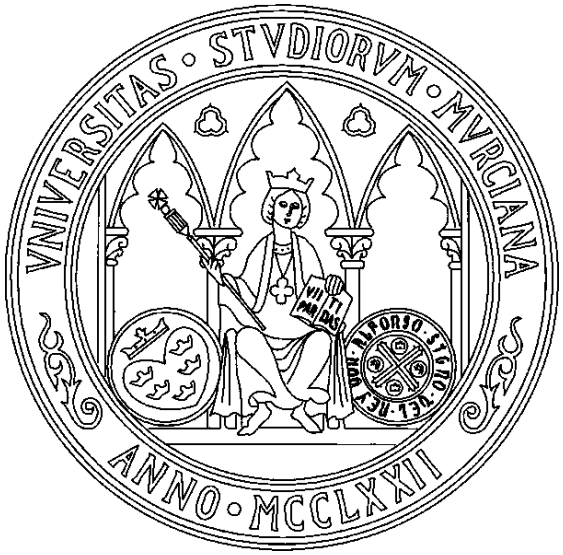

UNIVERSIDAD DE MURCIA

FACULTAD DE BIOLOGÍA

"Effect of 17α-ethinylestradiol on Immune and Reproductive System of the Gilthead Seabream (*Sparus aurata* L.). Functional Characterization of the G protein-coupled Estrogen Receptor"

"Efecto del 17α-etinilestradiol sobre el Sistema Inmunitario y Reproductor de la Dorada (*Sparus aurata* L.). Caracterización Funcional del Receptor de Estrógenos Asociado a Proteína G"

Dña. Isabel Cabas Sánchez

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Memoria que presenta

Dª. Isabel Cabas Sánchez

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INDEX

ABBREVIATIONS

11-KT	11-ketotestosterone
Ab	Antibody
Abs	Absorbance
AC	Adenylate cyclase
AGs	Acidophilic granulocytes
APC	Antigen presenting cell
B Ly	B lymphocytes
BPA	Bisphenol A
BSA	Bovine sérum albumin
Camp	Cyclic adenosine monophosphate
CCL4	CC chemikine ligand 4
cDNA	Complementary DNA
CFOS	Proto-oncogen *cfos*
COX	Cyclooxygenase
CREB	cAMP-response element binding protein
CSF	Colony stimulating fator
CTL	Cytotoxic T cells
CXC	Cis-X-cis chemokine
CYPs	Cytochrome P450 enzymes
dbcAMP	Cell-permeable cAMP analog 2′-dibutyryladenosine 3′,5′-cyclic
DES	Diethilestilbestrol
DHT	Dihydrotestosterone
Dmrt1	Double sex-and mab3-related transcription factor 1
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dpb	Days post-booster
dpp	Days post-priming
E_2	17β-estradiol
EAE	Experimental autoimmune encephalomyelitis
ED	Endocrine disruption
EDCs	Endocrine disruptor chemicals
EDTA	Ethylenediaminetetraacetic acid

EE_2	17α- ethinylestradiol
EGFR	Epidermal grown factor receptor
ELISA	Enzyme- linked immune sorbent assay
ER	Estrogen receptor
ERE	Estrogen response elements
ERK	Extracellular signal-regulated kinases
F	Forward oligonucleotide
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FSC	"Forward scater", cell size
FSH	Follicule stimulating hormone
FSH	Follicule stimulating hormone receptor
G	Gonad
G1	Specific agonist of G protein-coupled estrogen receptor
G7-	Acidophilic granulocyte-lacking cell suspensions acidophilus
G7+	Acidophilic granulocyte-enriched cell suspensions acidophilus
GDP	Guanosine diphosphate
GPCRs	G protein-coupled receptors
GPER	G protein-coupled estrogen receptor
GSI	Gonadosomatic index
GTHs	Gonadotropins
GTP	Guanosine triphosphate
Hf-FBS	Hormone free fetal bovine serum
HK	Head kidney
HSDs	Hydroxysteroid dehydrogenases
IFN	Interferon
Ig	Immunoglobulin
IGF-1	Insulin- like growth factor 1
IL	Interleukin
iNOS	Inducible NO synthase
IP	Propidium iodide
L	Liver

LH	Luteinizing hormone
LH-R	Luteinizing hormone receptor
LPS	Lipopolysaccharide
Ly	Lymphocytes
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MB	Body mass
MCSFR	Macrophage colony stimulating factor receptor
MFI	Mean fluorescence intensity
MG	Gonad mass
MHC	Major histocompatibility complex
MMP	Extracellular matrix metalloprotease
mRNA	Messenger RNA
MΦ	Macrophages
ns	Non-significant
NF-κB	Nuclear factor-κB
NRs	Nuclear receptors
Nt	Nucleotides
P	Progesterone
PAMP	Pathogen-associated molecular pattern
Pb	Base pairs
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PMA	Phorbol 12-myristate 13-acetate
Pre-SG	Pre-spermatogenesis
PRR	Pattern recognition receptor

PTG	Prostaglandin
PTGS2	Prostaglandin-endoperoxide synthase 2
R	Reverse primer
RC	Reproductive cycle
RNA	Ribonucleic acid
RNase	Ribonuclease
RNIs	Reactive nitrogen intermediates
ROIs	Reactive oxygen intermediates
rps18	Ribosomal protein S18 gene
RT	Reverse transcription
S	Spleen
SDS	Sodium dodecyl sulphate
SELE	E-Selectin
SERMs	Selective estrogen receptor modulators
SG	Spermatogenesis
SRD	Reductase
sRPMI	RPMI-1640 culture medium supplemented with 0.35% NaCl
SSC	"Side scatter", cellular granularity
StAR	Steroidogenic acute regulatory protein
T	Testosterone
T Ly	T lymphocytes
TCR	T cell receptor
TGFβ	Transforming growth factor β
Th	T helper lymphocytes
TLR	Toll-like receptors
TNF	Tumor necrosis factor
VaDNA	*Vibrio anguillarum* genomic DNA
VTG	Vitellogenin
WHO	World health organization
WWTP	Wastewater treatment plants

SUMMARY

Summary

During the development of this thesis, we have analyzed the effect of 17α-ethinylestradiol (EE_2), a potent synthetic estrogen, which is used in most oral contraceptives pills and with a widespread presence in the aquatic environment, on immune and reproductive system in the gilthead seabream (*Sparus aurata* L.). Moreover, we have determined the importance of the newly identified membrane estrogen receptor, called G protein-coupled estrogen receptor, GPER, on granulocyte biology. The gilthead seabream is a marine teleost fish with significant economic value in the Mediterranean aquaculture and has extensively being used as a model for studying the immune-reproductive interaction for being a protandrous hermaphrodite fish. The study was carried out in four consecutive stages.

In a first approach, we evaluated the effect of the exposure, through diet, to different concentrations of EE_2 on certain reproductive parameters such as the gonadosomatic index, serum levels of sex hormones and spermatogenesis, and some immune events that take place in the gonad. The results show that EE_2 interrupted spermatogenesis while promoting a testicular morphology similar to that seen in post-spawning stage but without release of sperm. Furthermore, it promoted the infiltration of acidophilic granulocyte, which are the functional equivalent of mammalian neutrophils, and B lymphocytes in the testis. This infiltration is accompanied by a dose-dependent increased expression of the genes encoding B lymphocyte markers, cytokines, chemokines and adhesion molecules, all known to be associated with leukocyte trafficking.

In a second stage of the study, and prompted by the above results, we evaluated the different degrees of sensitivity to low doses of EE_2 in specimens in two different stages of the reproductive cycle, i.e. after and during spermatogenesis. The results show that EE_2 provoked a strong estrogenic response, characterized by a strong induction of vitellogenin and by the alteration of serum levels of sex hormones. This latter observation t significantly correlated with a modulation in the expression profile of the genes encoding various enzymes involved in the synthesis of steroids. Interestingly, EE_2 wass able to modify the expression of immune-relevant genes relevant involved in the testicular physiology. We observed differences in all these effects that depended of the reproductive stage of the specimens.

Then, we aimed to investigate the capacity of EE_2 to modulate the innate immune response *in vivo* and *in vitro*. For this, specimens were bath-exposed to realistic concentrations of EE_2 and then immunized with hemocyanin in the presence of the adjuvant aluminum. The results indicate that EE_2 was able to inhibit in a dose-dependent manner the induction of interleukin-1β gene expression, but did not significantly alter the specific antibody titer. Moreover, *in vitro*, EE_2 was seen to inhibit both the production of reactive oxidative species and phagocytic activity of head kidney leukocytes and to alter the immune gene expression profile in primary macrophages. Thus, EE_2 treatment of activated macrophages, provoked decreased expression of pro-inflammatory genes and increased expression of genes encoding anti-inflammatory and tissue remodelling/repair enzymes. These results suggest that EE_2 might alter the capacity of fish to appropriately respond to infection, although this synthetic estrogen does not behave as an immunosuppressor.

Finally, a functional characterization of a recently identified membrane estrogen receptor, called GPER, was made. In a first step, we obtained the partial sequence of gilthead seabream GPER, which helped us to analyze the expression profile of GPER in gilthead seabream tissues and head kidney cell populations. We observe that GPER is expressed in reproductive and immune tissues and its expression was not modulated by specific activation thereof. Moreover, we found that acidophilic granulocytes are the head kidney cells with a higher level of expression of GPER and expressing it in a functional manner. Thus, the activation of this receptor slightly reduces the respiratory burst of these cells *in vitro* and drastically altered the expression profile of several genes encoding key pro-and anti-inflammatory mediators. Moreover, signaling by GPER *in vivo* slightly modulates the adaptive immune response. On the other hand, we found that the effects of a specific agonist of GPER (G1) in the induction of gene encoding prostaglandin-endoperoxide synthase 2, may be due, in part, to the activation of the cAMP/PKA/CREB signaling pathway, as we observed that a cAMP analogue was capable of mimicking these effects, while pharmacological inhibition of protein kinase A super-induced this expression. Moreover, G1 was able to promote the phosphorylation of CREB, an effect also mimicked by the cAMP analogue. Collectively, our results demonstrate for the first time that estrogens are able to modulate vertebrate granulocyte functions through GPER signalling and suggest therapeutic targets for several immune disorders where estrogens play a prominent role.

INTRODUCTION

0. OVERVIEW

In a world populated by approximately 7000 million of people, the demand for seafood, particularly fish, has increased fishing pressure, compromising its capacity for renewal. Extractive fishing hardly covers 70% of the annual total fishing volume (FAO, 2006), a situation to which aquaculture is seen as the only way to satisfy the demand in the near future. In Spain, located in 14th place world ranking of aquaculture producers and ranked 7th in the world ranking of exporters of fish, aquaculture accounts the 3% of world production and 25% of Europe, emerging as an area of economic activity of great strategic importance. 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, triggering stress in animals affecting the adult, juvenile and larvae. These conditions cause the development of infectious diseases that are responsible for substantial economic losses. Therefore, knowledge of the immune system of fish, in general, and of the species of crop, in particular, has become one of the primary objectives in research aquaculture.

Moreover, fish occupy a key phylogenetic position in the evolution of vertebrates representing the first animal group has an innate and adaptive immune system well structured, so that the study of the immunology of this group of vertebrates has a basic scientific interest. Thus, the vertebrate immune system has a common pattern but this does not exclude the existence of significant differences between individuals of the same species or between different species of vertebrates. The prevalence of innate immune response in fish versus the dominance of the adaptive response in higher vertebrates is the most important of these differences (Anderson et al., 2002).

There is increasing concern about the impact of several compound called “endocrine disruptor chemicals” (EDCs) able of mimicking the endocrine system and, therefore, may affect the immune and reproductive system. So, the evaluation of the possible effects of these compounds in fish immunity is an important topic, and, in particular, in species that are object to crop. Environmental estrogens are one of the principal groups of these EDCs and have been detected in aquatic environmental, but the evaluation of their effects on fish immune system is very limited. Moreover, it is a well know that estrogens are a key-modulator of immune system, acting through

classical nuclear estrogen receptor (ER). Nevertheless, some estrogen effects cannot be explained by this classical pathway, therefore, the importance of membrane ER, associated with rapid and non-genomic actions of estrogens, is recently taken into account.

The gilthead seabream (*Sparus aurata* L.) is a protandrous hermaphrodite seasonal breeding teleost with a bisexual gonad that offers an interesting model for studying immune-reproductive interactions. This is because the remodelling events of the gonad, especially during the post-spawning and testicular involution stages, compromise the immune system, and, importantly, a specific cell type present in this species, acidophilic granulocytes (AGs), cells that, however, do not express any of three nuclear ER identified in gilthead seabream.

The research group "Innate Immune System of Teleost Fish" which has developed this PhD thesis, has a long history in the study of the immune sytem and immure-reproduction of aquaculture interest Mediterranean species such as sea bream (*Sparus aurata* L.) and sea bass (*Dicentrarchus labrax* L.), species that are commercially important to the economy of the Region of Murcia and the national economy.

Located in the above framework, this thesis focuses on the study of effects of EE_2, apart from evaluating the expected estrogenic response, on the gonadal local immunity and in systemic immune response of seabream. On the other hand, we explore the ability of EE_2 exposure to reduce the capability of fish to properly response to an induced immune response by altering some leukocytes immune activities. Finally, we study the implication of a new identified membrane ER on immune system of seabream, showing the importance of this receptor in the regulation of the granulocytes biology.

1. IMMUNITY

Immunity is a reaction to foreign substances including microorganisms (viruses, bacteria, fungi, protozoa and multicellular parasites) and macromolecules (proteins and polysaccharides), without involving the pathological result of such a reaction (Abbas et al., 2001). The immune system is composed of cells and molecules that are responsible for immunity, and the collective and coordinated response against these foreign substances constitutes the immune response. Due to the wide variety of infectious agents, requires a variety of immune responses to combat each type of infection (Male and Roitt, 1996).

The immune response starts with the recognition of the pathogen or foreign material, and ends with the development of a mechanism able to remove it (Male and Roitt, 1996). The immune response can be divided into two branches: innate (natural or non-specific) and adaptive (acquired or specific). The fundamental difference between them is that the adaptive is highly specific for a particular pathogen and is most effective with each successive encounter with the same pathogen. Therefore, we can say that the two key features of the adaptive immune response are specificity and memory (Male and Roitt, 1996).

Innate immune response include physical barriers, phagocytic cells and eosiniohils, natural killer cells and various molecules from blood molecules (complement and acute phase proteins) (Male and Roitt, 1996; Abba et al., 2001; Mollen et al., 2006), acting first line of defense against infection until specific response is triggered.

Adaptive response including lymphocytes (Ly) and antibodies secreted and appears exclusively in vertebrates (Abbas et al., 2001). The Ly are capable of specifically recognizing individual pathogens for which there are two main categories of cells: T Ly (T cells) and B Ly (B cells). B cells are responsible for recognizing and combating extracellular pathogens and their products through their secretions, antibodies, which have the property of binding to a target molecule called an antigen. T cells, in turn, have a large number of activities. T helper Ly (Th) are involved in the regulation of development and production of antibodies (Ab) by B cells or interacting with phagocytic cells helping them to destroy the pathogens that they have

phagocytosed. Another type of T Ly, cytotoxic T cells (CTL) recognize and destroy virus-infected cells and tumor cells.

The innate and adaptive response acts in an integrated and coordinated form. There is considerable interaction between Ly and phagocytes (Male and Roitt, 1996). For example, some phagocytes capture and degrade antigens and present them to T cells attached to the surface major histocompatibility complex (MHC) in a form suitable for them to recognize. This process is called antigen presentation. In response, the Ly secrete soluble factors (cytokines) which activated phagocytes to destroy the pathogens that they have phagocytosed. 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 lymphocytes subsequently begin to generate the adaptive response.

1.1. Immune system of teleost fish

In teleost fish, the immune system shows similar characteristics to those of birds and mammals, displaying cellular and humoral responses that have the characteristics of specificity and memory (Van Muiswinkel, 1995). Teleost are the first animal group that have an innate and adaptive immune system well structured and differenciated. Its innate response comprises physical barriers barriers (epithelium and mucosa), cellular effectors (phagocytic cells and nonspecific cytotoxic) and humoral factors (complement and other acute phase proteins) and adaptive response comprises a cellular component (lymphocytes) and other humoral (antibodies). 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 poikilothermic) since the adaptive immune response is dependent on the temperature (Cuchens and Clem, 1997; Avtalion, 1981; Abruzzini et al., 1982; Clem et al., 1984; Clem et al., 1985; Clem et al., 1991).

1.1.1. Immune system organ

The organs and tissues of the immune system in teleosts have been classified, as in mammals, in primary and secondary organs (Zapata et al., 1996). Fish lack of bone marrow, being kidney, a primary organ, the par excellence hematopoietic organ. Kidney consists of two parts: the anterior or cephalic (head kidney, HK), with mainly

hematopoietic function, and subsequent or posterior, basically with excretory function. In gilthead seabream, HK is formed by precursor cells and by several leukocytes populations such as macrophages (MΦ), Ly and AGs. AGs are the major cell type participating in innate host responses, while the head kidney is the central immune organ that provides a source for AGs (Sepulcre et al., 2002; Chaves-Pozo et al., 2007). Regarding to secondary lymphoid organ, the spleen is the most important but presents few lymphocytes although may increase in number by administration of an antigen.

1.1.2. Innate immune system

The first line of defense of fish against invasion of microorganisms consists by physical and chemical barriers such as scales, skin and its secretion, mucus. The most important function of the mucus is to prevent attachment of bacteria, fungi or parasites to epithelial surfaces and digest microorganisms, thanks to a battery of lytic enzymes such as lysozyme.

Innate cellular response of the fish includes a variety of leukocytes, they include phagocytes (monocytes/ MΦ 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; Secombes and Fletcher. 1992; Sepulcre et al., 2002) and, moreover, can be the initiator of activation and regulation of the specific immune response (Clem et al., 1985; Clem et al., 1991; Vallejo et al., 1992). The process of phagocytosis in fish has the same steps as described for mammalian leucocytes, ending with two mechanisms responsible for the killing of phagocytized microorganisms: (i) production of reactive oxygen intermediates (ROIs) with a rapid and abrupt increase in the rate of oxygen consumption is known as explosion or respiratory burst and is independent of mitochondrial respiration, and (ii) the production of nitric oxide (NO) and other nitrogen reactive intermediates (RNIs). It is further known that ROIs produced by phagocytes of fish have bactericidal activity (Sharp and Secombes, 1993; Skarmeta et al., 1995).

Specifically, studies in gilthead seabream describe AGs as the more active and abundant phagocytic cell of the species (Sepulcre et al., 2002; Chaves-Pozo et al., 2004a). 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 ROIs production capabilities (Sepulcre et al., 2002;

Sepulcre et al., 2007), produce cytokines (see below) in response to several immunological stimuli (Chaves-Pozo et al., 2004a; Sepulcre et al., 2007) and express a broad range of Toll like receptors, TLRs (see below), although not TLR3 (Sepulcre et al., 2007).

Beside cellular effectors described above, there are a wide variety of substances (humoral effectors) that act on the innate defense of fish (Alexander and Ingram, 1992). These may be classified functionally into: (i) bacterial growth inhibitors such as transferrin, antiproteases and ceruloplasmin, (ii) viral replication inhibitors such as interferon, (iii) inhibitors of bacterial toxins, (iv) lysines such as lysozyme and chitinase, (v) agglutinins and precipitins such as lectins and C-reactive protein, and finally (vi) complement components that perform several functions, among which leukocyte chemotactic activity (Lamas and Ellis, 1994), opsonisation (Sakai, 1984a), inactivation of certain toxins (Von Eschen and Rudbach, 1974; Ellis, 1980; Sakai, 1984b), the bactericidal activity (Sakai, 1983), cytotoxicity and viral inactivation (Sakai, 1992).

