar el IGS para comprobar si el efecto previamente descrito en machos adultos (Rodenas y col., 2015) tiene lugar en este estadio del desarrollo.

En adultos, tanto el EE_2 como el Tmx, alteran *in vivo* la respuesta inmunitaria innata en peces vacunados mientras que sólo el Tmx modula la respuesta inmunitaria humoral inducida por un reto inmunitario (Rodenas y col., 2015). Respecto al compuesto G1, sabemos que la señalización *in vivo* a través del GPER modula la respuesta inmunitaria adaptativa (Cabas y col., 2013). Para analizar si estos efectos son independientes del estado de maduración de los ejemplares,

realizamos un patrón de inmunización en los juveniles de dorada similar al descrito previamente (Cabas y col., 2011, 2012; Rodenas y col., 2015). Así, los ejemplares fueron inmunizados/vacunados con hemocianina en presencia de aluminio, como adyuvante, o tratados con PBS, como control de la inmunización, tanto al finalizar el tratamiento (grupo A) como a los 120 días (grupo B; en este caso a animales que no habían sido inmunizados en el tratamiento) dpt. El *priming* se realizó 1 dpt en el grupo A y 120 dpt en el grupo B. El *booster* se llevó a cabo 14 dpt y 130 dpt, respectivamente. Se tomaron muestras de hígado (no tratados y tratados con EE_2, Tmx o G1, sin inmunizar) y de riñón cefálico, bazo y sangre (no tratados, inmunizados o no, y en los grupos tratados e inmunizados) que fueron procesadas para la extracción de ARN, producción de ROIs, análisis del título específico IgM frente al antígeno usado, análisis del porcentaje de células IgM+ y ensayo de proliferación 1 días después del *priming* (dpp) (2 dpt) y 16, 42, 107 y 196 días después del *booster* (dpb) (30, 56, 121 y 210 dpt) en el grupo A y 1 dpp (121 dpt) y 30 y 123 dpb (160 y 333 dpt) en el grupo B.

La inmunización produce el incremento de la expresión del gen *il1b* después del *priming* (Cabas y col., 2012; Rodenas y col., 2015). Sin embargo, este incremento no siempre se observa e incluso, a veces, la inmunización produce un descenso, posiblemente debido al diferente estado de maduración de los ejemplares y a las condiciones específicas del ambiente, como la temperatura del agua (Rodenas y col., 2015). De manera similar ocurre con los niveles de transcripción de la *il10* (Cabas y col., 2012, 2013a; Rodenas y col., 2015). Estos resultados sugieren la necesidad de hacer nuevos estudios para poder aclarar los efectos de la vacunación en el sistema inmunitario innato en peces hermafroditas durante su ciclo reproductor. El tratamiento con el EE_2, el Tmx o el G1 inhibe el incremento de los niveles de ARNm de *il1b* provocados por la inmunización, como se ha descrito en machos adultos (Cabas y col., 2012; Rodenas y col., 2015). Este hecho se observa cuando el *priming* se realiza 1 dpt (grupo A). Los niveles de ARNm de *il10*, sin embargo, sólo se ven inhibidos tras el tratamiento con el G1, como ya había sido descrito para adultos (Cabas y col., 2013). Sin embargo, cuando la inmunización se hizo 120 dpt, cuando los niveles de expresión de *vtg* habían vuelto a valores similares al grupo control no tratado, se produjo un efecto diferente al observado 1 dpt (valores de gen de la *vtg* altos). Así, la inmunización disminuyó los niveles de expresión de *il1b* mientras que incrementó los de *il10*. Por otro lado, sólo el G1 contrarrestó este efecto en el caso de los niveles de *il1b* mientras que el EE_2 y el Tmx inhibieron la inducción de la expresión de *il10* provocada por la inmunización. En contraposición a lo que se produce en adultos (Rodenas y col., 2015), el tratamiento con el EE_2, el Tmx y el G1 no altera la producción de ROIs en leucocitos de RC, estimulados o no, con un patrón molecular asociado a patógenos 16 dpb (30 dpt).