1.1.3. Adaptive immune system

Antibodies are key mediators of the adaptive immune response, together with its producing cells, Ly. *In vitro* studies have shown that fish have two cell populations which are equivalent to B and T cells of mammals. These studies using conjugated monoclonal antibodies (mAbs) against specific antigenic determinants on the surface of Ly and functional immunological assays. mAbs against immunoglobulin M (IgM) of teleost serum are capable of reacting with only one of the Ly populations (Lobb and Clem, 1982; DeLuca et al., 1983; Secombes et al., 1983; Navarro et al., 1993), suggesting that the surface Igs may be a marker for B cell-like cells and allowing the isolation of the two cell populations of lymphocytes of teleost: Ig+ e Ig-. Further, performing some functional studies, it have been shown that these two populations fo fish (Ig+ and Ig- Ly) have the functional characteristics of the B and T Ly of mammals, respectively (DeLuca et al., 1983; Sizemore et al., 1984; Miller et al., 1986; Marsden et al., 1995). It has been confirmed the existence of T cells in the teleost so genes coding the T cell receptor (TCR) (Haire etl al., 2000; Wang et al., 2001; Wermenstam and Pilstrom, 2001; Nam et al., 2003).

Likely mammalian, adaptive immune system of teleost presents memory (Van Muiswinkel, 1995). Thus, after a first contact with the antigen (primary response) produces a specific titer of antibodies in the serum, that is increased in a subsequent contact with the same antigen (secondary response), being this response dependent on temperature. The most common form of Ig in the serum of the teleost is a tetrameric form usually called IgM due to its high molecular weight and its polymeric structure. This Ig has several functions, including activation of the complement cascade that ends lysing the invading pathogen and binding to the surface antigens of the invading cells or virus-infected cells, thus indicating the target for subsequent action of cytotoxic and phagocytic cells.

As has been previously mentioned, the initiation of the adaptive immune response is controlled by MΦ which functions as antigen presenting cells (APC), degrading and presenting antigens together with MHC proteins to Ly so that they can recognize (Clem et al., 1985; Vallejo et al., 1992). Meanwhile, Ly may produce cytokines that activate MΦ after stimulation with an antigen (Graham and Secombes., 1988), showing, therefore, a coordinated and mutual control between the innate and adaptive response.

1.2. Regulatory molecules of the immune response

1.2.1. Cytokines

Cytokines are proteins (usually glycoproteins) with a low molecular weight (usually no more than 8-25kDa) that regulate all the important biological processes, including cell growth and activation, inflammation, tissue repair, fibrosis and morphogenesis. They are considered as a protein family from a functional point of view, since not all of them are chemically related (Feldmann, 1996). However, some cytokines share a high homology (about 30%), like interleukin-1β (IL-1β) and IL-1α, or tumoral necrosis factor-α (TNFα) and TNFβ. In addition, there are subfamilies with a really high structural homology (about 80%), like the interferon α (IFNα) subfamily with about 20 members.

Cytokines mediate effector phases in both innate and acquired immunity (Abbas et al., 2001). In the innate immunity, cytokines are produced mainly by mononuclear phagocytes and so are usually called monokines. Monokines are produced by

mononuclear phagocytes in response to microorganisms and upon T-cell antigen stimulation as part of acquired immunity. However, most of the cytokines involved in acquired immunity are produced by activated T lymphocytes and these molecules are referred to as lymphokines. Lymphokines present a double function, either regulating the proliferation and differentiation of different lymphocytes populations or participating in the activation and regulation of inflammatory cells (mononuclear phagocytes, neutrophils and eosinophils). Both lymphocytes and mononuclear phagocytes produce other cytokines known as colony stimulating factors (CSFs), which stimulate the proliferation and differentiation of immature leukocytes in the bone marrow. Some other cytokines known as chemokines are chemotactic for specific cell types.

Although cytokines are made up of a diverse group of proteins, they share some features (Abbas et al., 2001):

- They are produced during the effector stages of the innate and acquired immunity, and regulate the inflammatory and immune response.
- Their secretion is brief and auto-limited. In general, cytokines are not stored as pre-formed molecules, and their synthesis is initiated by a new genetic transcription.
- A particular cytokine may be produced by many different cellular types.
- A particular cytokine may act on different cell types.
- Cytokines usually produce different effects on the same target cell, simultaneously or not.
- Different cytokines may produce similar effects.
- Cytokines are usually involved in the synthesis and activity of other cytokines.
- Cytokines perform their action by binding to specific and high affinity receptors present on the target cell surface. This action can be autocrine, paracrine or endocrine.
- The expression of cytokine receptors is regulated by specific signals (other cytokines or even the same one).
- For many target cells, cytokines act as proliferation factors.

In fish, cytokines are grouped into growth factors (Grondel and Harmsen, 1984; Lawrence, 1996; Yin et al., 1997), pro-inflammatory cytokines (Jang et al., 1995a, b; Zou et al., 1999a, b; Fujiki et al., 2000), chemokines (Daniels., 1999; Fujiki et al., 1999;

Laing et al., 2002), immunosuppressive or anti-inflammatory cytokines (Sumathy et al., 1997; Laing et al., 1999; Harms et al., 2000) and IFNs (Congleton and Sun, 1996; Collet and Secombes, 2002; Hansen and La Patra, 2002).

1.2.2. Lipid mediators

Lipids, in addition to functioning as an energy source and as structural components of the cell membrane act as effectors and second messengers in a variety of biological processes (Cabral, 2005). These lipid mediators differ in the structural composition and exert multiple effects on cellular functions associated with homeostasis, immune response and inflammation.

Bioefectores lipids produced as a result of cutting the cell membrane phospholipids by the action of phospholipases. These enzymes are activated in response to a variety of extracellular stimuli such as bacterial peptides, cytokines, growth factors and mechanical trauma. Lipid mediators may act as second messengers intracellularly or extracellularly by signaling through G-protein coupled receptors. For their ability as immune effector molecules, lipids bioefectores complement the activities of proinflammatory and anti-inflammatory non-lipid immune modulators.

Arachidonic acid is the precursor of the immune active lipids, collectively called eicosanoids, which include prostanoids, leukotrienes lipoxins and endocannabinoids. Major producer of eicosanoids bioefectores are cells or derived from myeloid lineage such as platelets, monocytes, macrophages, neutrophils and mast cells (Harizi and Gualde, 2002), with the exception of leukotrienes that are produced by other types non-immune cell. Several enzymes regulate cellular levels of arachidonic acid and kept in esterified form until it is mobilized by phospholipases. Inflammatory stimuli induce the translocation of cytosolic phospholipase to the endoplasmic reticulum and nuclear membrane thereby releasing arachidonic acid from the lipid membrane of these organelles.

An important group of these lipid mediators are prostanoids which includes protagalandinas (PTG) and thromboxanes. These molecules are synthesized *de novo* by the action of two cyclooxygenase isoforms, COX-1 or COX-2 (PTGS2) from arachidonic acid released from cell membrane after being activated by various stimuli. These enzymes act upstream of a variety of isomerases whose action culminates in the

production of PGA2, PGD, PGE 2, PGI 2 and TXA. COX-2 is involved in the synthesis of pro-inflammatory prostaglandins. Its expression is very low or undetectable in the majority of cells and its expression increases significantly after stimulation, particularly in immune system cells (Smith et al., 1996).

The mode of action of these prostaglandins depending of the receptor with which interacts since the receptor of pro-inflammatory prostaglandins can activate opposite signalling pathways. In this way, they can act as anti-inflammatory or pro-inflammatory mediators depending on context.

1.3. Innate immune system receptors

The innate immune system uses a wide spectrum of receptors that recognize pathogen-associated molecular patterns (PAMPs) and have been called pattern recognition receptors (PRRs). These receptors may be present on the cell surface, into intracellular compartments, in the cytosol or may be soluble in blood or tissue fluids (Mendzhitoz and Janeway, 2000). The main functions of these receptors include opsonization, activation of complement cascades and coagulation, phagocytosis, activation of pro-inflammatory signaling pathways and the induction of apoptosis (Mendzhitoz and Janeway, 2000). The main group of membrane receptor PRRs are receptors similar to *Toll* proteins of Drosophila melanogaster ("Toll-Like Receptors", TLRs). These receptors differ among themselves in their specificity for the ligands. In gilthead seabream, the animal model of this study, AGs express a broad range of TLRs, although not TLR3 (Sepulcre et al., 2007), while TL9 is the only one which is expressed in the gonad (Chaves-Pozo et al., 2008a).

2. REPRODUCTIVE SYSTEM

In vertebrates there is a great diversity of breeding strategies that makes that gametogenic activity is constant throughout the year, or cyclic, in the case of vertebrates with seasonal breeding (Johnson and Nguyen, 1986; Sinha et al., 1988). In the latter case, the gonad undergoes significant fluctuations in the gametogenic activity as well as tissue remodeling processes.

2.1. Reproductive system of teleost fish

Fish are the group of vertebrates with greater diversity of reproductive strategies. So there are species that develop functional single sex throughout their life (gonochorists) and species with functional sexes throughout their life (hermaphrodites). Gonochorists species can be: i) primary or differentiated, if only develops a gonadal type, ii) undifferentiated, if all individuals first develop an ovary undifferentiated, which degenerates in half of the population to develop a functional testicle or iii) secondary, if develops a hermaphrodite gonad that subsequently evolves toward a single sex. Hermaphrodite species can be sequential, may be protogynous or protandrous according to the sex that develop earlier, female or male, respectively, or may be species ambisexuales if both gonads are developed simultaneously (Devlin and Nagahama, 2002).

Similarly to what happens with breeding strategies, reproductive cycles (RCs) are very diverse, depending on environmental factors such as photoperiod, temperature, osmolarity, availability of food, etc. These reproductive cycles can be classified based on the length of the laying period. So there are i) species such as salmonids, typical of cold weather, that lay for several weeks a year, ii) species such as gilthead seabream and common carp, typical of temperate weather, that lay for several months a year and iii) species typical of tropical climate that lay throughout the year. This behavior is reflected in the reproductive cycle, so in seasonal breading species, this cycle can be divided into four stages: gametogenic activity, spawning, post-spawning and resting while species that laying throughout the year lacking of post-spawning and quiescence stages (Scott et al., 1987; Miura, 1999a). However, despite this great diversity of strategies and reproductive cycles, morphological and functional characteristics of the gonad of teleosts are highly conserved even in hermaphroditic species (Le Gac and Loir, 1999).

2.2. Sex steroid hormones synthesis and hormonal regulation of reproductive processes

The regulation of the processes describe above depends largely on gonadotropins (GTHs), synthesized and secreted by the pituitary. In most teleost species studied, as well as other higher vertebrates, there is a duality of GTHs structurally homologous to the follicle stimulating hormone (FSH) and luteinizing hormone (LH) of mammals (Schulz et al., 2001). In gilthead seabream has been described during ontogeny and location of the cells producing FSH and LH from the pituitary (García-

Ayala et al., 2003). Moreover, receptor for these two hormones (FSH-R and LH-R) has been described in gilthead seabrem (Wong et al., 2003). However, these hormones do not act directly on spermatogenesis but induce the secretion of steroids and/or growth factors in different cell types of the gonad, which are involved in the complex network of cellular interactions that regulate testicular function (Miura et al., 1999b).

Thus, reproduction in fish is under the hormonal regulation of gonadal steroids (Fostier et al., 1983; Kime, 1993). Similar to other vertebrates, gametogenesis and sex behaviors in fish are directly controlled by sex steroid hormones. The synthesis of sex steroid hormones (steroidogenesis) mainly takes place in adrenal tissues and gonadal tissues (male testes and female ovaries) (Young et al., 2004). A schematic representation of the key steps involved in steroidogenesis in teleosts is shown in Figure 1. Cholesterol is the common precursor for all sex steroid hormones. The first rate-determining step involves the importation of cholesterol into the inner mitochondrial membrane. This step, which initiates steroidogenesis, is regulated by the steroidogenic acute regulatory protein (StAR), and the production of StAR is upregulated by GTH in fish (Bauer et al., 2000; Stocco, 2001; Kusakabe et al., 2002). Subsequent steps of the steroidogenic pathway are controlled by a number of steroidogenic enzymes including cytochrome P450 enzymes (cyp) and hydroxysteroid dehydrogenases (HSDs) (Miller, 1988; Senthilkumaran et al., 2004; Weltzien et al., 2004; Miller, 2005) that produced end-product hormones such as testosterone (T), dihydrotestosterone (DHT), 17β-estradiol (E_2) and estrone. DHT is one of the most physiologically important androgens in many male vertebrates (George and Wilson, 1994) with the exception of teleost fish, in which T and 11-ketotestosterone (11KT) are generally considered the major circulating male androgens, as well as the most potent ones (Borg, 1994). T levels increase in both females and males during gonadal development, while 11KT is considered to be a dominant androgen in males (Nagahama, 1983; Kime, 1993; Borg, 1994). On the other hand, E_2 has been considered to be the main hormone of female fish; however, recent studies have suggested that estrogens are ‘‘essential” for normal male reproduction (Miura et al., 1999b; Amer et al., 2001; Miura et al., 2002; Hess, 2003; Miura et al., 2003). The steroidogenic enzymes are primarily regulated at the transcriptional level under the control of the pituitary gland (Omura and Morohashi, 1995). Thus, any interference with their transcription may alter the production of sex hormones. Increasing evidence shows that genes regulating steroidogenesis are

important target sites for various endocrine disrupting chemicals (see below) (Thibaut and Porte, 2004; Sanderson, 2006).

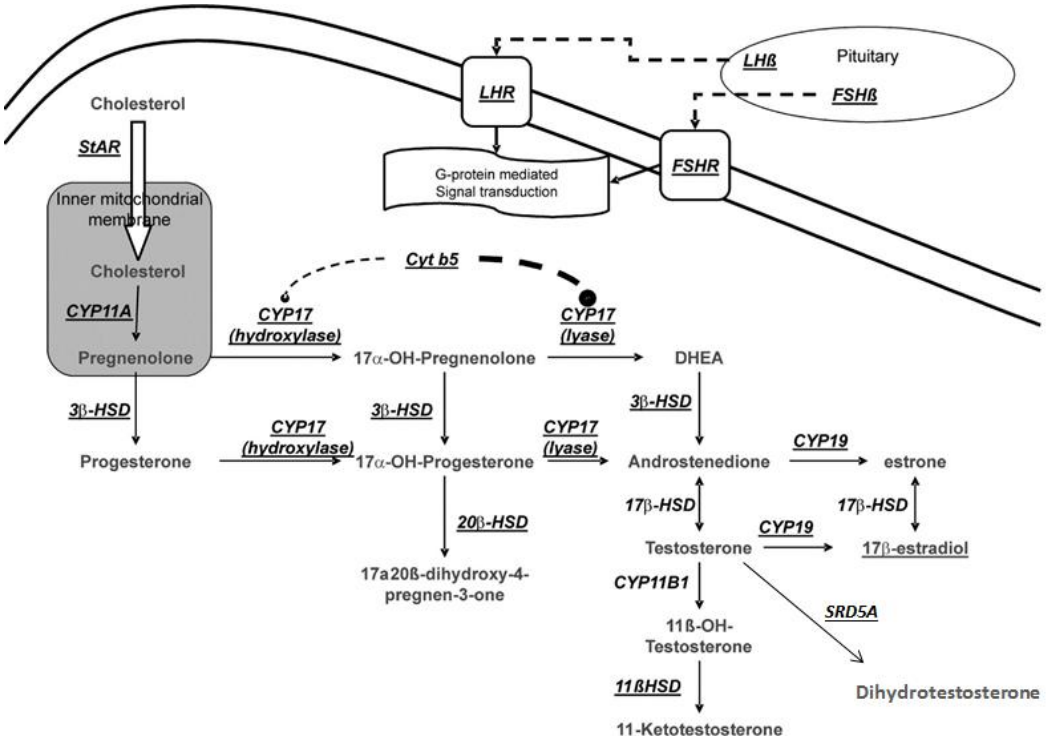

Figure 1. Schematic representation of the key steps involved in steroidogenesis in teleosts (Villeneuve et al., 2007).

2.3. Reproductive system of gilthead seabrem

The gilthead seabream is a protandrous hermaphrodite seasonal breeding teleost with a bisexual gonad that is composed of an ovarian area, mediodorsal, and testicular area, lateroventral. Specimens of gilthead seabream are developed, functionally, as males during the first and second RCs, although their gonads possess a non-developed ovarian area separated from the testicular area by connective tissue move to females (D□Ancona, 1941; Pascuali, 1941; Zohar et al., 1978; Chaves-Pozo et al., 2005; Liarte et al., 2007), but this depending on the natural environment of the populations studied. As have been described in this species, the RC of males consists of four stages as has been mentioned above: gametogenic activity (spermatogenesis), spawning, post-spawning and resting (Figure 2), in which the levels of E_2, T and 11KT varied (Chaves-Pozo et al., 2008b). Resting is replaced by a testicular involution stage when the fish are ready to undergo sex change (Chaves-Pozo et al., 2005a; Liarte et al., 2007). During

stages of post-spawning and quiescence, the gonad of gilthead seabream undergoes significant morphological changes, the result of tissue remodeling, leading to the elimination of the remaining sperm of previous cycle and germinal tissue reorganization (Chaves-Pozo et al., 2005a). These processes are correlated with a marked increase in serum levels of E_2 and the decrease in androgens, T and 11-KT, the main androgen in this species (Chaves-Pozo et al., 2008b).

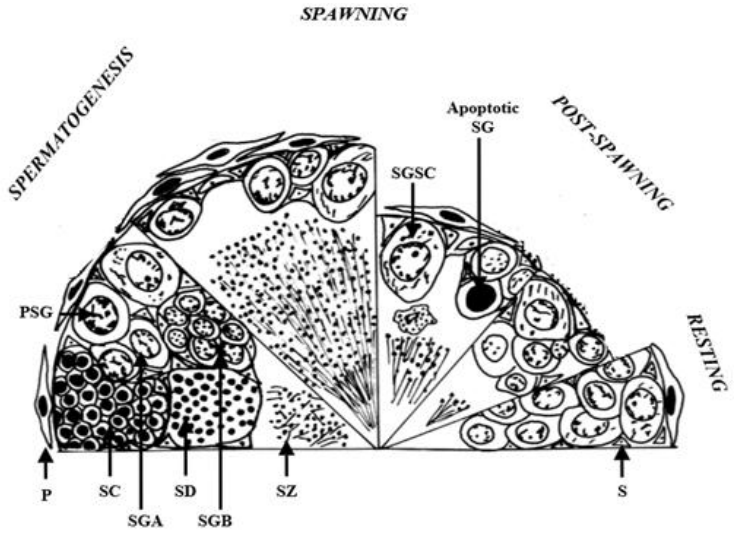

Figure 2. Schematic illustration of the changes in the testis of gilthead seabream. Germinal compartment. SGSC, spermatogonia stem cell. PSG, primary spermatogonia. SGA, spermatogonia A. SGB, spermatogonia B. SC, spermatocyte. SD, spermatid. (Chaves Pozo et al., 2008).

3. ESTROGEN RECEPTORS SIGNALLING AND EXPRESSION PATTERN

Traditionally, estrogens act via classical nuclear estrogens receptor, ERα and ERβ. Nevertheless, there are physiological responses to estrogens that cannot be explained by the activation of classical nuclear ERs. So, the relevance of membrane ERs has been increasing attention.

3.1. Nuclear estrogen receptor: ERs

The classical nuclear estrogens receptor, ERα and ERβ, both belong to the nuclear receptor superfamily (Evans and Bergeron, 1988). Nuclear receptors (NRs) have a DNA binding domain and a ligand binding domain (Wahli et al., 1991; Chawla et al., 2001).

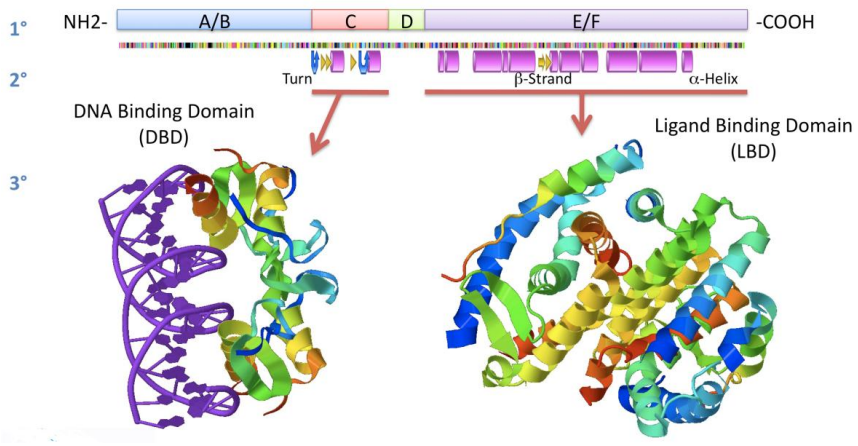

Figure 3. Schematic illustration of structure of nuclear receptors (NRs). Nuclear Receptor Resourece (Wahli et al., 1991; Chawla et al., 2001).

These ERs can shuttle between the cytoplasm and the nucleus and function as ligand-activated nuclear transcription factors that bind (direct or indirectly) *cis*-acting estrogen response elements (ERE) in the promoter regions of target genes and enhancer regions (Deroo and Korach, 2006) and forming the genomic actions of estrogens.

The expression of ERs is given in reproductive organs but also widely in immune cells (Straub, 2007). Moreover, in different mammalian models, the preponderance of ERα gene over the ERβ gene is accepted as being one of the mechanism that control the effects of E_2 on the immune system (Straub, 2007). In teleost, depending on the species studied, three or four different ERs genes have been described. Thus, in some species (gilthead seabream, atlantic croaker, zebrafish, goldfish) one ERα and two ERβ have been cloned, while in others (rainbow trout and Spinibarbus denticulatus) two ERα and two ERβ were found (Nagler et al., 2007; Iwanowicz and Ottinger, 2009. In order to determine whether immune tissues are potential targets for estrogens, several studies have looked at the expression of ERs in immune tissues. In immature and mature male and female channel catfish, for example, ERα is expressed in spleen, blood and head-kidney, while ERβ is only expressed in spleen (Xia et al., 2000). ERβ is expressed in the spleen and head-kidney of male and female common solea (Caviola et al., 2007). As has been mentioned above, in our animal model, gilthead seabream, three nuclear ERs, ERα, ERβ1 and ERβ2 have been described. These ERs are expressed in both reproductive and non-reproductive organs such as liver, brain, heart, kidney, intestine, gills, muscle and skin (Pinto et al., 2006). Moreover, ERs are expressed in the gilthead seabream HK cells populations. Thus,

gilthead seabream MΦ constitutively express only the ERα gene, although stimulation with phenol-extracted genomic DNA from the bacterium Vibrio anguillarum ATCC19264 cells (VaDNA) drastically up-regulates the expression of ERα, ERβ1 and ERβ2 genes, suggesting that the immune system is able to increase its sensitivity to E_2 during development of the immune response (Liarte et al., 2011a). HK Ly only express the ERα gene and interestingly, neither testicular nor HK AGs express any of the three nuclear ERs (Liarte et al., 2011a).

3.2. G protein-coupled estrogen receptors: GPER

G protein coupled receptors (GPCRs), also known as seven-transmembrane domain receptors, 7TM receptors, are membrane proteins that are involved in a broad range of biological processes, and a large number of clinically used drugs elicit their biological effects via a GPCR (approximately 40% of all modern medicinal drugs). And as a curiosity, the Nobel Prize in Chemistry 2012 was awarded jointly to Robert J. Lefkowitz and Brian K. Kobilka *"for studies of G-protein-coupled receptors"*

These receptors sense molecules outside the cell and activate inside signal transduction pathways and, ultimately, cellular responses. The ligands that bind and activate these receptors include light-sensitive compounds, pheromones, hormones and neurotransmitters. When a ligand binds to the GPCR it causes a conformational change in the GPCR, which allows it to act as a guanine nucleotide exchange factor. The GPCR can then activate an associated G-protein by exchanging its bound GDP for a GTP. The G-protein's α subunit, together with the bound GTP, can then dissociate from the β and γ subunits to further affect intracellular signaling proteins or target functional proteins directly depending on the α subunit type (Gαs, Gαi/o, Gαq/11, Gα12/13) (Dorsam and Gutkind, 2007).

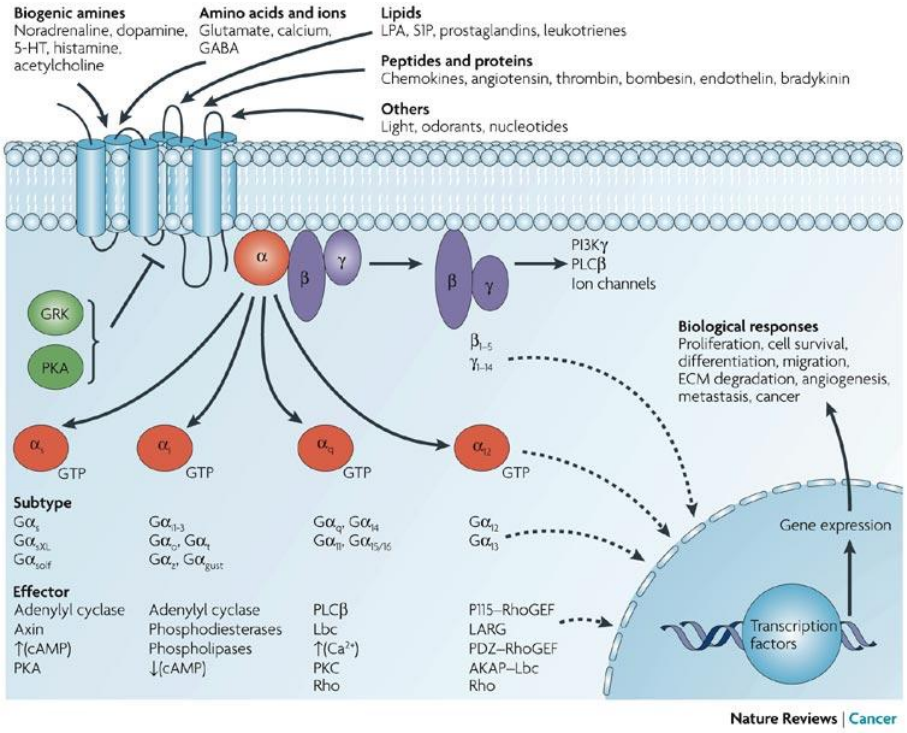

Figure 4. Diversity of G-protein-coupled receptor signalling. (Dorsam and Gutkind, 2007).

Previously rapid effects of estrogens had been identified but it was not until 2005, when an orphan GPCR, GPR30, now officially designated GPER (Alexander et al., 2011), was identified as an estrogen-binding intracellular membrane GPCR (Revankar et al., 2005; Thomas et al., 2005). It was later shown that GPER is activated by E_2 (Filardo et al., 2002; Funakoshi et al., 2006; Filardo et al., 2007). Up to now, the mechanisms described related with GPER activation include:

- Rapid activation of MAPKs, ERK-1 and ERK-2 activation through the transactivation of epidermal grown factor receptor (EGFR) (Filardo et al., 2000).
- PI3K signaling activation (Revankar et al., 2005).
- Activation of adenylate cyclase (AC) and subsequent cAMP activation (Filardo et al., 2002).
- Intracellular calcium mobilization (Revankar et al., 2005).

Most of these mechanisms have been in reviewed in (Prossnitz and Barton, 2009; Prossnitz and Maggiolini, 2009; Wang et al., 2001). The activation of GPER by G1, a GPER selective agonist (Bologa et al., 2006), does not trigger ERE-mediated activation but up-regulates the proto-oncogene CFOS by means of a non-genomic mechanism similar to E_2 (Albanito et al., 2007). It has been described that both the affinity and the

signaling pathway of GPER are conserved in both mammals and fish (Thomas et al., 2010). Moreover, environmental estrogens (see below) and selective estrogen receptor modulators (SERMs) such as tamoxifene (known antagonist of ERs) are able to bind GPER (Thomas and Dong, 2006; Watson et al., 2011). This latter fact is related with the development of cancer in other tissues (uterus and endometrium) when tamoxifene is used in breast cancer treatment (Goldstein, 2001).

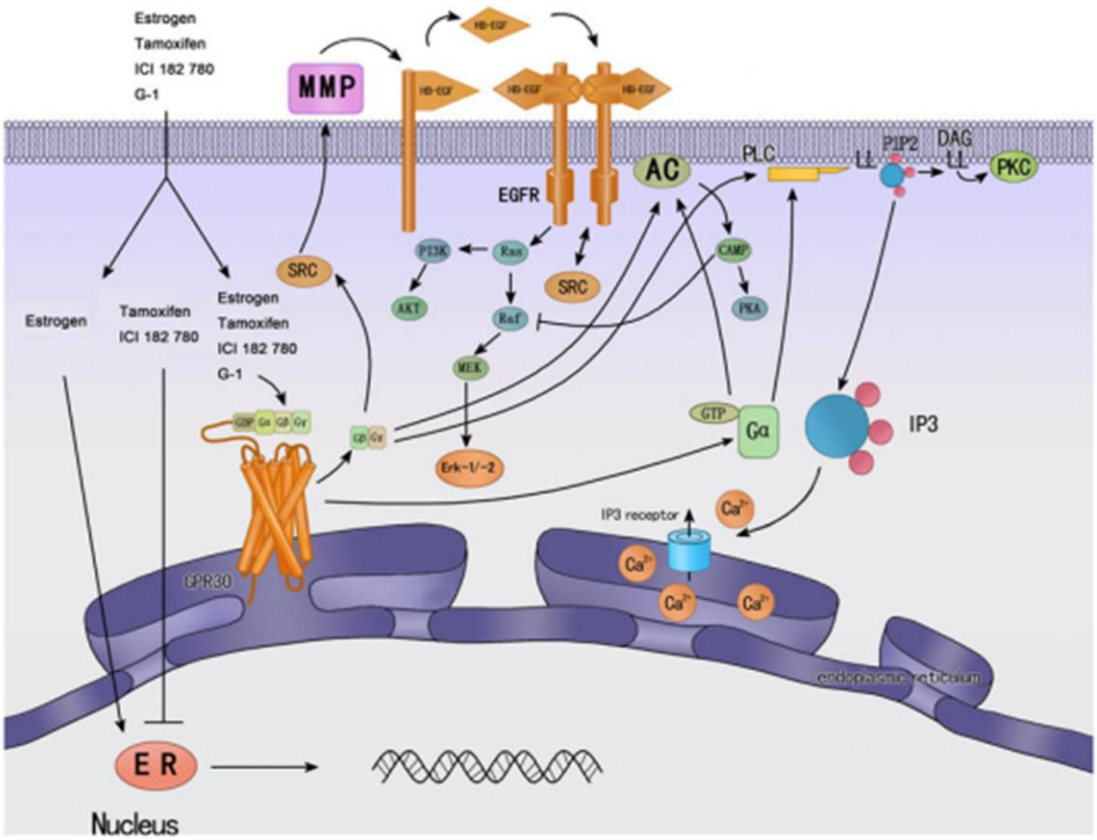

Figure 5. Cellular signaling mechanisms of GPER and classic nuclear estrogen receptors ERs (↓ activate; ⊥ inhibit). ERs are widely accepted as mainly mediating gene transcriptional regulation. Tamoxifen is an ER antagonist in some tissue, such as breast cancer, while has agonistic effects in other tissues, such as endometrium. GPER was found predominantly in the endoplasmic reticulum; estrogen and tamoxifen can bind GPER, and then activate multiple cellular effectors, such as ERK, PI3K, and PLC, and other rapid cellular processes. Most of them are mediated by transactivation of EGF-R (Wang et al., 2010).

On the other hand, it is know that increases in cellular cAMP stimulate protein kinase A (PKA) signalling. cAMP binds to the regulatory (R) subunits of PKA, thereby promoting their dissociation from the catalytic subunits. The liberated catalytic subunits enter the nucleus by passive diffusion and phosphorylate the cAMP-responsive element (CRE)-binding protein (CREB) at Ser133. Phosphorylated CREB (p-CREB) promotes

target gene expression at promoters containing CREs (Altarejos JY and Montminy M, 2011)

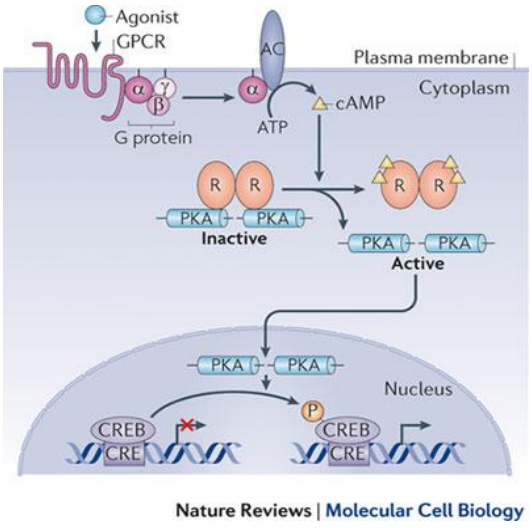

Figure 6. cAMP stimulates CREB phosphorylation (Altarejos JY and Montminy M, 2011).

Although from the identification of GPER there has been increasing attention to this receptor in recent years, there is not much information concerning the expression profile of GPER. The expression of GPER in sexual organs has not been analyzed in detail up to now. Moreover, the mRNA for GPER appears to be expressed extensively in most tissues as judged from the overall reports (Mizukami, 2010; Olde and Fredrik Leeb-Lundberg, 2011). In mammals, the expression of GPER in MΦ and Ly, among other immune cells, has also been described (Owman et al., 1996; Blasko et al., 2009; Rettew et al., 2010; Brunsing and Prossnitz, 2011). Moreover, in human, it has been described that GPER is expressed in haematopoietic stem cells but not in mature megakaryocytes (Di Vito el al., 2010). In fish, GPER has been detected in Atlantic croaker and zefrafish gonads (Pang et al., 2008; Liu et al., 2009; Pang and Thomas, 2009). Recently, it has been review the involvement of GPER action in regulation of physiological responses and disease, so, GPER is implicated in neuroendocrine and cerebral functions, immune cell function, endocrine regulation and metabolism, cardiovascular and kidney function, and reproductive functions. Collectively, these studies suggest the therapeutic potential of regulating GPER activity as a novel approach for the treatment of these conditions (Prossnitz and Barton, 2011).

Overall, physiological responses to estrogens are often categorized as rapid/non-genomic or genomic, although is much evidence that these artificially defined categories

are connected (Moriarty et al., 2006; Ma and Pei, 2007). Moreover, rapid signaling events initiated by GPER have been shown to regulate gene expression (Prossnitz and Maggiolini, 2009). Thus, it is possible to stablish four estrogen and estrogen receptor signaling pathways (Prossnitz and Barton, 2011) as is shown in Figure 8.

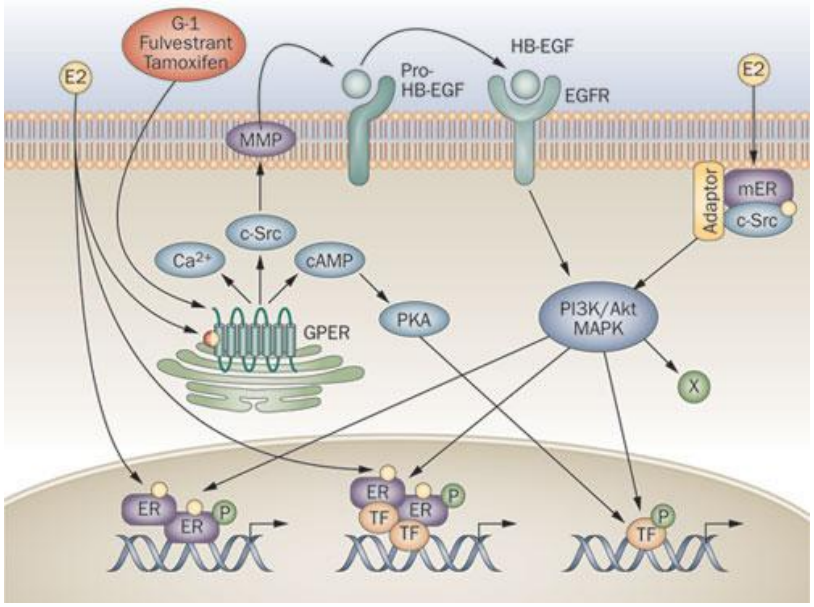

Figure 7. Nongenomic and genomic estrogen signaling pathways. Endogenous estrogens including 17β-estradiol are nonselective activators of the three known ERs, ERα, ERβ and GPER. 17β-Estradiol activates nuclear ERs, inducing receptor dimerization and binding of receptor dimers to the promoters of target genes. Alternatively, activated ERs modulate the function of other classes of TFs through protein–protein interactions. Subpopulations of ERs at the plasma membrane activated by E_2 interact with adaptor proteins (adaptor) and signaling molecules such as c-Src, which mediates rapid signaling via PI3K–Akt and MAPK pathways. E_2, 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 GPER, which is predominantly localized intracellularly. GPER activation stimulates cAMP production, calcium mobilization and c-Src, which activates MMPs. These MMPs cleave pro-HB-EGF, releasing free HB-EGF that transactivates EGFR, which in turn activates MAPK and PI3K–Akt pathways that can induce additional rapid (nongenomic) effects (X), or genomic effects regulating gene transcription. E_2-mediated transcriptional regulation may involve phosphorylation (P) of ER or other TFs that may directly interact with ER, or bind independently of ER within the promoters of target genes. 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).

4. IMMUNE-REPRODUCTIVE INTERACTIONS

It is widely accepted that females of all ages experience significantly lower rates of infection and resultant mortality than men. This significant difference in the inflammatory response of women compared with that of men has long been noted (Grossman, 1985; Olsen and Kovacs, 1996). This heightened inflammatory response is advantageous in response to infection and sepsis but is unfavorable in immune responses against self, leading to an overall increased rate of autoimmune diseases in women compared to men (Olsen and Kovacs, 1996; Tabal, 1981). There is still the unresolved paradox with respect to theimmunomodulating role of estrogens. On one side, it is recognized inhibition of bone resorption and suppression of inflammation in several animal models of chronic inflammatory diseases. On the other side, it is observded the immune supportive role in trauma/sepsis and the proinflammatory effects in some chronic autoimmune diseases in humans.

Immune-reproductive interactions can be viewed from two points of view, one where certain populations of immune cells, or the immune mediators that they produce, present in the gonad, are capable of regulating the physiology of the same. Furthermore, steroid hormones are capable of regulating activities and physiology of immune cells. It is known that, in mammalian, exist populations of testicular macrophages, lymphocytes and mast cells. The resident leukocyte populations in rat testis interact and regulate testicular function. Thus, macrophages regulate Leydig cell development, spermatogenesis and steroidogenesis through the production of protein factors or soluble lipid (Hedger, 1997; Nes et al., 2000). Furthermore, factors produced by T cells and macrophages, along with the production of transforming growth factor (TGF)-β, activin or Fas ligand by Sertoli cells, guarantee testicular immunosuppression and the inhibition of cytotoxic T cells preventing the development of autoimmune reactions in the testis (Hedger, 1997). Moreover, steroid and pituitary hormones regulate the functions and responses of no-testicular leukocytes. Thus, changes in E_2 plasma levels along the ovarian cycle of hamster have been correlated with variations in the production of IL-1 by peritoneal macrophages (Yoshida et al., 1996). Moreover, in human neutrophils, the E_2 regulates its own receptors (Molero et al., 2002).

These mentioned evidence, along with many others, show that estrogen has an immune modulatory role (Straub, 2007). Nevertheless, the immune-modulating role of estrogens is controversial due to the fact that the effects of estrogens differ, depending

on the immune stimulus (foreign antigens or autoantigens) and subsequent antigen-specific immune response, the cell types involved, the target organ and its specific microenviroment, the concentration of estrogens and the variability in expression of estrogen receptor genes depending on the microenvironment and the cell type. These findings reinforce the concept that estrogens have antiinflammatory but also proinflammatory roles depending on above-mentioned influencing factors (Straub, 2007).