No hemos encontrado estudios que hayan evaluado la influencia de ninguno de estos compuestos en la función de las células B en peces aunque sí que hay estudios que establecen la influencia del E_2 en este tipo celular, pero con resultados contradictorios dependiendo de la concentración, la estimulación y el periodo de exposición (Shelley y col., 2013). Para evaluar de manera más precisa el impacto de estos compuestos en la respuesta inmunitaria adaptativa humoral durante la ontogenia, los juveniles de dorada fueron inmunizados cuando el sistema inmunitario adaptativo es funcional (Mulero y col., 2008). En concreto, nos hemos centrado en los posibles efectos de estos compuestos en el porcentaje de células B IgM^+ y en la producción de anticuerpos. Los Ly de dorada expresan REs (Liarte y col., 2011a) y, en concreto, los linfocitos B además expresan GPER (Cabas y col., 2015) por lo que tanto los estrógenos naturales como los sintéticos podrían modular directamente su función en el transcurso de una respuesta inmunitaria, pudiendo además ser vulnerables a DEs de carácter estrogénico (Milla y col., 2011). El Tmx aumenta el porcentaje de células IgM^+ en RC y en bazo tras el cese de los tratamientos de los juveniles de dorada, al igual que ocurre en adultos (Rodenas y col., 2015). Además, el incremento en el número de células IgM^+ que proliferan en presencia del antígeno hemocianina en combinación con ADN genómico de *Vibrio anguillarum* en peces vacunados y tratados con Tmx tras el cese del tratamiento, sugiere un papel esencial de este compuesto en la homeostasis de la respuesta inmunitaria. También hemos observado que el Tmx produce un incremento en el título de IgM específica contra el antígeno usado en juveniles vacunados a 107 dpb cuando la inmunización se realizó 1 dpt. Sin embargo, el título de IgM cae en juveniles vacunados y tratados con Tmx a 196 dpb o a 213 dpb cuando el *priming* se realizó 1 o 120 dpt, respectivamente. Además, ni el EE_2 ni el G1 modificaron el porcentaje de células IgM^+ tras el cese de los tratamientos aunque sí que produjeron el mismo efecto que el Tmx en la alteración del título de IgM específica cuando el *priming* se realizó 1 dpt, pero sólo el EE_2 cuando se realizó 120 dpt.

Podemos concluir que el EE_2 y el Tmx también actúan como DEs en juveniles de dorada y, como en el caso de los adultos, aunque no actúan como sustancias inmunosupresoras, modulan la capacidad de los ejemplares de responder apropiadamente frente a una infección. Nuestros resultados sugieren que el efecto de los MSREs en la respuesta humoral adaptativa difiere entre adultos y juveniles de dorada dependiendo del compuesto usado y del tiempo de exposición así como de cuándo se haya realizado la vacunación.

3.3 Los estrógenos estimulan la producción de anticuerpos a través de la señalización por el receptor de estrógenos asociado a proteína G, GPER1.

Tras observar los efectos producidos por diferentes DEs en juveniles de dorada, quisimos profundizar en los posibles efectos adversos del EE2 sobre el sistema inmunitario innato y, más concretamente, sobre la población de Ly y la producción de anticuerpos. Los resultados obtenidos están recogidos en el capítulo 3.

Ejemplares de dorada (63.2 ± 7.9 g) fueron tratados con EE_2 a la concentración previamente usada para adultos y juveniles de dorada (5 µg EE_2/g comida,) durante 76 días. Posteriormente, los ejemplares fueron alimentados con dieta comercial durante 23 días más.

Para profundizar en los posible efectos derivados de la administración del EE_2 sobre la población de linfocitos y la producción de anticuerpos, realizamos una única inmunización similar a lo descrito previamente (Cabas y col., 2011, 2012; Rodenas y col., 2015, 2016) al final del tratamiento. Así, los ejemplares fueron inmunizados/vacunados con hemocianina en presencia de aluminio, como adyuvante, o tratados con PBS, como control de la inmunización. Se tomaron muestras de hígado (no tratados y tratados sin inmunizar), de RC, bazo y sangre (no tratados y tratados, inmunizados y sin inmunizar) que fueron procesadas para el análisis de los niveles en suero de Vtg, extracción de ARN, análisis del porcentaje de células Zap70, IgM y Pax5 positivas a 1, 9 y 23 días post-inmunización (dpi) y para ensayos de proliferación, análisis del título de IgM total y del título específico de Ig M frente al antígeno usado y frente a otro antígeno modelo como la lisozima.

En primer lugar, comprobamos que este compuesto incrementa de manera significativa los niveles en suero de Vtg a los 76 días de tratamiento, como previamente habíamos descrito para los niveles de transcripción de *vtg* en hígado, tanto en adultos como en juveniles de dorada (Cabas y col., 2012, 2013a; García-Hernández y col., 2016; Rodenas y col., 2015, 2016). Tras el cese del tratamiento, los niveles de Vtg disminuyen de manera progresiva aunque aun permanecen alterados a los 23 días post-tratamiento (dpt).

La inmunización produce el incremento de la expresión del gen *il1b* (Cabas y col., 2012; Rodenas y col., 2015, 2016) que permance alterado hasta 9 dpi. Sin embargo, el EE_2 no modula su expresión, en contraposición a lo que previamente habíamos descrito tras 110 dt (Rodenas y col., 2016).

No hemos encontrado referencias en la bibliografía que hayan evaluado la influencia del EE_2 sobre las células T y B. Como precedente sabemos que, tras la exposición a efluentes con alta capacidad estrogénica, se produce la reducción del

número de Ly circulantes, indicando inmunosupresión (Filby y col., 2007), y tras el tratamiento con MSREs y E_2 se produce la disminución del peso y células del timo (Bernardi y col., 2014). En mamíferos, las células T tienen diferentes funciones, mediando la respuesta humoral y celular.

En este trabajo demostramos que el EE_2 provoca un incremento del porcenje y la capacidad de proliferación de los Ly T determinado por la expresión de una proteína que se asocia a la cadena Z del receptor de las células T (Zap70) que juega un papel esencial en los eventos involucrados en iniciar la respuesta dependiente de células T a través del receptor de antígenos (Wang y col., 2010). Este marcador ha sido usado para identificar células T también en carpa (Piazzon y col., 2015) y en pez cebra (Yoon y col., 2015). Además participa en la señalización a través del receptor de células B (BCR) en leucemia linfoide crónica (Wang y col., 2016), con lo que Zap70 es considerado como un candidato para posibles tratamientos. En peces, se ha descrito que las células T tienen funciones similares a las que se conocen en mamíferos (Nakanishi y col., 2015).

En el porcentaje de células IgM positivas no observamos cambios tras el tratamiento con EE_2, en contraposición a lo que previamente habíamos observado (Rodenas y col., 2015, 2016). Sin embargo, la exposición a este compuesto modula el porcentaje de células IgM y Pax5 positivas, así como su capacidad de proliferación, indicando que el EE_2 podría modular la diferenciación de células B, ya que Pax5 es el principal factor de transcripción que juega un papel esencial en la diferenciación de células B en vertebrados hacia células plasmáticas.

Los niveles de anticuerpos están relacionados con la resistencia a enfermedades y podrían usarse como marcadores de resistencia a enfermedades genéticas (Kachamakova y col., 2006). En general, las condiciones ambientales están consideradas como factores importantes en la regulación de la producción de anticuerpos en animales (González y col., 1988). En este contexto, las hormonas esteroideas (incluyendo a E_2) actúan directamente en la supresión de la producción de IgM por los linfocitos B *in vitro* (Hou y col., 1999) o estimulan su producción. Ademas, el dietiletilbestrol y el bisfenol A, otros DEs de carácter estrogénico, incrementan la producción de anticuerpos IgM *in vitro*. Estos resultados sugieren que los xenoestrógenos están involucrados en la producción de anticuerpos y podrían estar involucrados en el desarrollo de enfermedades autoinmunes (Inadera, 2006; Murakami y col., 1992). Del mismo modo, en este estudio hemos observado que el EE_2 induce de manera significativa la producción de anticuerpos IgM.