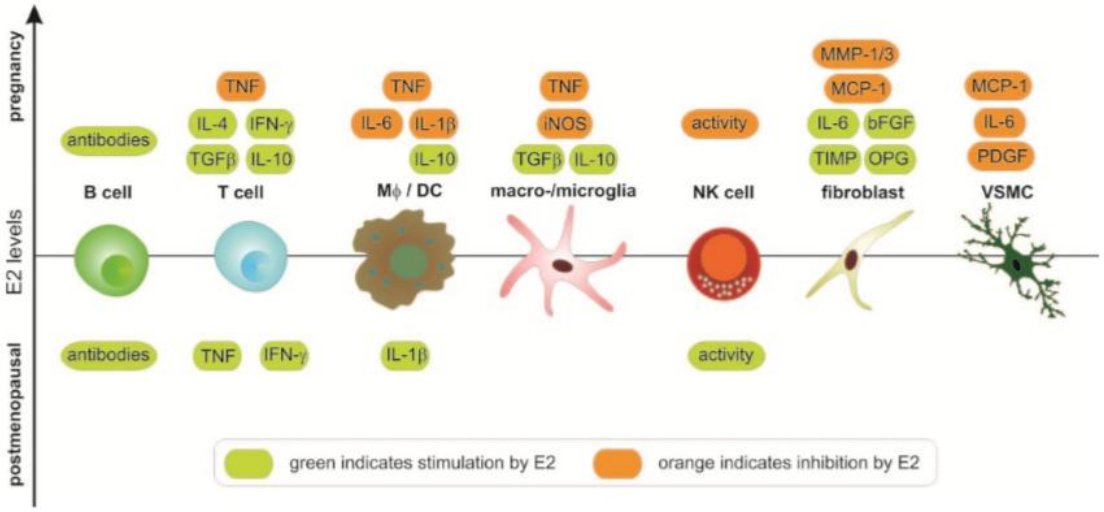

Figure 8. Influence of estrogens on important pro- and antiinflammatory pathways in different cell types. On the y-axis, the concentration of estrogens is given. Depending on the concentration of estrogens, factors in green boxes are stimulated, and factors in orange boxes are inhibited by estrogens. DC, Dendritic cell; MΦ, monocyte/macrophage; NK cell, natural killer cell; OPG, osteoprotegerin; PDGF, platelet-derived growth factor; VSMC, vascular smooth muscle cell (Straub, 2007).

The main effects of estrogens on the immune response involve enhancing the immune/inflammatory response by activating the nuclear factor κB (NFkB) signalling pathway (Cutolo et al., 2004). Furthermore, using ER knock-out mice, researchers have shown that ER participates in the stimulation of interleukin (IL)-10 and IgM production. In accordance with these roles, a number of epidemiological studies have highlighted the relationship between plasma estrogen levels, IL production, and autoimmune disorders linked to some diseases (Cutolo et al., 2006). However, E_2 also has an inhibitory effect on bone resorption and the suppression of inflammation in several animal models of chronic inflammatory diseases (Straub, 2007). Because of the significant involvement of estrogen to these disorders, it is crucial to consider not only

signaling through classical ERs but also by GPER, to properly design possible therapeutic targets and to take into account the possible unwanted effects of certain estrogen therapies.

4.1. Immune-reproductive interactions in teleost fish

In the testis of teleosts there are leukocytes whose type, abundance and location vary depending on the stage of the RC and the species to be studied (Billard, 1983; Scott and Sumpter, 1989; Besseau and Faliex, 1994; Bruslé-Sicard and Fourcault, 1997; Lo Nostro el al., 2004). Thus, in the gametogenic activity and spawning stages some macrophages have been described in the interstitial tissue of the rainbow trout testis (Loir et al., 1995), whereas in the post-spawning stage a high population of phagocyte cells has been described in several teleost fish (Henderson, 1962; Shrestha and Khanna, 1976; Scott and Sumpter, 1989; Loir et al., 1995). In general, these leukocytes are not considered as a testicular leukocyte population, but infiltrate in the organ, to a greater or lesser extent, depending on the stage of the RC.

As in mammals, in teleost, pituitary and steroids hormones are able to regulate the functions and reactions of leukocytes. In carp, *Cyprinus carpio,* intraperitoneal injections of $E_{2,}$ progesterone (P) and 11KT inhibit the phagocytosis, respiratory burst and the production of NO in a dose dependent manner of HK macrophages (Watanuki et al., 2002). However, these hormones inhibiting the phagocytic activity of carp macrophages from HK, although only P and 11KT inhibit the NO production and none of them has an effect on the respiratory burst (Yamaguchi et al., 2001). In goldfish, E_2 administration supress the immune system and increase their susceptibility to infection by trypanosomes (Wang and Belosevic, 1994).

In gilthead seabram, previous studies in our laboratory have suggested that sex hormones might be key regulators of leukocyte functions in the gonad. Thus, AGs infiltration in the testis, orchestrated by gonadal factors including sex steroid hormones, has been observed to be related to: (i) post-spawning and testicular involution stages, (ii) E_2 and T hormone peak, (iii) increase in the expression of gonadal aromatase, the enzyme that transforms to T to E_2 and, (iv) experimental induction of increase of E_2 serum levels in spermatogenically active males which also occurs acceleration of the final events of spermatogenesis and inhibition of the proliferation of spermatogonia in early stages. However, when AGs infiltrate the testis, they show heavily impaired

reactive oxygen intermediate production and phagocytic activity (hardly 1% of the testicular AGs are able to phagocytise) while the production of IL-1β is sharply induced. Interestingly, it is the gonad itself which actively regulates the presence of these immune cells in the testis by stimulating their extravasation from the blood (Chaves-Pozo et al., 2003, 2005a, 2005b, 2007, 2008a). These data, together with the expression pattern of cytokines and metalloproteinase (MMPs) (Chaves et al., 2008c) by this cell type, suggested that AGs are essential for testicular tissue formation, remodelling and cell renewal (Chaves-Pozo et al., 2010a). Moreover, MΦ and Ly have also been observed in the interstitial tissue (Liarte et al., 2007; Chaves-Pozo et al., 2008a). However, the number of testicular macrophages remains steady throughout the RC when the specimens are males, while no data related to lymphocytes are available (Chaves-Pozo et al., 2008a). These observations which, taken together, suggest that the presence of immune cells and cytokines in the gilthead seabream gonad guarantees and modulates the reproductive functions.

On the other hand, E_2 are able to regulate gilthead seabream primary MΦ, cells known to be key cell type in the innate immune reponse, activation (Liarte et al., 2011a, b) and to profoundly alter the gene expression profile of these cells, especially gene ontology category immune-related processes and pathways (Liarte et al., 2011b). Moreover, *in vitro* long term treatment of HK leukocytes with E_2 revealed a suppressive effect on the production of ROIs and the VaDNA-stimulated production of IL-1β (Chaves-Pozo et al., 2003). However, short term treatment with higher concentrations of E_2 inhibited the phagocytic capability of HK leukocytes, while the percentage of phagocytic cells and the VaDNA-stimulated production of ROIs and cell migration activity remained steady (Liarte et al., 2011a).

5. ENDOCRINE DISRUPTION

We live in a world in which man-made chemicals are part of everyday life. Some of these chemical pollutants can affect the endocrine (hormone) system, being the endocrine system a series of ductless glands that secrete hormones (molecule produced by an endocrine gland that travels through the blood to produce effects on distant cells and tissues) directly into the blood to regulate various body functions. Therefore, these chemical pollutants can interfere with hormonally-controlled processes of humans and

wildlife, resulting in adverse effects on health, growth, development and reproduction of individuals, their progeny or of populations are. These compounds are called endocrine disrupters chemicals (EDCs). The World Health Organization (WHO) defines EDCs as "*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*" (WHO/IPCS 2012). The phenomenon causing by the EDCs, alteration of endocrine function, is called endocrine disruption (ED). EDCs are present in materials (i.e. packaging), goods (i.e. electronics, furniture, household cleaners), personal care products (i.e. cosmetics, lotions, soaps, shampoos), and pharmaceuticals (typically the active pharmaceutical ingredient). These chemicals come from a variety of sources, enter the environment during production, use or disposal of chemicals or products, and have a range of behaviours in the environment. Some of these EDCs are persistent in the environment, bioaccumulate through food webs to high concentrations in wildlife and humans, and can be transferred to the developing fetus and the newborn through the placenta or breast milk, respectively. There is abundant evidence of ED in humans and wildlife such as alteration in female and male reproductive health and sex ratio, and increase detection of thyroid-related disorders, hormone-related cancers, neurodevelopmental disorders, adrenal disorders, metabolic disorders, bone disorders, immune function disorders and diseases and even population declines (WHO/IPCS 2012).

The EDCs can be classified according to different criteria such as common chemical properties and structural features, use and occurrence, pollution sources, mechanism of action (mimicking, antagonizing or interfering hormones), origin, etc. According to its origin, the EDCs may be classified of biological origin or anthropogenic;

- Biological origin: natural hormones (E_2, estriol, estrone, progesterone, T) and phytoestrogens (isoflavones and components of soybean).
- Anthropogenic origin: pharmaceutical drugs, pesticides, herbicides, fungicides, personal care products, metals and industrial chemicals.

Focusing in pharmaceutical drugs, the hormone present in them, along with naturally-produced hormones, are excreted by women and men and not fully removed through the sewage treatment process. As a result, they are found in the effluents being

discharged from sewage treatment works into receiving waters (Monteiro and Boxall, 2010). Fish species, freshwater and saltwater fish and aquatic invertebrates living exposed to municipal waste water untreated and/or treated water, both in coastal areas and in inland tanks are exposed to different types of EDCs because they are not completely destroyed in the existing sewage treatment plants therefore are found in low concentrations in aquatic environments (Kidd et al., 2007).

5.1. Enviromental estrogens or xenoestrogens

Environmental estrogens (EEs) are one important group belonging to EDCs and one of the most studied EDCs. They can be:

- Natural: E_2, estrone, estriol and phytoestrogens
- Synthetic: Diethilestilbestrol (DES), somes pesticides, bisphenol A (BPA), 17α-ethinylestradiol (EE_2)

They behave like estrogens and therefore present estrogenic activity. Are substances which bind and activate the ERs (Filby et al., 2007) and inducing the expression of estrogen-dependent genes. 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).

These substances, whether natural or synthetic, generated both in human and in animals of different species, impaired growth, increased incidence of problems related to the male reproductive tract, reduced fertility, loss of mating efficiency, behavioral abnormalities, metabolic disorders evident from birth demasculinization, feminization and immune system disorders and even increased incidence of various cancers (Colborn et al., 1993). Another factor to consider is that act differently depending on the state of development (development, puberty, sexual maturity) and sex of the target species exposure (Serrano et al., 2001). Moreover, recent evidence suggests that endocrine disruption as a consequence of estrogen exposure may result, on occasions, in the near collapse of wild fish populations (Kidd et al., 2007).

5.2. 17α-ethinylestradiol

EE_2 is a synthetic estrogen with structural homology to the female sex hormone E_2, differring in the presence of an ethinyl group at C17 (identif de EE_2) (Figure 9). EE_2

is a pharmaceutical compound, derived from estrone, being the active component of most of the modern hormonal contraceptives pills, also widely used in estrogen replacement therapies and treatment of breast cancer.

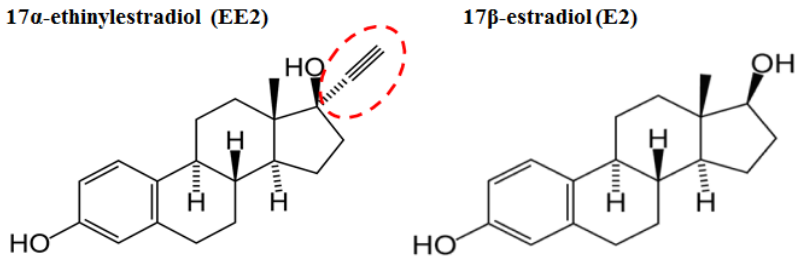

Figure 9. Structure of 17α-ethinylestradiol and 17β-estradiol. The circle indicates the ethinyl group at C17 in the EE_2 molecule.

Anticonceptive pills are currently used by more than 100 million women worldwide. After absorption in the small intestine, EE_2 is carried in the hepatic portal vein to the liver. A portion is directly absorbed into the bloodstream (peak plasma concentrations occur initially at 2 to 3 hours after oral ingestion) but the majority is metabolized in conjugated forms that are released through the gallbladder back into the intestines. Liver metabolism creates conjugated metabolites of EE_2 which are polar and soluble, making excretion easier. Approximately 60% of orally administered EE_2 is inactivated by the liver via first-pass effect through enterohepatic circulation. The major metabolic pathway of EE_2 degradation is aromatic hydroxylation, but a wide variety of hydroxylated and methylated metabolites are formed. These are present as free metabolites and as conjugates with glucuronide and sulphate. The sulfate and glucuronide metabolites re-enter the lumen of the bowel, where they can be excreted or the bowel flora can deconjugate the metabolites, which are reabsorbed causing an increase in circulating EE_2 (peak plasma concentrations at 12 hours) (Hardman et al., 1996). EE_2 is orally bioactive because it is removed more slowly from circulation than E_2, an effect that is essential for improved oral effectiveness and biological efficacy. Finally, the conjugates metabolites of EE_2 are excreted in feces or urine (Orme et al., 1983). Elimination phase half life is reported between 13 and 27 hours (Hardman et al., 1996). Thes conjugated metabolites of EE_2 can be subsequently deconjugation processes during the treatments undergoing wastewater (Desbrow et al., 1998). Thus, this compound will be discharged in its active form to the waterways through effluents from wastewater treatment plants (WWTP). Effluents from WWTPs constitute the major

source of estrogen contamination of aquatic environments (Ingerslev et al., 2003). EE_2 has been widely present in aquatic environment where can reach levels found 1-10ng/L but has come to be detect up to 47 ng/L (Desbrow et al., 1998; Ternes et al., 1999; Johnson et al., 2005; Hinteman et al., 2006; García-Reyero et al., 2011). EE_2 has a number of features (listed below) which make it one of the most potent estrogen:

- Low susceptibility to metabolic breakdown (due to ethinyl group at C17) and high persistence in the environment (Lai et al., 2002a).
- High tendency to bioconcentrate in the body (Larsson et al., 1999).
- 10-50 times more potent *in vivo* than E_2 (Thorpe et al., 2003; Nash et al., 2004).
- High affinity for ERs (Tilton et al., 2005).
- Lipophilic substance, longer half-life and tendency to bioaccumulate, reaching 650-fold increases in whole body tissues (Lai et al., 2002b; Gibson et al., 2005; Fenlon et al., 2010).
- Subjected to the enterohepatic circulation, amplifying their toxic effect due to continuous redosing (Schultz et al., 2003)

Due to its estrogenic activity, effects of EE_2 on the reproductive performance of animals have been widely studied. Nevertheless, there are few studies about effects of EE_2 on the fish immune response. It has been found that *in vitro* treatment with high doses of EE_2 reduces the phagocytic activity of tilapia head kidney leukocytes (Law et al., 2011). The addition of EE_2 to weakly estrogenic effluents results in lymphopenia in the fathead minnows (Filby et al., 2007) while EE_2 treatment during early developmental stages reduces the number and size of the splenic melanomacrophages center and impairs the expression of IGF-I and IGF-II in immune organs in fathead minnows (Shved et al., 2007) and increases TNFα and IL-1β mRNA levels in zebrafish (Jin et al., 2010).

Finally, taking into account that immune tissue and cells express both classical ERs and membrane ER, and that EDCs are able to bind to both ERs and mER, the importance of endocrine disruption, by environmental estrogens, in fish inmunity should not be underestimated.

OBJETIVES

This work has the following specific objectives:

1. To evaluate the ability of EE_2 to provoke an estrogenic response *in vivo* in male gilthead seabream.

2. To evaluate the ability of EE_2 to alter the physiology and the local immune response of the gonad.

3. To evaluate the ability of EE_2 to alter the systemic immune response, analysing immune activities of head kidney leukocytes, the expression profile of macrophages and the *in vivo* capabilities to respond to an immune challenge.

4. To perform a functional characterization of the G protein-coupled estrogen receptor, GPER, both *in vitro* and *in vivo.*

CHAPER I

DIETARY INTAKE OF 17α-ETHINYLESTRADIOL PROMOTES LEUKOCYTES INFILTRATION IN THE GONAD OF THE HERMAPHRODITE GILTHEAD SEABREAM

ABSTRACT

A wide variety of chemicals discharged from industrial and municipal sources have been reported to disrupt the endocrine system of animals, which may be exposed via the food chain and contaminated water. 17α-ethinylestradiol (EE_2), a drug used in oral contraceptives and hormone replacement therapy, has a widespread presence in the aquatic environment. Current knowledge on the sensitivity of marine fish to estrogenic environmental chemicals is limited. We report here the effects of dietary intake of EE_2 on gilthead seabream, a marine hermaphrodite teleost, focusing on the immune events that take place in the gonad. When seabream males were fed with 5, 50, 125 and 200 μg EE_2/g food for 7, 14, 21 and 28 days an infiltration of acidophilic granulocytes and B lymphocytes occurred in the testis as the same time that spermatogenesis is disrupted. Moreover, the dietary intake of EE_2 promoted a dose-dependent up-regulation of the expression of genes coding for cytokines, chemokines and adhesion molecules correlated with a leukocyte infiltration.

1. INTRODUCTION

A wide variety of chemicals discharged from industrial and municipal sources have been reported to disrupt the endocrine system of animals, which may be exposed via the food chain and contaminated water (Jones et al., 2004). Endocrine disrupting chemicals (EDCs) mimicking or antagonizing the action of hormones are a particular cause for concern. Recent evidence indicates that endocrine disruption as a consequence of estrogen exposure may result, on occasions, in the near collapse of wild fish populations (Kidd et al., 2007). As global consumption of pharmaceuticals rises, an inevitable consequence is an increased level of contamination of surface and ground waters with these biologically active drugs, and thus in turn a greater potential for adverse effects in aquatic wildlife (Corcoran et al., 2010). 17α-ethinylestradiol (EE_2), a pharmaceutical compound used for oral contraceptives and hormone replacement therapy, has a widespread presence in the aquatic environment (Ternes, et al., 1999), where it reaches concentrations of 0.5 to 62 ng/l in European sewage and superficial waters (Kuch and Ballschmiter, 2000; Johnson et al., 2005; Hinteman et al., 2006). Several fish species have been bath-exposed to environmental concentrations of EE_2 to assess any effects on reproduction (Lai et al., 2002a; Peters et al., 2007; Lange et al., 2008; Xu et al., 2008; Hashimoto et al., 2009; Hogan et al., 2010; Kaptaner and Unal, 2010; Marlatt et al., 2010). However, little attention has been paid to other physiological processes, such as immune responses that are known to be affected by estrogens (Lai et al., 2002a). Moreover, the resistance of EE_2 to degradation, which could support its bioaccumulation throughout the food chain as it has been predicted by some food-web models (Lai et al., 2002b), should not be underestimated and, for instance, the determination of the impact of the intake of low levels of EE_2 with the food on fish reproduction but also on other physiological processes such as immune responses is mandatory.

The gilthead seabream (*Sparus aurata* L.) is a seasonally breeding, marine, protandrous hermaphrodite teleost with a bisexual gonad, which, during the male phase, has a functional testicular area and a non-functional ovarian area. The testis undergoes abrupt morphological changes especially after spawning, including a massive infiltration of acidophilic granulocytes (AG) (Chaves-Pozo et al., 2003, 2005a; Liarte et al., 2007). These immune cells are produced in the head-kidney, the main haematopoietic organ in fish, but, when they infiltrate the testis, they show heavily

impaired functions (Chaves-Pozo et al., 2005b). Interestingly, it is the gonad itself which actively regulates the presence of these immune cells in the testis by stimulating their extravasations from the blood (Chaves-Pozo et al., 2005b; Chaves-Pozo et al., 2008a). Endogenous increases of 17β-estradiol (E_2) in serum are correlated with AG migration into the gonad after spawning (Chaves-Pozo et al., 2008b, 2010), while exogenous E_2 accelerates the final events of spermatogenesis, inhibits the proliferation of spermatogonia in early stages, and induces AG infiltration (Chaves-Pozo et al., 2007). However, AG does not express any of the three estrogen receptors (ERs) (ERa, ERb1, ERb2) known in gilthead seabream (Pinto et al., 2006; Liarte et al., 2011a). Interestingly, macrophages (MΦ) and lymphocytes (Ly) are always present in the interstitial tissue of the gilthead seabream gonad (Liarte et al., 2007) and express ERa (Liarte et al., 2011a). Moreover, MΦ are known to be a key cell type in the immune-modulatory role played by E_2 in the gilthead seabream gonad (Chaves-Pozo et al., 2010a; Liarte et al., 2011).

In the present study, gilthead seabream were fed for 7, 14, 21 and 28 days with pellet diet containing EE_2 ranging from an environmental concentration (5μg/g EE_2) to non-environmental ones (50, 125 and 200μg/g EE_2) in order to asses the potential risks to wild gilthead seabream populations focusing on the local immune regulation of the spermatogenesis.