Por otro lado, hasta que se identificó a GPER como un receptor de estrógenos en 2005, se pensaba que los estrógenos sólo mediaban su acción a través de la vía clásica de señalización a través de RE nucleares como REα y REβ. Así, aunque la información es escasa a cerca de las posibles aplicaciones terapeúticas de

GPER, hay estudios que relacionan a este receptor con la atrofia del timo provocado por estrógenos (Wang y col., 2008a), la producción de células T en el timo (Isensee y col., 2009), un fenotipo supresor en las células T CD4 positivas (Brunsing y col., 2013) y en la inducción de IL10 en la población Th17 (Brunsing y Prossnitz, 2011). Además, la señalización de GPER *in vivo* modula la producción de anticuerpos IgM específicos en suero de peces vacunados (Cabas y col., 2013a).

Como conclusión podríamos decir que los estrógenos pueden modular la población de linfocitos en vertebrados. Además, tras la activación de GPER con G1, su agonista selectivo, observamos un incremento en la producción de anticuerpos, similar al que produce EE_2, lo que nos sugiere la implicación de la señalización de GPER en la producción de anticuerpos.

3.4 GPER1 regula las funciones de los neutrófilos humanos.

Este capítulo, incluido como último en esta Tesis Doctoral, fue desarrollado cronológicamente en tercer lugar, con motivo de una estancia pre-doctoral en el Departamento de Patología y Diagnóstico de la Universidad de Verona (Italia).

Se sabe que los estrógenos son moduladores clave en diferentes aspectos de la fisiología humana y animal, incluida la del sistema inmunitario (Edwards y col., 2005). Aunque los estrógenos actúan principalmente por mecanismos genómicos clásicos, mediados por los REs nucleares, recientemente se ha descrito que también pueden activar rutas rápidas de transducción a través de mecanismos no genómicos. Así, en el año 2000, se identificó un receptor asociado a proteína G, GPER1, previamente denominado GPR30, como un receptor que media efectos no genómicos de los estrógenos (Revankar y col., 2005; Thomas y col., 2005; Prossnitz y col., 2009). Sin embargo no existe mucha información sobre el papel de este receptor en inmunidad y, especialmente, esta información es muy escasa en neutrófilos aunque se sabe que la línea celular similar a neutrófilos HL-60 expresan funcionalmente tanto REs nucleares (REa y REb) como GPER1 (Blesson y Sahlin., 2012). En estudios previos en dorada (Cabas y col., 2013a), demostramos que los GAs, el equivalente funcional de los neutrófilos de mamíferos, expresan GPER1, a nivel de ARNm y de proteína, y que los estrógenos son capaces de modular las funciones de estas células a través de la ruta GPER1/AMPc/potein quinasa A/CREB (Cabas y col., 2013), utilizando G1, un agonista específico del GPER1. Debido a que muchos de los efectos de los estrógenos no pueden ser explicados por la activación de los REs clásicos y a la implicación clara de los estrógenos en ciertos desórdenes inmunitarios, nos pareció muy interesante investigar la relevancia de GPER1 en la inmunidad con la posibilidad de diseñar nuevas dianas terapeúticas. En este estudio, nos centramos en caracterizar funcionalmente el GPER1 en neutrófilos humanos con el fin de determinar si este podría ser utilizado como herramienta para tratar

enfermedades autoinmunes e inflamatorias crónicas en las que los estrógenos y los neutrófilos tienen un papel fundamental. Los resultados obtenidos están recogidos en el capítulo 4 (Rodenas y col., 2016).

A partir de sangre periférica de donantes sanos, aislamos neutrófilos mediante gradiente de ficoll en condiciones libres de endotoxinas. Además purificamos eosinófilos, monocitos y células dendríticas. Tras la purificación, fueron tratados o no con diferentes estímulos como el factor estimulante de colonias de granulocitos (G-CSF), interferon gamma (IFNγ), factor de necrosis tumoral α (TNFα), IL10, lipopolisacárido (LPS) y G1. Tras centrifugar, los sobrenadantes fueron procesados para el análisis de la liberación de citoquinas como IL8 y el antagonista del receptor de la interleuquina (IL1RA). Los *pellets* los usamos para la extracción de ARN y posterior análisis de expresión de genes y para el análisis de proteínas mediante western blot en las distintas poblaciones aisladas anteriormente. Así evaluamos la expresión de GPER1 y su vía de señalización. Además los resultados fueron confirmados mediante citometría de flujo, usando un péptido de bloqueo que nos asegura que los resultados observados son específicos de nuestro marcaje. Por último, tras la activación de los neutrófilos con N-formilmeteonina-leucil-fenilalanina (fMPL) o LPS analizamos la producción de radicales libres de oxígeno.

En primer lugar comprobamos que los neutrófilos humanos expresan GPER1. Mediante western blot, observamos que diferentes tipos celulares inmunitarios humanos entre los que se incluyen los neutrófilos (monocitos, eosinófilos y células dendríticas) muestran una banda correspondiente a la proteína GPER. Además, mediante citometría de de flujo, confirmamos que los neutrófilos expresan GPER ya que se marcan con un anticuerpo contra el GPER1, de manera dosis dependiente. Esta tinción es específica ya que la pre-incubación del anticuerpo GPER1 con un péptido de bloqueo específico reduce este marcaje. Aunque originalmente se describió que el GPER se localiza en el retículo endoplasmático en diferentes líneas celulares de cáncer (Revankar y col., 2005), nosotros hemos comprobado que el receptor se expresa en la membrana plasmática, al igual que ha sido descrito en eosinófilos humanos (Tamaki y col., 2014). Además, tras la activación *in vitro* de GPER1 con diferentes citoquinas y LPS no se producen cambios significativos en su expresión.

GPER1 parece ser funcional en neutrófilos humanos como evidencia su activación tras el tratamiento con G1 en la inducción de la expresión del pro-oncogen C-FOS, un conocido marcador de la activación por el mecanismo de señalización de GPER1 (Albanito y col., 2007), el incremento de la explosión respiratoria de los neutrófilos provocada por diferentes agentes como LPS y N-formilmeteonina-leucil-fenilalanina (fMLP), el incremento de la expresión de CD11b y la disminución de CD62L, dos clásicos marcadores asociados con la activación de

neutrófilos *in vitro* (García-Vicuna y col., 1997), y la inducción de la expresión de varios genes que codifican para marcadores clave pro-inflamatorios como *IL1B*, *COX2*, *GCSF* y *CXCL8*. Sin embargo, la activación inducida por la señalización de GPER1 no es idéntica a la activación observada con LPS ya que aunque G1 y LPS estimulan la liberación de la citoquina pro-inflamatoria CXCL8, G1 no induce la liberación de la citoquina anti-inflamatoria IL1RA. Por otra parte, la activación de GPER1 con G1 en GAs de dorada no incrementa, de manera consistente, la explosión respiratoria y muestra un efecto anti-inflamatorio con la inducción de genes que codifican para COX2, prostaglandina D2 sintetasa (PTGDS), e IL-10 (Cabas y col., 2013). Otra de las diferencias de la activación de GPER1 es que mientras que G1 produce un incremento de la explosión respiratoria de los neutrófilos, los estrógenos reducen la liberación del anión superóxido en neutrófilos humanos (Bekesi y col., 2000, 2007). Sin embargo, este efecto del incremento en la producción de ROS es similar al que produce el para-nonilfenol, descrito como agonista del GPER1 (Prossnitz y col., 2015), y el Tmx (Corriden y col., 2015). Todos estos resultados sugieren que hay diferencias en el efecto de la señalización del GPER1 en los neutrófilos de vertebrados y que, aunque los efectos de la activación del GPER son muy complejos, la señalización a través de este receptor sugiere un papel fundamental de los estrógenos en la homeostasis de la respuesta inmunitaria.

Otra interesante observación derivada del presente estudio es que la estimulación de neutrófilos humanos con el agonista específico G1 aumenta su viabilidad, teniendo en cuenta que los estrógenos retrasan la apoptosis en neutrófilos humanos (Molloy y col., 2003). Sin embargo, la estimulación con IL-5 de GPER1 en eosinófilos humanos inhibe la apoptosis espontánea a través de la inhibición de caspasa-2 mientras que promueve la apoptosis dependiente de caspasa-3 (Tamaki y col., 2014). Por lo tanto, la regulación de la viabilidad de neutrófilos humanos mediante GPER1 requiere nuevas investigaciones.