2. MATERIAL AND METHODS

2.1. Animals and experimental design

Healthy specimens of gilthead seabream (*Sparus aurata* L., Actinopterygii, Perciformes, Sparidae) were bred and kept at the Centro Oceanográfico de Murcia (IEO, Mazarrón, Murcia). The experiment was conducted to test the effects of the dietary intake of 5, 50, 125 and 200μg EE_2 (purity 98%; Sigma) /g food for 0, 7, 14, 21 and 28 days.

The experiment was performed using mature gilthead seabream males (n= 400) at the spermatogenesis stage with a body weight of 320g kept in $2m^3$ tanks with the water temperature ranging from 14.6 to 17.8ºC. Specimens were kept with a flow-through circuit, a suitable aeration and filtration system and natural photoperiod. The environmental parameters, mortality and food intake was recorded daily. The EE_2 was

incorporated in the commercial food (44% protein, 22% lipids, Skretting, Spain) at doses of 0, 5, 50, 125 and 200μg/g food, using the ethanol evaporation method (0.3l ethanol/kg of food) as described elsewhere (Shved et al., 2007). The specimens were fed three times a day *ad libitum* and fasted for 24 h before sampling. Specimens (n=6 fish/group and time) were anesthetized with 40 ppm of clove oil. Afterwards decapitated, weighed, and the gonads were removed and weighed, while serum samples from trunk blood were obtained by centrifugation and immediately frozen and stored at -80ºC until use. The gonads were processed for light microscopy and gene analysis as described below. The experiments described comply with the Guidelines of the European Union Council (86/609/EU), the Bioethical Committee of the University of Murcia (Spain) and the Instituto Español de Oceanografía (Spain) for the use of laboratory animals.

2.2. Gonadosomatic index

As an index of the reproductive stage, the gonadosomatic index (GSI) we calculated as 100*[MG/MB] (%), where MG is gonad mass (in grams) and MB is body mass (in grams).

2.3. Analytical techniques

Serum levels of E_2, testosterone (T), and 11-ketotestosterone (11KT) were quantified by ELISA analysis following the method described by Rodriguez et al. (2000). Steroids were extracted from 1 to 2μl of serum in 0.6ml of methanol (Panreac). E_2 and T standards were purchased from Sigma-Aldrich. The 11KT standard, mouse anti-rabbit IgG monoclonal antibody (mAb), and specific anti-steroid antibodies and enzymatic tracers (steroid acetylcholinesterase conjugates) were obtained from Cayman Chemical. Microtiter plates (MaxiSorp) were purchased from Nunc. A standard curve from 6.13x 10^{-4} to 2.5ng/ml (0.03-125pg/well) was established in all the assays. Standards and extracted serum samples were run in duplicate. The lower limit of detection for all the assays was 12.21pg/ml. The intra-assay coefficients of variation (calculated from sample duplicates) were 6.5 ± 1.0% for E_2, 18.5 ± 2.5% for T, and 24.5± 14.3% for 11KT assays. Details on cross-reactivity for specific antibodies were provided by the supplier (0.01% of anti-11KT reacts with T; 2.2% of anti-T reacts with 11KT; and 0.1% of anti-E_2 reacts with T).

2.4. Light microscopy and immunocytochemical staining

The gonads were fixed in Bouin's solution, embedded in paraffin (Paraplast Plus; Sherwood Medical, Athy, Ireland), and sectioned at 5μm. After dewaxing and rehydratation, some sections were stained with hematoxylin-eosin in order to determine the reproductive stage and the degree of development of each specimen. The sections used to localize AG with a mAb specific to gilthead seabream AG (G7) (Sepulcre et al., 2002) and B Ly with a commercial mAb specific to immunoglobulin M (IgM, Aquatic Diagnostic) (Sepulcre et al., 2011) or to analyze cell proliferation with a commercial mAb specific to proliferating cell nuclear antigen (PCNA, Sigma) were subjected to immunocytochemistry following an indirect method previously described (Chaves-Pozo et al., 2005a). Rabbit polyclonal anti-PCNA antibodies cross-react with PCNA from all vertebrate species that have been investigated so far, including fish (Kilemade et al., 2002). The antibodies were used at the optimal dilution of 1:100, 1:250 or 1:1000, respectively. Some other sections were subjected to immunocytochemistry following an indirect method or the avidin-biotin complex technique in order to localize Mc by using an antibody against seabream macrophage colony stimulating factor receptor (MCSFR) (Roca et al., 2006), which specifically stained Mc (Mulero et al., 2008). The specificity of the reactions was determined by omitting the first antiserum. No immunostaining was observed when the first antiserum was omitted.

2.5. Analysis of gene expression

Total RNA was extracted from gonad fragments with TRIzol Reagent (Invitrogen) following the manufacturer's instructions, and quantified with a spectrophotometer (NanoDrop, ND-1000). After determining that the gonad of all the animals from the same group, were in the same histological stage, the RNA of 6 fish per group were pooled using the same amount of RNA from each specimen. The RNA was then treated with DNase I, amplification grade (1 unit/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 1μg of total RNA, at 50ºC for 60 min.

The expression of genes coding for tumor necrosis factor α (TNFα), CC chemokine ligand 4 (CCL4), CXC chemokine interleukin 8 (IL8), leukocyte adhesion molecule E-selectin (SELE), heavy chain of immunoglobulin M (IGHM) and heavy chain of immunoglobulin T (IGHT) was analyzed by real-time PCR performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core

Reagents (Applied Biosystems) as previously described (Chaves-Pozo et al., 2008a). For each mRNA, gene expression was corrected by the ribosomal protein S18 gene (*rsp18*) content in each sample. The gilthead seabream specific primers used are shown in Table 1. In all cases, each PCR was performed with triplicate samples and repeated at least twice.

Gene	Accession number	Name	Sequence (5′-3′)	Use
tnfa	AJ413189	FE1	TCGTTCAGAGTCTCCTGCAG	Real time PCR
		RE2	CATGGACTCTGAGTAGCGCGA	
ccl4	AM765840	F1	GCTGTGTTTGTGCTGATGCT	Real time PCR
		R1	GCTGGCTGGTCTTTTGGTAG	
il8	AM765841	F2	GCCACTCTGAAGAGGACAGG	Real time PCR
		R2	TTTGGTTGTCTTTGGTCGAA	
sele	AM749963	F1	GACAGTGAGCAGGCGTACAA	Real time PCR
		R1	ATCGCTTCATGATCCACACA	
ighm	AM493677	F1	CAGCCTCGAGAAGTGGAAAC	Real time PCR
		R1	GAGGTTGACCAGGTTGGTGT	
ight	FM145138	F1	TGGCAAATTGATGGACAAAA	Real time PCR
		R1	CCATCTCCCTTGTGGACAGT	
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC	Real time PCR
		R	CTTCTGCCTGTTGAGGAACC	

Table 1. Gene accession numbers and primer sequences used for gene expression analysis. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html)

2.6. Calculation and statistics

Mean ± SEM were calculated for GSI and inter-group differences between GSI were assessed by one way analysis of variance (ANOVA) followed by Tukey's post hoc test. All gene expression data were analyzed by one-way ANOVA and Tukey multiple range tests to determine differences between groups. The quantification of anti-IgM and G7 stained areas were calculated as the mean value ± SEM of the stained area / total area of 20 randomly distributed optical areas at 100x magnification. The stained areas were measured by image analysis using a Nikon eclipse E600 light microscope, an Olympus SC30 digital camera (Olympus soft imaging solutions GMBH), and Leica Qwin software (Leica microsystems). Differences were analyzed by one-way ANOVA and Tukey's post hoc test. All statistical analyses were carried out using SPSS 12.0 (SPSS, Chicago, IL, USA). The critical value for statistical significance was taken as $p \leq 0.05$.

3. RESULTS

3.1. EE_2 promotes fish mortality and decreases the gonadosomatic index

All the concentrations of EE_2 tested induced the death of seabream males, reaching 38% at the end of the trial for the two higher doses assayed (125 and 200μg EE_2/g food) (Fig. 1a). At the end of the experiment, the GSI was much lower in all the treated groups than in the control (Fig. 1b).

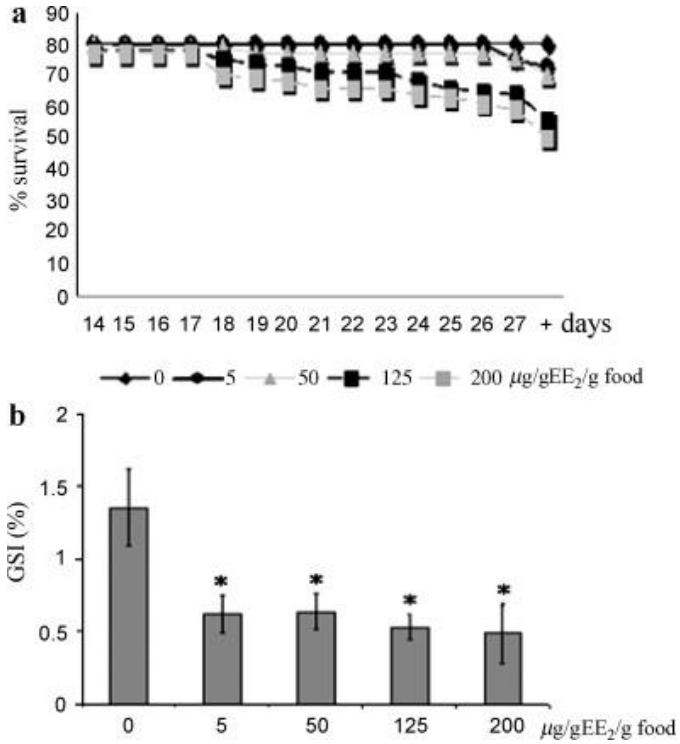

Fig. 1. Effects of EE_2 dietary intake on fish survival (a) and gonadosomatic index (b) after 28 days of 0, 5, 50, 125 and 200 μg EE_2/g food. Asterisks denote statistically significant differences compared with control, determined by ANOVA and Tukey post hoc test ($P < 0.05$).

3.2. EE_2 modifies the serum levels of sex steroids

The fish fed with EE_2 showed increased E_2 serum levels at all time points analyzed, although the effect was particularly evident for the groups fed with 125 and 200 μg/g EE_2 (Fig. 2a). In contrast, T levels (Fig. 2b) decreased with all the EE_2 concentrations used after 14 days of exposure, while 11KT decreased at all the concentrations and times of exposure, except at 5μg EE_2/g food after 7 days of exposure (Fig. 2c).

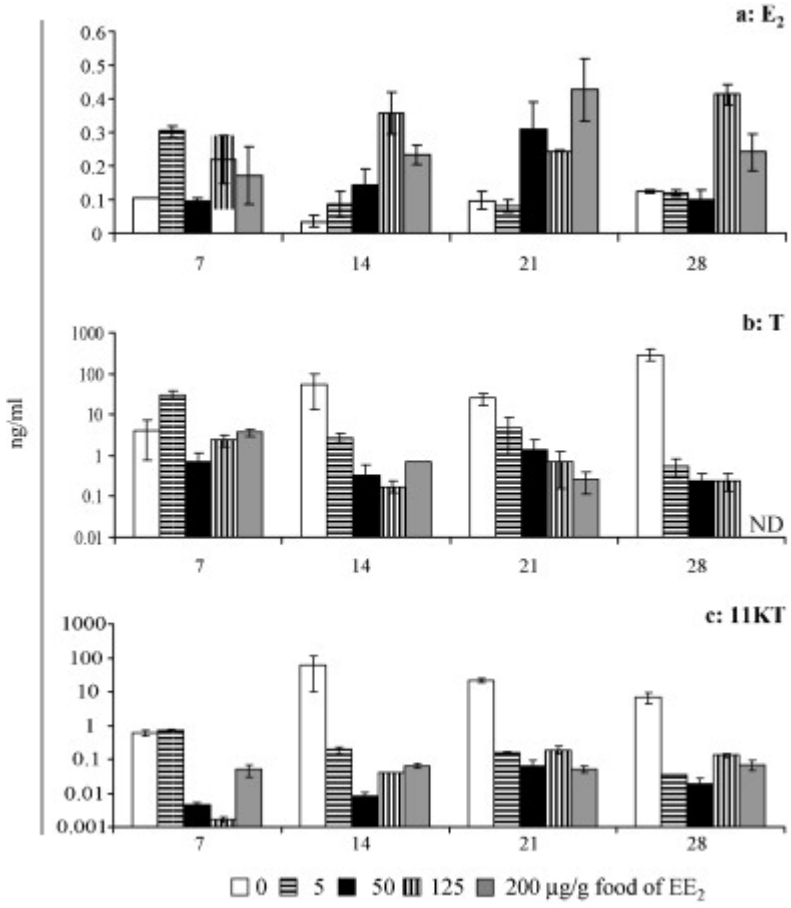

Fig. 2. Effects of EE_2 dietary intake on 17β-estradiol (E_2) (a), testosterone (T) (b), and 11-ketotestosterone (11KT) (c) serum levels. Data were obtained from a pool of serum obtained after mixing the same amount of serum from 6 fish/group and represent the means ± S.E.M. of duplicate samples. ND: non-detected.

3.3. Although some germ cells proliferate after EE_2 dietary intake, spermatogenesis is disrupted

The testes of control fish were in mid-spermatogenesis throughout the experimental period showing different, but always low, amounts of spermatozoa (Fig. 3a) and the proliferative activity rate (data not shown) that we have previously described for the gilthead seabream testis (Chaves-Pozo et al., 2010a). In general, a degenerative process in the testis was observed which drastically reduced the germinal epithelium to be formed by pre-meiotic germ cells and increased the amount of free spermatozoa from day 7 onwards of 50, 125 and 200µg EE_2/g food dietary intake (Fig. 3b) and 14 days of 5µg EE_2/g food dietary intake. Interestingly, there was an increase of the interstitial tissue (Fig. 3c) and the connective tissue around the blood vessels at day 21. At the end

of the trial, day 28, the testis showed a degenerated morphology. These results were consistently found in all individuals of each treatment group.

3.4. Acidophilic granulocytes and IgM-positive cells but not MCSFR-positive cells are recruited to the gonad after EE_2 dietary intake

In relation with the disruption of the spermatogenesis, no proliferation of immune cells was observed (data not shown).

AG infiltrated the gonad after 14 days of 200μg EE_2/g food dietary intake or after 21 and 28 days with the other concentrations assayed (Figs. 3d-g). They occupied the same positions in the testis as after the physiological AG infiltration that occurred during the post-spawning stage (Chaves-Pozo et al., 2010a), such as the connective and the interstitial tissues (Fig. 3d). In contrast to what happened during post-spawning, on day 28 of EE_2 exposure, the AG appeared in large clusters close to very vacuolated cells in the interstitial tissue (Fig. 3e), close to degenerative areas (Fig. 3f) and close to the empty areas surrounded by epithelium (Fig. 3g).

MCSFR was detected in the Sertoli cells that formed the cysts and in the Sertoli cells that limited the lumen of the efferent duct (Fig. 3d) and in Mc located in the interstitial tissue (Fig. 3h) as we have previously described (Chaves-Pozo et al., 2010a). However, the MΦ abundance and localization was unaltered by EE_2 exposure.

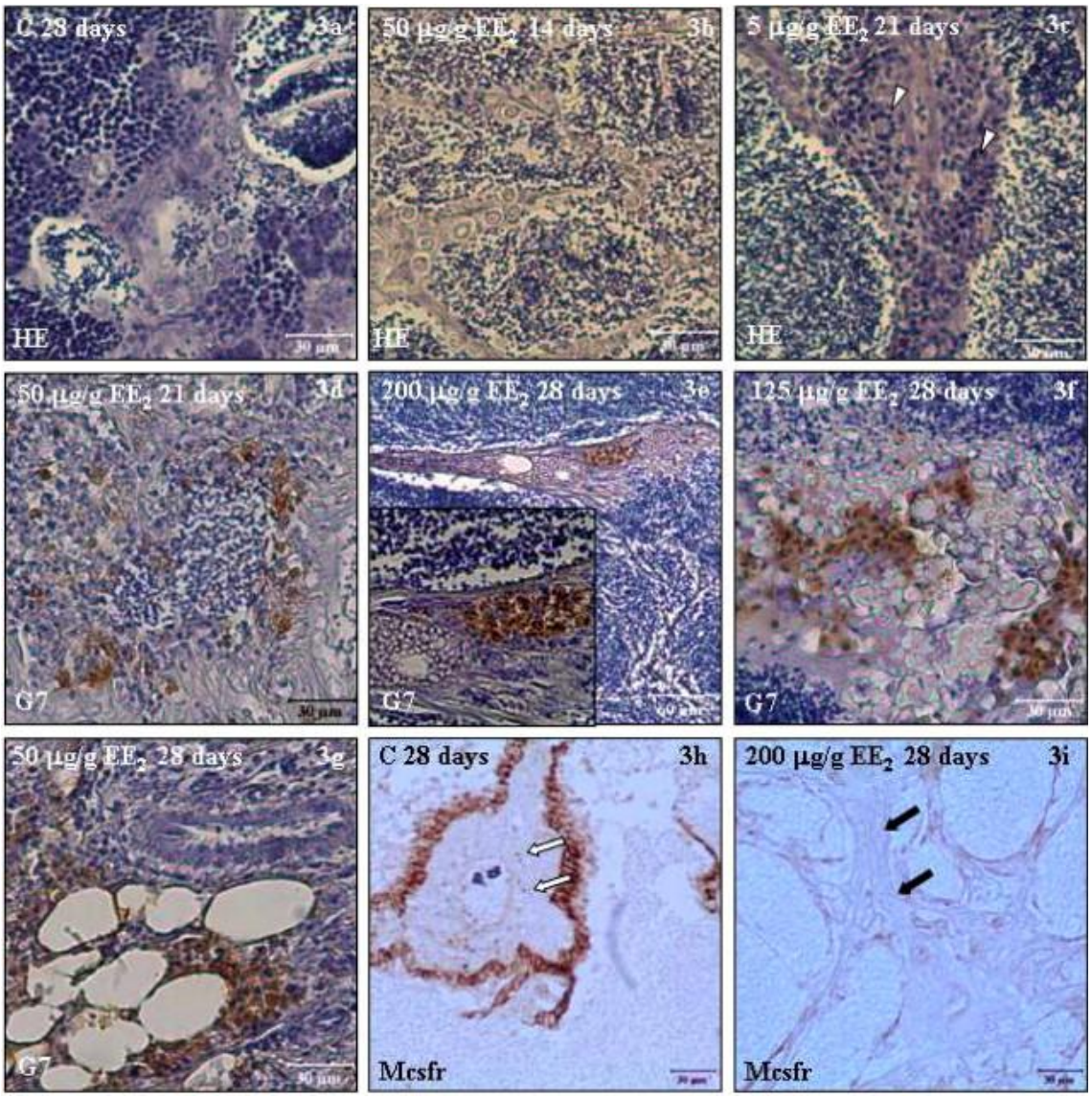

Fig. 3. Paraffin embedded sections of the testis of gilthead seabream in mid-spermatogenesis subjected to vehicle (control, C) (a, h) or 5 (c), 50 (b, d, g), 125 (f) or 200 (e, i) µg EE_2/g food dietary intake during 14 (b), 21 (c, d) or 28 (a, e–i) days stained with hematoxylin–eosin (a–c) or stained with the serum against gilthead seabream acidophilic granulocytes (G7) (d–g) or against gilthead seabream Mcsfr (h–i). Mid-spermatogenesis testis (a). Large amounts of free spermatozoa in the lumen of the tubules (b, c), along with a reduction in the germinal epithelium formed by spermatogonia stem cells and cysts of primary spermatogonia (b, c), were observed and an increase in the interstitial tissue (white arrow heads) (c). After EE_2 exposure, acidophilic granulocytes were observed in the interstitial tissue (d) and close to a very vacuolated cell type in the interstitial tissue (e), to degenerative areas (f) and to empty areas surrounded by an epithelium (g). The cells stained with anti-Mcsfr after EE_2 dietary intake were the Sertoli cells that limited the lumen of the tubules after spermatozoa are released from the cyst and macrophages located in the interstitial tissue (white arrows) (h), but were not presented in the enlarged interstitial tissue (black arrows) (i). Scale bar = 10 µm (inset e), 30 µm (a–d, f–i) and 60 µm (e).