El siguiente paso fue el de explorar la ruta de señalización aguas abajo de la activación del GPER1. Analizamos, mediante western blot, la activación de la adenil ciclasa (Filardo y col., 2002), p38 MAPK (Sathya y col., 2015) y ERK (Filardo y col., 2002; Albanito y col., 2007) ya que en células cancerígenas humanas la activación del GPER1 promueve la activación de esta ruta. Hasta la fecha no se había explorado esta vía de señalización en células inmunitarias de mamíferos aunque sabemos que la activación del GPER1 con su agonista G1 señaliza mediante la ruta adenil ciclasa/cAMP/PKA/CREB en granulocitos de dorada (Cabas et al., 2013). De la misma manera, GPER1 señaliza a través de esta vía en neutrófilos aunque también lo hace a través de p38 MAPK y ERK, como ha sido previamente demostrado en líneas celulares de cáncer (Filardo y col., 2002; Albanito y col., 2007; Sathya y col., 2015). La vía de señalización de GPER1 es diferente a lo descrito para REs ya que inhiben la vía NF-κB (Kalaitzidis y col., 2005) incrementando los niveles de IKBα (Xing y col., 2012).

Por lo tanto, nuestros resultados ponen de manifiesto la importancia de los efectos mediados por estrógenos a través de GPER1 en neutrófilos humanos en condiciones fisiológicas y patológicas. Además, la regulación de los neutrófilos, vía GPER1, debe de tenerse en cuenta en el contexto de disrupción endocrina ya que xenoestrógenos, como el bisfenol A, algunos pesticidas o el EE_2, son sustancias naturales o sintéticas que tienen una gran estabilidad en el medio ambiente provocando efectos tóxicos en el contexto de autoinmunidad (Chighizola y col., 2012).

En conclusión, hemos descrito, por primera vez, que los neutrófilos humanos expresan constitutivamente un receptor de estrógenos asociado a proteína G, GPER1, que es funcional y que su agonista selectivo, G1, regula la viabilidad y promueve la activación celular. La activación de GPER1 promueve a los neutrófilos humanos hacia un fenotipo pro-inflamatorio. Además, ya que los estrógenos juegan un papel fundamental en desórdenes autoinmunes y en enfermedades inflamatorias crónicas, nuestro estudio sugiere que el GPER1 es una prometedora diana terapéutica.

4. CONCLUSIONES

I. El EE_2 y el Tmx administrados en dieta pueden ser considerados disruptores endocrinos en dorada ya que provocan un aumento del nivel de expresión del gen que codifica para la vitelogenina en hígado, siendo este aumento independiente del estado de desarrollo de los ejemplares tratados y mucho mayor en el caso del EE_2. Sin embargo, el G1 no modifica dicho nivel de expresión génica en ninguna de las dosis utilizadas.

II. El efecto disruptor provocado por la administración en dieta de EE_2 y de Tmx desaparece cuando la administración de dicho compuestos cesa.

III. El EE_2, el Tmx y el G1 administrados en la dieta no se comportan como sustancias inmunosopresoras en dorada a las dosis testadas.

IV. El EE_2, el Tmx y el G1 disminuyen la capacidad de los ejemplares de responder a un estímulo inmunitario *in vivo* ya que inhiben la producción del gen que codifica para la citoquina pro-inflamatoria IL-1β. Además, apenas afecta a la capacidad de los fagocitos de producir ROS.

V. El efecto del EE_2, el Tmx o el G1 en la respuesta inmunitaria adaptativa humoral difiere entre adultos y juveniles de dorada.

VI. El EE_2 y el Tmx incrementan la producción de anticuerpos en peces vacunados durante el tratamiento. Sin embargo, sólo el Tmx incrementa la producción de anticuerpos y el número de linfocitos B IgM-positivos tras el cese del tratamiento.

VII. La administración de EE_2 en dieta incrementa la abundancia y la proliferación de linfocitos T y linfocitos B-IgM positivos en la respuesta inmunitaria primaria. Además, incrementa la producción de anticuerpos naturales a través de la señalización por GPER1.

VIII. Los neutrófilos humanos expresan de manera funcional un GPER1 que regula sus funciones a través de las rutas de señalización AMP/PKA/CREB, p38 MAPK y ERK.