Moreover, EE_2 promoted the recruitment of IgM-positive (IgM+) B Ly from day 7. Thus, although the number of B Ly varied between specimens in control fish (Figs. 4a, c), the EE_2 dietary intake promotes an increase in the amount of IgM+ B Ly in the testis (Figs. 4b, d) from day 7 onwards. In all EE_2 concentrations used and times of exposure, IgM+ B Ly appeared in the interstitial tissue of the testis (Figs. 4b, d), and between the spermatozoa (Fig. 4e). Interestingly, in contrast to controls, IgM was also detected in the plasma of the main blood vessels in the testis of EE_2-treated fish from day 14 onwards (Fig. 4f).

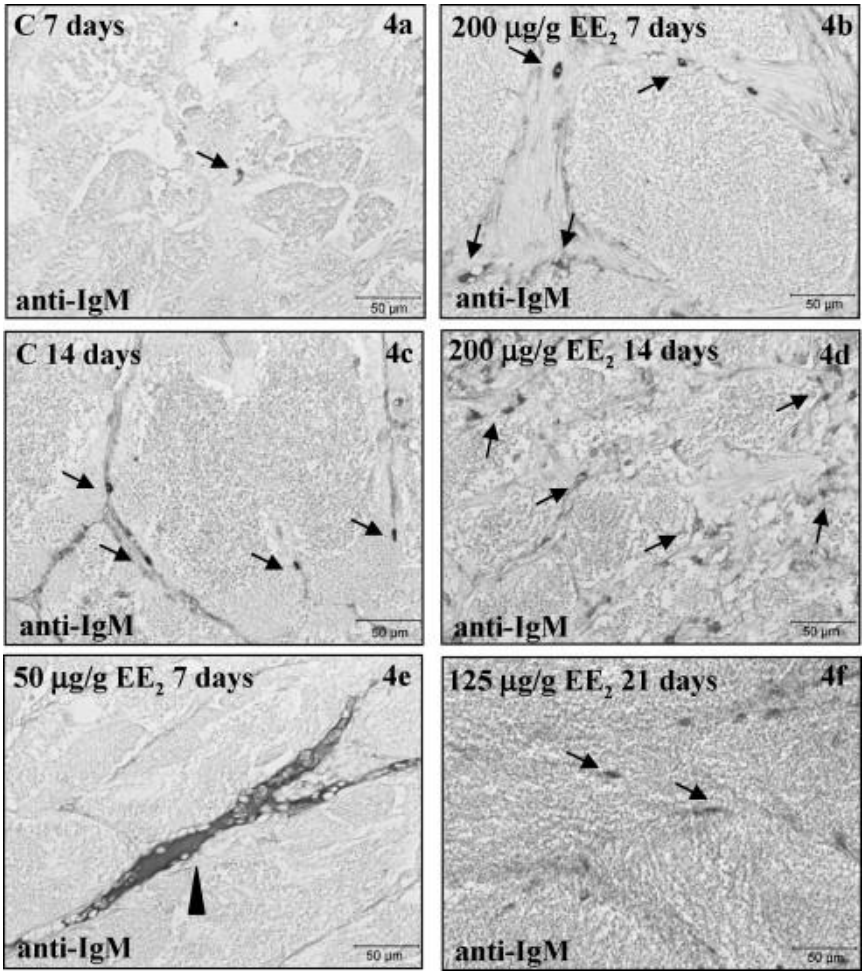

Fig. 4. Paraffin embedded sections of the testis of gilthead seabream gonad subjected to vehicle (control, C) (a, c) or 50 (e), 125 (f) or 200 (b, d) µg EE_2/g food dietary intake during 7 (a, b, e), 14 (c, d) or 21 (f) days immunostained with the serum against IgM. Although variable amounts of IgM+ B lymphocytes (Ly) were presented in the testicular interstitial tissue of control (a, c), the amount of IgM+ B Ly (arrows) (b, d) increased at day 7 onwards when compared with controls (a, c). IgM was also detected in the plasma of the main blood vessels (arrow head) in the testis of EE2 exposure fish (e). IgM+ B Ly (arrows) appeared in the interstitial tissue (b, d), and between the spermatozoa (f). Scale bar = 50 µm.

The quantification of G7 (Fig. 5a) and anti-IgM (Fig. 5b) infiltration at 28 days of dietary intake of EE_2 showed a dose-dependent increased number of IgM+ cells (B Ly) and G7+ cells (AGs). Thus, we observed 20.2, 17.8, 32.2 and 24.5 fold increases of IgM^+ cells and 3.36, 91.5, 168 and 97.7 fold increases of G7+ cells compared with controls after 28 days of exposure with 5, 50, 125 and 200µg EE_2 /g food, respectively.

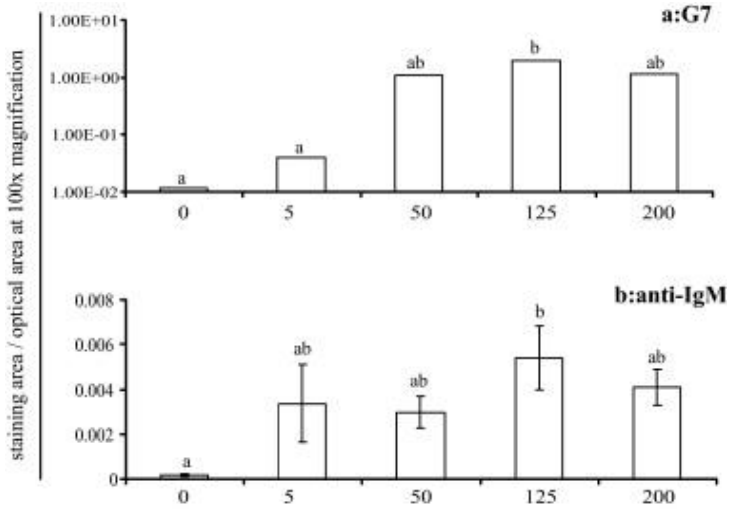

Fig. 5. The mean of the staining area with G7 (a) or anti-IgM (b) per optical area at 100× magnification from control and EE_2 treated groups after 28 days of exposure. Data represent the mean ± SEM for (n = 6) fish/group. Different letters denote statistically significant differences between the groups according to a Tukey post hoc test.

We also analyzed the expression of the genes coding for IgM and IgT (Fig. 6) which are known to be expressed in two subpopulations of B Ly. Our data showed that both genes are expressed in the gonad and their mRNA levels were mainly up-regulated in a dose dependent manner. However, we also observed some down-regulation of the IgM mRNA levels after 14 days of exposure with 5µg EE_2/g food (Fig. 6a) and in the IgT mRNA levels after 14 and 21 days of exposure with 5 and 50µg EE_2 /g food, respectively (Fig. 6b). Interestingly, we observed some variations on the gene expression levels of both, IgM and IgT, in control fish (Fig. 6).

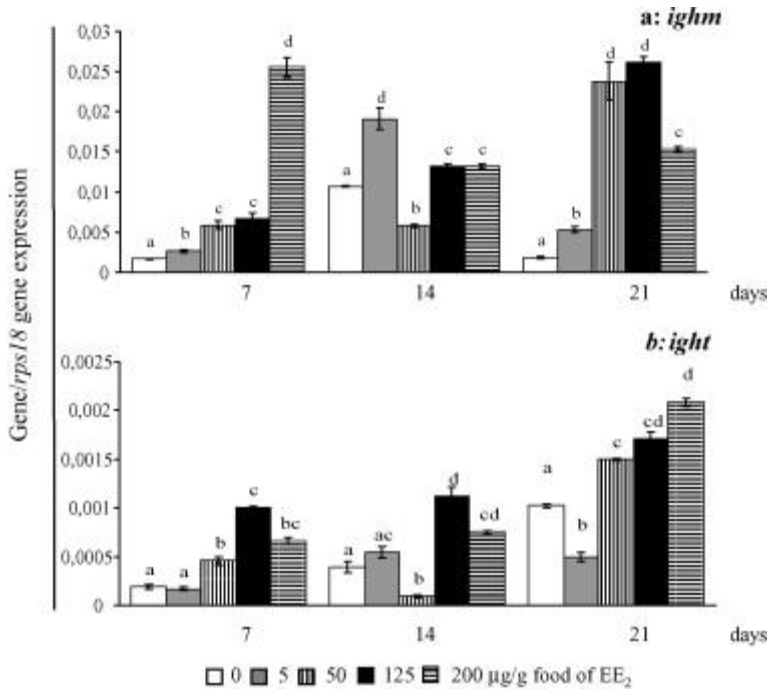

Fig. 6. Expression of genes coding for IgM and IgT in the gonad. The mRNA levels of ighm (a) and ight (b) were studied by real-time RT-PCR in the gonad from control and EE_2 treated groups. Data represent means ± S.E.M. of triplicate samples. Total mRNA was obtained after mixing the same amount of mRNA from 6 fish/group. Different letters denote statistically significant differences between the groups according to a Tukey test ($P < 0.05$).

3.5. EE_2 induces the expression of chemokines and adhesion molecule

We next analyzed the expression of genes coding for TNFα, CCL4, IL8 and SELE, since these molecules were found to regulate leukocytes trafficking in the gilthead seabream (Roca et al., 2008). The results showed that the mRNA level of these four genes was significantly higher after 7, 14 and 21 days of EE_2 exposure. Most of them showed a dose-dependent increase after EE_2 exposure, reaching their highest levels (between 10 and 16-fold increase) in the fish fed 200µg EE_2/g food during 14 days. However, after 21 days of EE_2 exposure, the highest expression levels (between 10 and 20 fold increase) were recorded in the fish fed 125µg EE_2/g food (Fig. 7).

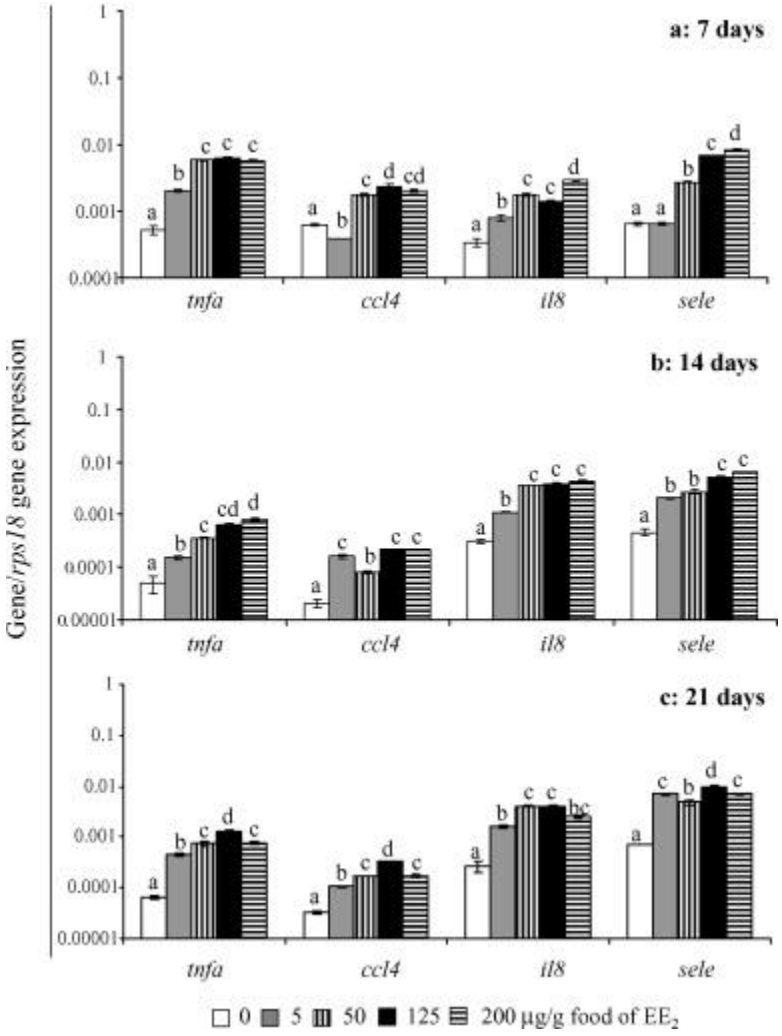

Fig. 7. Expression of genes coding for TNFα, CCL4, IL8 and SELE in the gonad. The mRNA levels of the genes were studied by real-time RT-PCR in the gonad from control and EE_2 treated groups after 7 (a) 14 (b) and 21 (c) days of exposure. Data represent means ± S.E.M. of triplicate samples. Total mRNA was obtained after mixing the same amount of mRNA from 6 fish/group. Different letters denote statistically significant differences between the groups according to a Tukey test ($P < 0.05$).

4. DISCUSSION AND CONCLUSIONS

Much concern has been expressed concerning the hazards of pharmaceutical compounds in the environment, and it is frequently recommended that more research be done in this area (Jones et al., 2004). Pollution by pharmaceuticals such as EE_2 can occur in very low and difficult to detect concentrations but they have potential for producing environmental harm. Thus, EE_2 has a strong estrogenic potency 10-50 fold higher than that of the natural estrogen E_2 mainly due to its longer half-life and tendency to bioaccumulate, reaching 650-fold increases in whole body tissues (Lai et al., 2002a;

Gibson et al., 2005; Fenlon et al., 2010). Although, it is known that estrogen might affect other physiological processes, such as immune responses (Lai et al., 2002a), little attention has been paid to these effects. Current knowledge about the sensitivity of marine fish to estrogenic environmental chemicals is still limited and the present study is intended to fill some gaps in this knowledge by providing data about the effects of EE_2 dietary intake on gilthead seabream, a marine seasonal breeder, hermaphrodite protandrous species, focusing on the immune events that take places in the gonad, concomitantly with testicular development during the male phase (Liarte et al., 2007; Chaves-Pozo et al., 2007, 2009, 2010).

The mortality of fish exposed to EE_2 increased from day 8 onwards. Although EE_2-treated fish showed a lack of appetite, as occurs in gilthead seabream treated with E_2 (Condeca and Canario, 1999), the more toxic effects of EE_2 are evident from the mortality rates observed in this experiment that do not occur with E_2 treatment (Chaves-Pozo et al., 2007). A widely recorded effect of estrogenic compounds is the modification of steroidogenic sex hormone levels in different tissues of fish species (Peters et al., 2007; Hogan et al., 2010; Marlatt et al., 2010). As expected, our data show that EE_2 modify the E_2 serum levels, however as the gilthead seabream is a hermaphroditic specie, this modification was completely different from the effect seen in the gonochoristic fish species (Peters et al., 2007; Swapna and Senthilkumaran, 2009; Hogan et al., 2010). After EE_2 treatment, as also occurs with E_2 treatment (Chaves-Pozo et al., 2007), no induction of spawning was observed even though the gonad appeared full of free spermatozoa. Moreover, the GSI decreased at all the EE_2 concentrations used after 28 days of dietary intake, as occurs in most of the experiments performed using bath exposure (Saaristo et al., 2009; Hogan et al., 2010).

Our main result shows that EE_2 promotes an inflammatory process in the gonad. Large amounts of AG were observed in the same location as they occupy in the testis during the development of the gonad or after E_2 treatment of gilthead seabream males (Chaves-Pozo et al., 2007, 2009). IgM+ B Ly also infiltrated the gonad and were located in the interstitial tissue and between the spermatozoa. Moreover, expression of the genes coding for IgM and IgT were increased after EE_2 dietary intake mainly in a dose dependent manner. We also recorded some variability in the amount of IgM+ B Ly and IgMh and IgT genes expression in control fish, probably due to their involvement in IgM and IgT secretion into the semen at certain point throughout spermatogenesis as

occur in other fish species (Lahnsteiner and Radner, 2010). Although, we can not ascertain that IgT positive (IgT+) cells, a specific B Ly subpopulation for mucosal immune responses (Zhang et al., 2010), infiltrated the gonad, we can concluded that there were present in the gonad and an increase in the IgT gene expression was induced by EE_2 dietary intake. Further studies will be needed in order to determine the role of different B Ly subpopulations in the physiology of the gonad.

Taking into account that the recruitment of leukocytes, AG and Ly, might be orchestrated by signals from the injured tissue and the degenerative process and that AG do not express any of the three ER known in the gilthead seabream and Ly only express the ERα (Liarte et al., 2011a), we analyzed the gene expression pattern of the genes encoding TNFα, CCL4, IL8 and SELE, since they have been found to regulate AG and Ly infiltration in this species (Roca et al., 2008; Chaves-Pozo et al., 2010a). Our data showed that the EE_2-triggered leukocytes infiltration of the gonad correlated with the induction of these genes. Thus, IgM+ B Ly infiltrated the gonad from day 7 onwards, and from day 14 onwards they were numerous in number, while AG appeared first at day 14 in the gonads exposed to the highest doses of EE_2, which showed a strong induction of these genes. Interestingly, leukocytes were present in the gonad of fish exposed to all the doses of EE_2 assayed after 21 days of treatment. Taken together, these data suggest that independently of ER gene expression in the different cell types, a high level of TNFα, CCL4, IL8 and SELE gene expression is also involved in leukocytes migration into the gonad in response to estrogenic agents. However, we cannot disregard other chemokines or adhesion molecules described in mammals, but not in gilthead seabream, which might also be involved in leukocytes infiltration. In this sense, further work is needed to clearly identify the molecules involved in this physiological inflammatory process in the gonad.

Recently, gilthead seabream MΦ have been described as a key cell type in the immune-regulatory role of estrogens (Liarte et al., 2011a, b). This prompted to us to investigate whether Mc also infiltrate the gonad to dampen the inflammatory response triggered by EE_2, since they are able to block leukocytes phagocytic ability and capacity upon E_2 stimulation (Liarte et al., 2011a). Using an antibody against gilthead seabream MCSFR, which is expressed in leukocytes from the monocyte/macrophage lineage (Roca et al., 2006), but also in certain non-mononuclear phagocytic cells, such as Sertoli cells and granulose cells (Chaves-Pozo et al., 2008a), we founded that MCSFR positive

cells were unaltered in the interstitial tissue of the gonad, ruling out that EE_2 also promotes Mc infiltration on the gonad.

In conclusion, EE_2 promoted the recruitment of AG and IgM-positive B Ly but this was not the case for interstitial MCSFR-positive cells that corresponded to testicular Mc. Interestingly, EE_2 exposure up-regulates the expression of genes coding for IgM and IgT and for TNFα, CCL4, IL8 and SELE, which were found to regulate leukocytes trafficking in the gilthead seabream (Roca et al., 2008). Concomitantly with leukocytes recruitment, EE_2 also disrupts spermatogenesis.

CHAPER II

THE EFFECT OF 17α-ETHYNYLESTRADIOL ON STEROIDOGENESIS AND GONADAL CYTOKINE GENE EXPRESSION IS RELATED WITH THE REPRODUCTIVE STAGE IN MARINE HERMAPHRODITE FISH

ABSTRACT

Pollutants have been reported to disrupt the endocrine system of animals, which may be exposed through contaminated water or through the food chain. Although 17α-ethynylestradiol (EE_2), a drug used in hormone therapies, is widely present in the aquatic environment, current knowledge on the sensitivity of marine fish to estrogenic pollutants is limited. We report the effect of the dietary intake of 5 μg EE_2/g food on different processes of testicular physiology, ranging from steroidogenesis to pathogen recognition, at both pre-spermatogenesis (pre-SG) and spermatogenesis (SG) reproductive stages, of gilthead seabream (*Sparus aurata* L.), a marine hermaphrodite teleost. A differential effect between pre-SG and SG specimens was detected in the sex hormone serum levels and in the expression profile of some steroidogenic-relevant molecules, vitellogenin, double sex- and mab3-related transcription factor 1 and some hormone receptors. Interestingly, EE_2 modified the expression pattern of some immune molecules involved in testicular physiology. These differences probably reflect a developmental adjustment of the sensitivity to EE_2 in the gilthead seabream gonad.

1. INTRODUCTION

A wide variety of chemicals discharged from industrial and municipal sources have been reported to disrupt the endocrine system of animals, which may be exposed via the food chain or directly through contaminated water (Jones et al., 2004). Endocrine disrupting chemicals (EDCs) mimicking or antagonizing the action of hormones are a particular cause for concern. Recent evidence suggests that endocrine disruption as a consequence of estrogen exposure may have very serious consequences for the wild fish populations (Kidd et al., 2007). An inevitable consequence of the increasing consumption of pharmaceuticals is an increased level of contamination of surface and ground waters by these biologically active drugs, accompanied by a greater potential for adverse effects on aquatic wildlife (Corcoran et al., 2010). Most of the estrogenic activity is adsorbed on sediments, and routes of exposure may include benthic food chain transfer (Matthiessen et al., 2002). Marine sediments may alter the expression of genes that are biomarkers for fish endocrine disruption (Ribecco et al., 2011). 17α-ethynylestradiol (EE_2), a pharmaceutical compound used in oral contraceptives and hormone replacement therapy with a strong affinity for estrogen receptors (ER) (Tilton et al., 2005), has a widespread presence in the aquatic environment (Ternes et al., 1999), where it reaches concentrations of 0.5 to 62 ng/l in European sewage and superficial waters (Johnson et al., 2005; Hinteman et al., 2006). Several fish species have been bath-exposed to environmental concentrations of EE_2 to ascertain any effect on reproduction (Soffker et al., 2012). Moreover, the resistance of EE_2 to degradation, which could encourage its bioaccumulation throughout the food chain, as predicted by several food-web models (Lai et al., 2002b), should not be underestimated. The determination of the impact of even low concentrations of EE_2 on fish reproduction is therefore advisable.

Reproduction in fish is subject to hormonal regulation by gonadal steroids (Fostier et al., 1983; Klim et al., 1993). Dihydrotestosterone (DHT) is one of the most physiologically important androgens in many male vertebrates (George et al., 1994), with the exception of teleost fish, in which testosterone (T) and 11-ketotestosterone (11KT) are generally considered the major and most potent circulating male androgens (Borg, 1994). T levels increase in both females and males during gonadal development, while 11KT is considered to be the dominant androgen in males (Kime et al., 1993; Borg, 1994). While 17β-estradiol (E_2) has been considered to be the main hormone of

female fish, recent studies have suggested that estrogens are ‘‘essential” for normal male reproduction (Miura et al., 1999b; Amer et al., 2001; Hess, 2003). However, little is known on the local immune regulation that takes place in the fish testis that provides protection for the developing male germ cells, while permitting qualitatively normal inflammatory responses and protection against infection (Chaves-Pozo et al., 2008a).

The gilthead seabream (*Sparus aurata* L.) is a seasonally breeding, marine, protandrous hermaphrodite teleost, which has recently been used to describe the biological effects of contaminated marine sediments in light of its importance as a commercial food and its use as sentinel fish for environmental studies and monitoring (Ribecco et al., 2011). The gilthead seabream is common in the Mediterranean Sea and, due to its euryhaline and eurythermal habits, the species is found in both marine and brackish water environments such as coastal lagoons and estuarine areas, particularly during the initial stages of its life cycle (FAO 2005-2013). Levels of some xenobiotics are much higher in the Mediterranean Sea than in other seas and oceans (Aguilar et al., 2002) since, among other reasons, it has limited exchange of water with the Atlantic Ocean, and is surrounded by some of the most heavily populated and industrialized countries in the world. The reproductive cycle of gilthead seabream is divided in four stages: spermatogenesis (SG), spawning, post-spawning and resting (Chaves-Pozo et al., 2005a), in which the levels of E_2, T and 11KT (Chaves-Pozo et al., 2008b), as well as the gene expression and production of several cytokines (Chaves-Pozo et al., 2008a) vary. Interestingly, in male gilthead seabream, E_2 serum levels increase after spawning when a massive infiltration of acidophilic granulocytes (AGs, the professional phagocytes in the gilthead seabream) into the gonad takes place (Sepulcre et al., 2002, 2007; Chaves-Pozo et al., 2010a), although this cell type does not express any of the three known nuclear ER, namely ERa, ERb1 and ERb2 (Liarte et al., 2011a). Moreover, AG infiltration also occurs in the testis of specimens during the morphogenesis process (Chaves-Pozo et al., 2008c). These data, together with the expression pattern of cytokines and metalloproteinase (MMPs) by this cell type, suggested that AGs are essential for testicular tissue formation, remodelling and cell renewal (Chaves-Pozo et al., 2010a). We have recently reported that EE_2 dietary intake disrupts spermatogenesis and promotes leukocyte infiltration in the gonad by up-regulating the expression of several genes involved in regulating leukocyte trafficking in the testis of SG stage fish (Cabas et al., 2011). Moreover, bath-exposure to EE_2 might alter the capacity of

gilthead seabream to appropriately respond to infection although this synthetic estrogen does not behave as an immunosuppressor (Cabas et al., 2012). Furthermore, it is known that the ability to respond to sex hormones or endocrine disruptors depends on the maturation stage of the fish (Schulz et al., 2008).

In this framework, the present study tries to fill in some gaps in this knowledge by providing data about the effects of EE_2 on the local immune regulation that takes place in the gonad in two different physiological stages of the reproductive cycle of a marine hermaphrodite fish, the gilthead seabream. Moreover, we analyse the gene expression profile of some reproductive molecules with the idea of using them as biomarkers of endocrine disruption. With this aim in mind, gilthead seabream specimens, in pre-SG and SG stages, were fed for 28 days with a pellet diet containing 5 μg EE_2/g food, in order to determine whether EE_2 promotes an estrogenic response by measuring the sperm quantity and quality, the serum levels of the main sex hormones and the gene expression of *vtg*.

2. MATERIAL AND METHODS

2.1. Animals and experimental design

Healthy specimens of gilthead seabream (Actinopterygii, Perciformes, Sparidae) were bred and kept at the Centro Oceanográfico de Murcia (Instituto Español de Oceanografía, Mazarrón, Murcia, Spain).

The experiment was performed using 30 pre-SG specimens (June, with a body weight of 110 ± 20 g, 14-months old) and 30 SG specimens (November, with a body weight of 405 ± 25 g, 19-months old) of gilthead seabream males. The fish were kept in 2 m^3 tanks with a flow-through circuit, suitable aeration and filtration system and natural photoperiod. The water temperature ranged from 14.6 to 17.8 ºC. The environmental parameters, mortality and food intake was recorded daily. The EE_2 was incorporated in the commercial food (44 % protein, 22 % lipids, Skretting, Spain) at doses of 0 (control) and 5 μg/g food, using the ethanol evaporation method (0.3 l ethanol/kg of food) as described elsewhere (Shved et al., 2007). The specimens were fed *ad libitum* three times a day for 28 days and fasted for 24 h before sampling. Sampling was carried out after 7 and 28 days of EE_2 exposure (n=6 fish/group). For this, specimens were anesthetized with 40 μl/l of clove oil and the urogenital pore was dried

before collecting sperm as described below. The specimens were then decapitated, weighed, and the livers and gonads were removed and processed for gene analysis, as described below. Serum samples from trunk blood were obtained by centrifugation and immediately frozen and stored at - 80 ºC until use. The experiments complied with the Guidelines of the European Union Council (86/609/EU), the Bioethical Committee of the University of Murcia (Spain) and the Instituto Español de Oceanografía (Spain) for the use of laboratory animals.

2.2. Measurement of sperm quantity and quality

Stripped sperm was obtained by gentle abdominal massage, collecting and measuring the sperm in the genital pore with a syringe as the semen flowed out (urine-contaminated samples were discarded). The total semen from 6 fish of each group was used immediately to determine cell concentration and motility. To determine the sperm concentration, semen was diluted in 1% formol (Panreac) and 5% $NaHCO_3$ (Sigma) in water at a ratio of 1:400 and the spermatozoa were counted using a Newbauer chamber. Motility was analysed activating 1 µl of sperm (diluted on Ringer 200 mOsm solution at the optimal dilution of 1:5) with 20 µl of seawater (Chereguini et al., 1997). The duration of sperm motility was determined by measuring the time elapsing between sperm activation and the cessation of cell displacement using a light microscope at 400x magnification. The motility index was expressed on a relative scale of 0 to 5 (Sánchez-Rodríguez, 1995).

2.3. Analytical techniques

Serum levels of E_2, T, and 11KT were quantified by ELISA following the method previously described (Rodríguez et al., 2000). Steroids were extracted from 20 µl of serum in 0.6 ml of methanol (Panreac).The methanol was then evaporated at 37 ºC and the steroids were resuspended in 400 µl of reaction buffer [0.1 M phosphate buffer with 1 mM EDTA (Sigma), 0.4 M NaCl (Sigma), 1.5 mM NaN_3 (Sigma) and 0.1 % albumin from bovine serum (Sigma)]. Then, 50 µl were used in each well so that 2.5 µl of serum were used in each well for all the assays. E_2 and T standards were purchased from Sigma-Aldrich. The 11KT standard, mouse anti-rabbit IgG monoclonal antibody (mAb), and specific anti-steroid antibodies and enzymatic tracers (steroid acetylcholinesterase conjugates) were obtained from Cayman Chemical. Microtiter plates (MaxiSorp) were purchased from Nunc. A standard curve from 6.13×10^{-4} to 2.5

ng/ml (0.03-125 pg/well) was established in all the assays. Standards and extracted serum samples were run in duplicate. The lower limit of detection for all the assays was 12.21 pg/ml. The intra-assay coefficients of variation (calculated from duplicate samples) were 2.18 ± 1.95 % for E_2, 1.37 ± 1.61 % for T, and 10.43± 2.26% for 11KT for the pre-SG specimens. Details on cross-reactivity for specific antibodies were provided by the supplier (0.01 % of anti-11KT reacts with T; 2.2 % of anti-T reacts with 11KT; and 0.1 % of anti-E_2 reacts with T).

2.4. Analysis of gene expression

Total RNA was extracted from liver and gonad fragments with TRIzol Reagent (Invitrogen) following the manufacturer's instructions, and quantified with a spectrophotometer (NanoDrop, ND-1000). The RNA of 6 fish per group was pooled using the same amount of RNA from each specimen. The RNA was then treated with DNase I (amplification grade, 1 unit/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-dT_{18} primer from 1μg of total RNA, at 50 ºC for 50 min. Real-time PCR performed with an ABI PRISM 7500 (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) was then used to analyse the expression of the genes coding for (i) steroidogenesis-related molecules: steroidogenic acute regulatory protein (*star*), cholesterol side chain cleavage cytochrome P450 (*cyp11a1*), 3β-hydroxysteroid deshydrogenase (*hsd3b*), aromatase (*cyp19a1a*), steroid 11-beta-hydroxylase (*cyp11b1*), 5α reductase (*srd5a*), and 11β-hydroxysteroid deshydrogenase (*hsd11b*), (ii) the hepatic vitellogenin (*vtg*); (iii) the testicular specific protein, double sex-and mab3-related transcription factor 1 (*dmrt1*); (iv) hormone receptors: estrogen receptor α (*era*), follicle stimulating hormone (FSH) receptor (*fshr*) and luteinizing hormone (LH) receptor (*lhr*) and; (v) immune- relevant molecules: interleukin 1β (*il1b*), tumour necrosis factor α (*tnfa*), transforming growth factor β1 (*tgfb1*), matrix metalloproteinase (*mmp*) 9 and 13 (*mmp13*), toll-like receptor 9 (*tlr9*), major histocompatibility complex I α protein (*mhc1a*), CC chemokine ligand (*ccl4*), CXC chemokine interleukin 8 (*il8*), leukocyte adhesion molecule E-selectine (*sele*) and heavy chain of immunoglobulin M (*ighm*) and T (*ight*). For each mRNA, gene expression was normalized to the ribosomal protein S18 gene (*rsp18*) content in each sample using the comparative Ct method ($2^{-\Delta\Delta Ct}$) (where Ct

is a cycle threshold). The gilthead seabream specific primers used are shown in Table 1. In all cases, each PCR was performed in triplicate

Gen	Accession Number	Name	Nucleotide sequence (5´→3´)
star	AM905934	F1 R1	ACATCGGGAAGGTGTTCAAG TCTCTGCAGACACCTCATGG
cyp11a1	FM159974.1	F R	CGCTGCTGTGGACATTGTAT CATCATGTCTCCCTGGCTTT
hsd3b	HS985587	F R	GGAGGACAAACTGGTGGAGG ACATTCTCCGTTCCGGTGAC
cyp19a1a	AF399824	F2 R2	CAATGGAGAGGAAACCCTCA ATGCAGCTGAGTCCCTGTCT
cyp11b1	FP332145	F R	GCTATCTTTGGACCCCATCA CTTGACTGTGCCTTTCAGCA
srd5a	AM958800	F R	TGCACTTTCGTGACTCTGCT TTTCGCACAAGACGTCCAGA
hsd11b	AM973598	F R	AGACATGGGCAACGAGTCAG TCCACATCTCCCTCCCACAT
vtg	AF210428	F1 R1	CTGCTGAAGAGGGACCAGAC TTGCCTGCAGGATGATGATA
dmrt1	AM493678	F R	GATGGACAATCCCTGACACC GGGTAGCGTGAAGGTTGGTA
era	AF136979	F R	GCTTGCCGTCTTAGGAAGTG TGCTGCTGATGTGTTTCCTC
fshr	AY587262	F2 R2	TCCCACTACGGATCCTCATC AACGGGAACAGTCAGTTTG
lhr	AY587261	F2 R2	ATACACGACCACGCATTCAA CGCCGGTAACTTCTTGAGAG
il1b	AJ277166	F2 R3	GGGCTGAACAACAGCACTCTC TTAACACTCTCCACCCTCCA
tnfa	AJ413189	FE1 RE3	TCGTTCAGAGTCTCCTGCAG CATGGACTCTGAGTAGCGCGA
tgfb1	AF424703	F R	AGAGACGGGCAGTAAAGAA GCCTGAGGAGACTCTGTTGG
mmp9	AM905938	F1 R1	GGGGTACCCTCTGTCGATTT CCTCCCCAGCAATATTCAGA
mmp13	AM905935	F R	CGGTGATTCCTACCCATTTG TGAGCGGAAAGTGAAGGTCT
tlr9	AY751798	F2 R2	GGAGGAGAGGGACTGGAT GATCACACCGTCACTGTCTC
mhc1a	AY292461	F R	CCAGAGCTTCCCTCAGTGTC CATCTGGAAGGTTCCATCGT
ccl4	AM765840	F1 R1	GCTGTGTTTGTGCTGATGCT GCTGGCTGGTCTTTTGGTAG
il8	AM765841	F2 R2	GCCACTCTGAAGAGGACAGG TTTGGTTGTCTTTGGTCGAA
sele	AM749963	F1 R1	GACAGTGAGCAGGCGTACAA ATCGCTTCATGATCCACACA
ighm	AM493677	F1 R1	CAGCCTCGAGAAGTGGAAAC GAGGTTGACCAGGTTGGTGT
ight	FM145138	F1 R1	TGGCAAATTGATGGACAAAA CCATCTCCCTTGTGGACAGT
rps18	AM490061	F R	AGGGTGTTGGCAGACGTTAC CTTCTGCCTGTTGAGGAACC

Table 1. Gene accession numbers and primer sequences used for gene expression analysis. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html).

2.5. Calculation and statistics

The mean ± SEM were calculated for the stripped sperm volume, concentration and motility index, and values of the same were compared between the control and treated groups throughout the experiment by means of two-way analysis of variance (ANOVA) following by a Post-hoc Waller-Duncan test ($P \leq 0.05$). All gene expression data were analyzed by a Student *t*-test to determine differences between the two groups selected. The critical value for statistical significance was taken as $P \leq 0.05$. The asterisks mean: *P <0.05, **P <0.01 and ***P <0.001. All statistical analyses were carried out using the GraphPad Prism 5 program.

3. RESULTS

3.1. EE_2 reduces the stripped sperm volume and motility in specimens in the spermatogenesis stage

In the SG stage, the dietary intake of EE_2 decreased the stripped sperm volume and motility but did not modify the stripped sperm concentration (Table 2). No data are presented for pre-SG specimens as no stripped sperm was obtained.

	Sperm volume (ml)		**Sperm concentration (cell/ml)**		**Sperm motility index**	
	Days of diet-exposure		Days of diet-exposure		Days of diet-exposure	
Treatment (EE_2)	**7**	**28**	**7**	**28**	**7**	**28**
0 µg/g food	0.63±0.19	1.80±0.38	$(3.28\pm0.76)\times10^9$	$(6.46\pm1.22)\times10^9$	2.08±0.48	2.43±0.23
5 µg/g food	0.25±0.09	1.30±0.79*	$(1.58\pm0.97)\times10^9$	$(2.57\pm1.45)\times10^9$	1.46±0.70	0.95±0.48*

Table 2: Effects of EE_2 (different doses and exposure times) on sperm volume (ml), concentration (cells/ml) and motility index. Data represent means ± SEM of six independent fish per group. Asterisks denote statistically significant differences between treatment and control groups according to a Student t test ($P \leq 0.05$). Letters denote statistically significant differences between different concentrations of EE_2 in the same time-exposure groups according to two-way analysis of variance (ANOVA) following by Waller-Duncan test.

3.2. EE_2 modifies serum sex steroid hormonal levels and modulates the gene expression profile of some steroidogenic enzymes

We have previously demonstrated in SG specimens of gilthead seabream that 5 μg EE_2/g food promoted an increase in E_2 and T serum levels after 7 days of treatment, while E_2 serum levels were similar and T and 11KT serum levels were lower than those of control specimens after 28 days of treatment (Cabas et al., 2011). In contrast, in pre-SG specimens, the dietary intake of 5 μg EE_2/g food did not significantly modify the E_2 serum levels (Fig. 1a), while T (Fig. 1b) and 11KT (Fig. 1c) levels decreased at the end of the experiment.

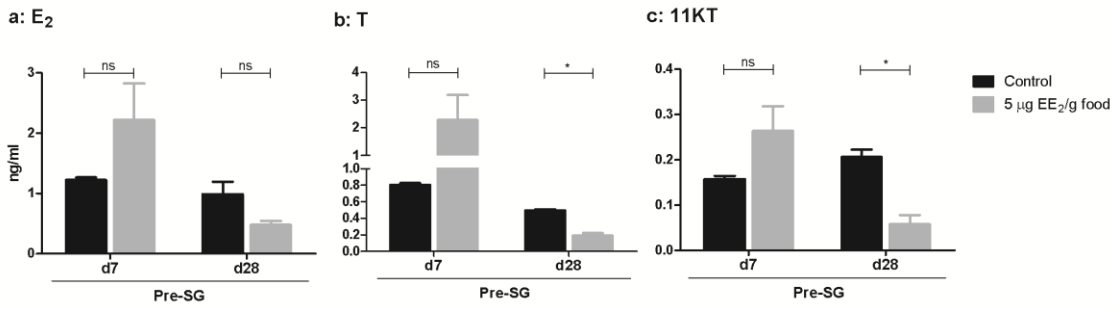

Figure 1. Modulation of serum sex hormone levels by in vivo EE_2 exposure. 17β-estradiol (E_2) (a), testosterone (T) (b) and 11-ketotestosterone (11KT) (c) serum levels were determined in gilthead seabream specimens in the pre-SG stage after the dietary intake of 0 (control) and 5 μg EE_2/g food for 7 and 28 days. The same amount of serum from 6 fish/group was mixed and the serum levels (ng/ml) were analyzed by ELISA. The data represent means ± S.E.M. in duplicate. The asterisks denote statistically significant differences compared with the untreated control group according to Student t-test. *p <0.05, **p <0.01 and ***p <0.001. ns, not significant.

We next investigated the gonadal gene expression of several steroidogenic enzymes (Fig. 2). First, it was observed that the gene expression levels of *star* (Fig. 2a), *cyp11a1* (Fig. 2b), *hsd3b* (Fig. 2c) and *srd5a* (Fig. 2f) were higher in SG than in pre-SG specimens. In contrast, the gene expression levels of *cyp19a1a* (Fig. 2d), *cyp11b1* (Fig. 2e), and *hsd11b* (Fig. 2g) were lower in SG specimens. EE_2 inhibited the expression of *star* (Fig. 2a), *cyp11a1* (Fig. 2b) and *cyp11b1* (Fig. 2e) after 7 and 28 days of exposure and the expression of *hsd3b* (Fig. 2c) and *srd5a* (Fig. 2f) only after 7 days, in both pre-SG and SG specimens. However, a differential regulation in the expression of *cyp19a1a* was observed (Fig. 2d): it was inhibited in pre-SG specimens after 28 days of exposure

to EE_2 and up-regulated in SG specimens after 7 and 28 days of exposure. Moreover, EE_2 did not modify the expression levels of *hsd11b* (Fig. 2g) in pre-SG specimens, but increased its expression in SG specimens at both sampling times.