Appendixes

Publications derived from the Thesis

Rodenas MC, Cabas I, Abellán E, Meseguer J, Mulero V, García-Ayala (2015). Tamoxifen persistently disrupts the humoral adaptive immune response of gilthead seabream (*Sparus aurata* L.). Developmental and Comparative Immunology 53, 283-292.

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Rodenas MC, Tamassia N, Cabas I, Calzetti F, Meseguer J, Cassatella MA, García-Ayala A, Mulero V (2017). G protein-coupled estrogen receptor 1 regulates human neutrophil function. Biomedicine Hub 2, 1-13.

Rodenas MC, Cabas I, García-Alcázar A, Meseguer J, Mulero V, García-Ayala (2017). Estrogens promote the production of neutralizing natural antibodies through G protein-coupled estrogen receptor signaling pathway in fish. Under review.

Other publications/collaborations related to the Thesis

Cabas I, **Rodenas MC**, Meseguer J, Mulero V, García-Ayala A (2012). El 17alpha-etinilestradiol altera in vivo e in vitro el perfil de expression génica de citoquinas y quimioquinas de los fagocitos profesionales de dorada. Avances en Endocrinología Comparada, 14, 213-215. ISBN 978-84-96703-42-1. Book chapter.

Cabas I, **Rodenas MC**, Abellán E, Meseguer J, Mulero V, García-Ayala A (2013). Estrogen signalling through the g protein-coupled estrogen receptor, GPER, regulates granulocyte activation in fish. Journal of Immunology, 191, 4628-4639.

García-Hernández MP, **Rodenas MC**, Cabas I, García-Alcázar A, Chaves-Pozo E, García-Ayala A (2016). Tamoxifen disrupts the reproductive process in gilthead seabream males and modulates the effects promoted by 17α-ethynylestradiol. Comparative Biochemistry and Physiology, Part C 179, 94–106.

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Participation in (inter) national conferences

Rodenas MC, Gómez-González NE, Cabas I, García-Hernández MP, Mulero V, Chaves-Pozo E, García-Alcázar A, García-Ayala A. Los disruptores endocrinos: un problema para la salud de especies acuícolas. El caso del etinilestradiol. CONAMA. Madrid, 2016. National. Oral.

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Gómez-González NE, **Rodenas MC**, García-Alcázar A, Sepulcre MP, Mulero V, García-Ayala A. GB10, a monoclonal antibody specific to gilthead seabream mast cells. VI Iberian Congress of Ichthiology. Murcia, 2016. International. Poster.

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Rodenas MC, Cabas I, Arizcun M, Meseguer J, Mulero V, García-Ayala A. Persistence of disruption in the adaptive immune response in weaned gilthead seabream (*Sparus aurata* L.) larvae after discontinued exposure to estrogenic and/or anti-estrogenic compounds. The 13th ISDCI Congress. Murcia, 2015. International. Oral.

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García Henández MP, **Rodenas MC**, Cabas I, García-Alcázar A, Chaves-Pozo E, García-Ayala A. Impact of tamoxifen on the gonad immune response in gilthead seabream. Interaction with EE_2 and reversivility of effects. The 13th ISDCI Congress. Murcia, 2015. International. Poster.

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Rodenas MC, Cabas I, Abellán E, Mulero V, García-Ayala A, Chaves-Pozo E. Effects of dietary intake of 17α-etynylestradiol and tamoxifen on the gonad of gilthead seabream. 26th Conference of European Comparative Endocrinologists. Zurich, 2012. International. Poster.

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Chaves-Pozo E, Cabas I, **Rodenas MC**, Abellán E, García-Alcázar A, Mulero V, García-Ayala A Immune-reproductive interactions on the gonad of the teleostean fish. 26th Conference of European Comparative Endocrinologists. Zurich, 2012. International. Oral.

Rodenas MC, Cabas I, García-Alcázar A, García-Ayala A, Meseguer J. Efecto del 17α-etinilestradiol sobre la respuesta inmunitaria de dorada Sprus aurata L. XIII Congreso Nacional de Acuicultura. Barcelona, 2011. Internacional. Poster.

Short stays at international research centres

Research stay in the Department of Pathology of the Medical School, University of Verona (Italy) during 4 months under the supervisión of Dr. Marco Antonio Cassatella.