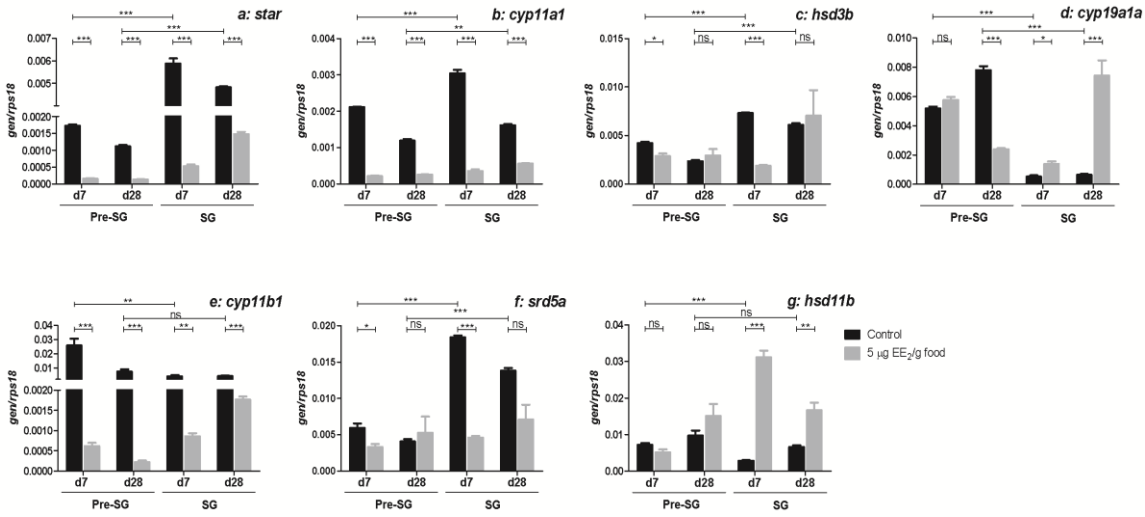

Figure 2. EE_2 modulates the expression of genes coding for steroidogenic-relevant molecules in the gonad. Specimens at both pre-SG and SG stage were treated with 0 (control) and 5 µg EE_2/g food for 7 and 28 days. Afterwards, the mRNA levels of *star* (a), *cyp11a1* (b), *hsd3b* (c), *cyp19a1a* (d), *cyp11b1* (e), *srd5a* (f) and *hsd11b* (g) were determined in the gonad by real-time RT-PCR. Data represent means ± S.E.M. of triplicate samples. Total mRNA was obtained after mixing the same amount of mRNA from 5 to 6 fish/group. The asterisks denote statistically significant differences from untreated control group for each time point, according to Student t-test. *p <0.05, **p <0.01 and ***p <0.001. ns, not significant.

3.3. EE_2 increases the expression profile of the hepatic vtg gene

The expression of *vtg*, a gene induced by activation of nuclear ER (Sumpter and Jobling, 1995), was slightly higher in pre-SG than in SG specimens. Moreover, the *vtg* expression levels were significantly up-regulated in the liver at both reproductive stages and EE_2 exposure times assayed (Fig. 3a).

3.4. EE_2 modulates the expression of testicular specific protein, dmrt1, and some hormone receptor genes in the gonad

As expected, expression of the gene that codes for *dmrt1* was higher in the gonad of SG than in pre-SG specimens (Fig. 3b). EE_2 decreased the *dmrt1* expression levels in SG specimens after 7 days of exposure, but the effect disappeared after 28 days. No

significant changes were observed in the *dmrt1* expression levels in pre-SG specimens after 7 or 28 days of exposure.

Interestingly, the mRNA expression levels of *era* (Fig. 3c), *fsrh* (Fig. 3d) and *lhr* (Fig. 3e) were higher in the gonad of pre-SG than SG specimens except for the *era* levels at day 28 of treatment. EE_2 increased the *era* expression levels in the gonad of pre-SG specimens after 7 and 28 days or only after 28 days of exposure in SG (Fig. 3c). In pre-SG specimens, EE_2 decreased the *fshr* (Fig. 3d) and *lhr* (Fig. 3e) expression levels after 7 days of exposure, but increased *lhr* expression after 28 days (Fig. 3e). Nevertheless, in SG specimens, *fshr* expression levels increased only after 28 days of EE_2 dietary intake (Fig. 3d), while the *lhr* expression levels were slightly lower after 7 days of EE_2 exposure (Fig. 3e).

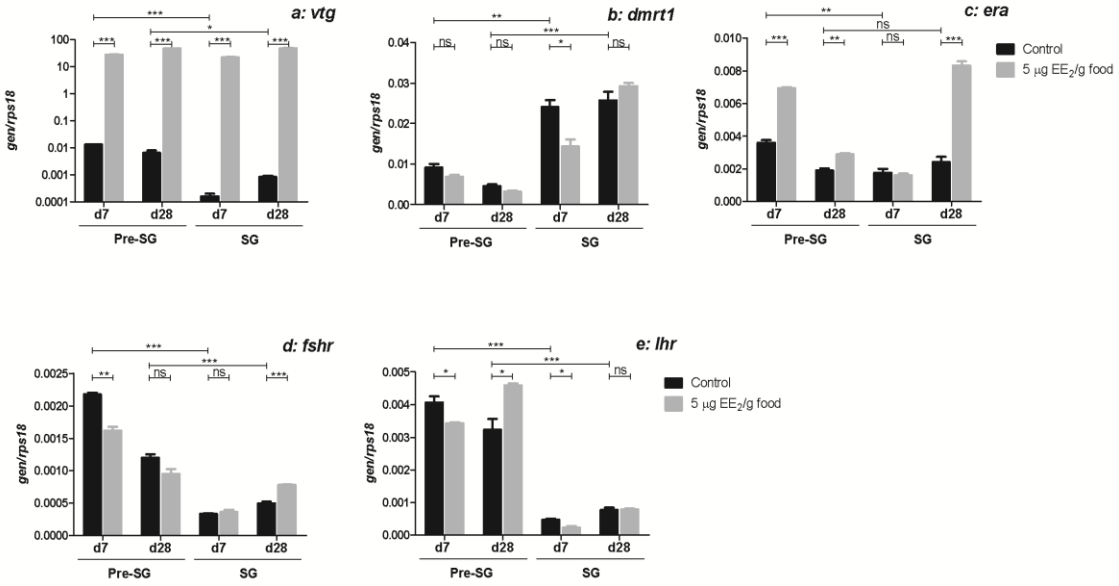

Figure 3. EE_2 promotes an estrogenic response and modulates the expression of genes coding for hormone receptors. Specimens at both pre-SG and SG stages were treated with 0 and 5 µg EE_2/g food for 7 and 28 days. Afterwards, the mRNA levels of *vtg* were determined in the liver (a) and the mRNA levels of *dmrt1* (b), *era* (c), *fshr* (d) and *lhr* (e) in the gonad by real-time RT-PCR. Data represent means ± S.E.M. of triplicate samples. Total mRNA was obtained after mixing the same amount of mRNA from 5 to 6 fish/group. The asterisks denote statistically significant differences from untreated control group for each time point, according to Student t-test. *p <0.05, **p <0.01 and ***p <0.001. ns, not significant.

3.5. EE_2 modifies the gene expression of molecules relevant in the immune response in the gonad

To explore the local immune regulation that occurred in the gonad, we analysed the expression of genes coding for several pro- and anti-inflammatory cytokines, MMPs, molecules related with pathogen recognition, antigen presentation, leukocyte recruitment, and B lymphocytes markers (Figs. 4, 5). Interestingly, almost all of the immune-related genes analyzed showed higher expression levels in pre-SG specimens than in SG, except those for *tnfa* (Fig. 4b), *mmp9* (Fig. 4d) and *mhc1a* (Fig. 4g), which were more highly expressed in SG specimens on at least one of the times analysed.

EE_2 was seen to differently modulate the expression levels of these immune-related genes in pre-SG and SG specimens. Thus, EE_2 inhibited the expression level of *il1b* after 7 days and increased it after 28 days in pre-SG specimens, while no significant differences were observed in SG specimens (Fig. 4a). Moreover, the expression levels of *tnfa* (Fig. 4b) and *mmp9* (Fig. 4d) increased after EE_2 exposure in both pre-SG and SG specimens, the increase being more pronounced in SG specimens. In contrast, the expression levels of *tgfb1* (Fig. 4c), *mmp13* (Fig. 4e) and *tlr9* (Fig. 4f) were increased by EE_2 in pre-SG specimens at both times analysed, and only after 28 days of exposure in SG specimens. As regards the gene related with antigen presentation that codes for MHC Iα protein, named *spau-UAA* following the accepted nomenclature (Klein et al., 1990), EE_2 increased the *mhc1a* mRNA levels in both pre-SG and SG specimens at both times analysed, except after 7 days of exposure in pre-SG specimens where a decrease was observed (Fig. 4g).

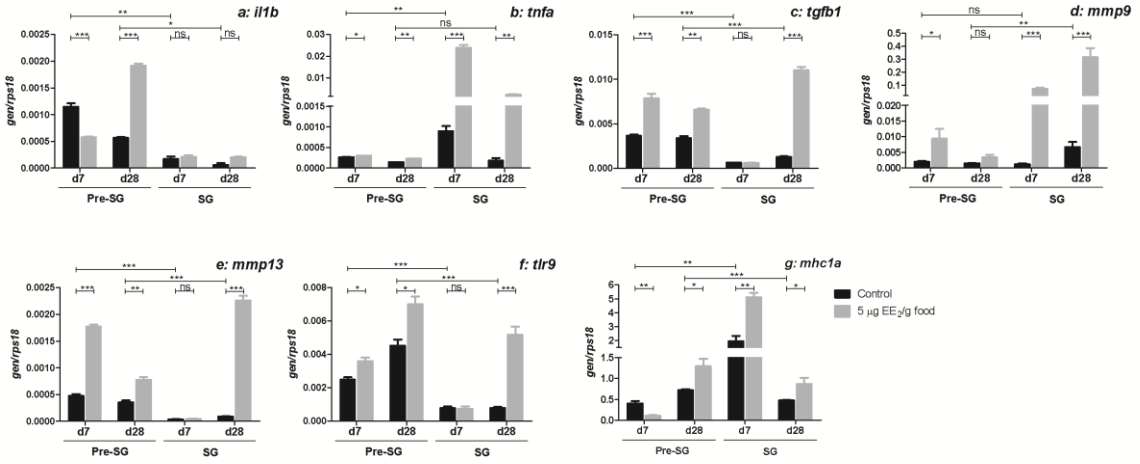

Figure 4. EE_2 modulates the expression of genes coding for immune-relevant molecules in the gonad. Specimens at both pre-SG and SG stage were treated with 0 and 5 µg EE_2/g food for 7 and 28 days. Afterwards, the mRNA levels of *il1b* (a), *tnfa* (b), *tgfb1* (c), *mmp9* (d), *mmp13* (e), *tlr9* (f), and *mhc1a* (g) were determined in the gonad by real-time RT-PCR. Data represent means ± S.E.M. of triplicate samples.

Total mRNA was obtained after mixing the same amount of mRNA from 5 to 6 fish/group. The asterisks denote statistically significant differences from untreated control group for each time point, according to Student t-test. *p <0.05, **p <0.01 and ***p <0.001. ns, not significant.

We previously demonstrated that the dietary intake of EE_2 promoted an up-regulation in the gonad of the genes coding for *ccl4*, *il8* and *sele*, and the B lymphocyte markers, *ighm* and *ight*, in SG specimens after 7, 14 and 21 days of exposure, which occurred simultaneously with an infiltration of AGs and lymphocytes (Cabas et al., 2011). Here, we explore the differential regulation by EE_2 of the expression of *ccl4* (Fig. 5a), *il8* (Fig. 5b), *sele* (Fig. 5c), *ighm* (Fig. 5d) and *ight* (Fig.5e), in pre-SG and SG specimens, after 7 and 28 days of exposure. As mentioned above, the expression levels of all these genes were higher in pre-SG than in SG specimens (Fig. 5 and Cabas et al., 2011). Similarly to what was previously observed after 7 days of EE_2 exposure in SG specimens, EE_2 increased the expression levels of all these genes in the gonad of SG specimens after 28 days. Nevertheless, in pre-SG specimens, EE_2 modulated the expression levels of these genes in a different way. Thus, although *ccl4*, *sele* and *ight* expression levels increased after certain times of EE_2 exposure, the expression levels of *ccl4* decreased and the transcription of *il8* and *ighm* were unchanged after 7 days of EE_2 exposure. Moreover, *sele* and *ighm* expression levels fell after 28 days of EE_2 exposure.

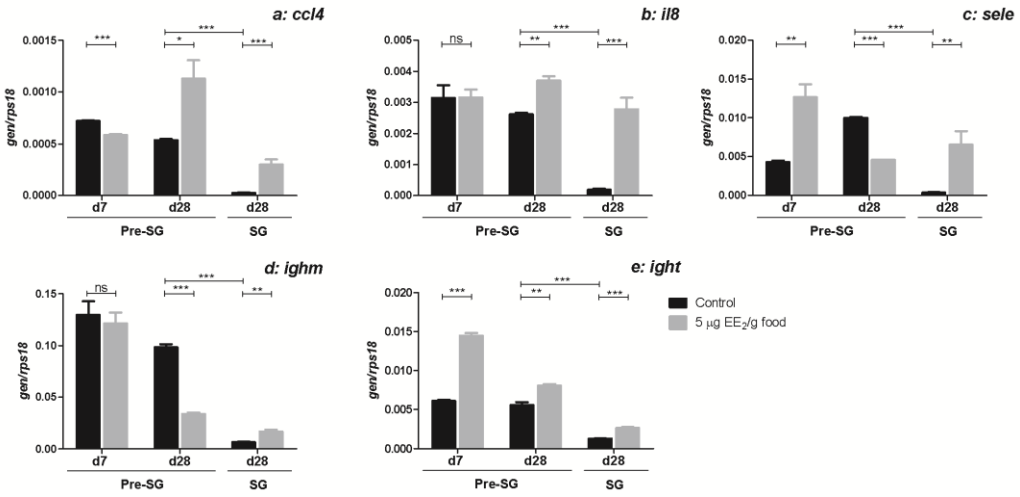

Figure 5. EE_2 modulates the expression of genes involved in regulating leukocyte trafficking and lymphocytes B markers. Specimens at both pre-SG and SG stage were treated with 0 and 5 µg EE_2/g food for 7 and 28 days and 28 days, respectively. Afterwards, the mRNA levels of *ccl4* (a), *il8* (b), *sele* (c), *ighm* (d) and *ight* (e) were determined in the gonad by real-time RT-PCR. Data represent means ± S.E.M. of triplicate samples. Total mRNA was obtained after mixing the same amount of mRNA from 5 to 6

fish/group. The asterisks denote statistically significant differences from untreated control group for each time point, according to Student t-test. *p <0.05, **p <0.01 and ***p <0.001. ns, not significant.

4. DISCUSSION

The inevitable consequence of the ever increasing consumption of pharmaceuticals is an increased level of contamination of surface and ground waters by biologically active drugs, which might have adverse effects in aquatic wildlife (Corcoran et al., 2010). EE_2 is an environmental estrogen considered as an EDC with strong estrogenic effects and a widespread presence in the aquatic environments. Fish represent the animal group most affected by EDC exposure since they are continuously and directly exposed to these contaminants. Most authors agree that EE_2 promotes an immature stage of the male gonads by blocking its development or by inducing the ablation of post-meiotic germ cells when immature fish or spermatogenically active fish are treated, respectively (Lange et al., 2008; Xu et al., 2008). Moreover, in gonochoristic fish species, a widely observed effect of estrogenic compounds is the modification of sperm quality, steroidogenic sex hormone levels and hepatic Vtg production (Sumpter and Jobling, 1995; Hogan et al., 2010). However, little is known about these effects in hermaphroditic fish species.

In the gilthead seabream, EE_2 promotes an estrogenic response as seen from the decrease in sperm quality (sperm volume and the motility index) and an increase in *vtg* gene expression levels. Interestingly, in the gilthead seabream E_2 serum levels show a response to EE_2 exposure related with the reproductive stage. Thus, in SG specimens the E_2 serum levels increased upon 7 days of EE_2 exposure (Cabas et al., 2011), while in pre-SG specimens no significant differences were observed, probably due to the fact that E_2 serum levels are already very high in the pre-SG specimens (1.2 ng/ml) compared with SG specimens (0.1 ng/ml) (Cabas et al., 2011). Moreover, the effect of the dietary intake of EE_2 on T and 11KT serum levels is similar in both stages although less pronounced in pre-SG than in SG specimens (Cabas et al., 2011). The modification of sex hormone levels detected in the serum of pre-SG and SG gilthead seabream was in concordance with the transcript regulation of the most relevant steroidogenic molecules involved in their production. Thus, EE_2 down-regulated the transcripts of *star*, *cyp11a1*, *hsd3b*, *cyp11b1* and *srd5a* (steroidogenic molecules involved in androgen production) in the gonad of both pre-SG and SG specimens while the *cyp19a1a* (steroidogenic

enzyme involved in estrogen production) expression levels were down-regulated in the gonad of pre-SG males and up-regulated in the gonad of SG males.

To assess whether any testicular reproductive parameters could be used as markers of endocrine disruption in gilthead seabream, the gene expression levels of the testicular specific protein, *dmrt1*, and of some hormone receptor genes were analysed in both stages of the reproductive cycle. The *dmrt1* expression levels in pre-SG specimens were not affected by the dietary intake of EE_2, while in SG specimens, EE_2 was seen to lower *dmrt1* expression levels after 7 days, but this effect had disappeared by the end of the treatment. As regards *era* gene, EE_2 treatment increased its expression in the gonad of pre-SG and SG fish, although at different time points. We have previously recorded increases in *era* gene expression upon E_2 treatment in endothelial cells and macrophages *in vitro* (Liarte et al., 2011a; 2011c) and upon EE_2-bath exposure in head-kidney leukocytes *in vivo* (Cabas et al., 2012). Although *era* gene expression was seen to have increased in all the analyses carried out and could well be used as biomarker of endocrine disruption (Carnevali et al., 2011), the magnitude of the response differed from that observed for *vtg* gene expression and the time at which the effect appear became evident varied between stages and between tissues. Furthermore, these differences suggest changes in the sensitivity to estrogens during sexual maturation and point to the need for further studies to clearly determine life stages that are susceptible to estrogenic pollutants in fish. Moreover, EE_2 dietary intake down-regulated the *fshr* and *lhr* expression levels, except in the gonad of SG fish treated for 28 days, while the *dmrt1* expression levels remained similar to those of the control fish. Interestingly, all these data agree with the disruption of spermatogenesis and the recrudescence of the testicular area of the gonad and the non-induction of the sex change previously observed in SG gilthead seabream gonad upon EE_2 dietary intake (Cabas et al., 2011).

A relevant role for immune molecules in the regulation of spermatogenesis and/or steroidogenesis has been described in various vertebrates including fish (O'Bryan et al., 2008; Chaves-Pozo et al., 2008a). In the gilthead seabream, the testis undergoes abrupt morphological changes especially after spawning, including a massive infiltration of AGs (Chaves et al., 2003, 2005a, 2010; Liarte et al., 2007). AGs are produced in the head-kidney and when they infiltrate the testis, they show heavily impaired functions (Chaves-Pozo et al., 2005b). Interestingly, the expression of genes coding for pro-inflammatory and anti-inflammatory mediators, innate immune

receptors, lymphocyte receptors, anti-bacterial and anti-viral proteins and molecules related to leukocyte infiltration show a testicular pattern that depends on the reproductive stage of the gilthead seabream specimens (Chaves-Pozo et al., 2008a) and which guarantees and modulates reproductive functions. In addition, endogenous increases of E_2 in serum are correlated with AG infiltration into the gonad after spawning (Chaves-Pozo et al., 2010a), while the dietary intake of EE_2 by SG specimens of gilthead seabream induced the recruitment of AGs and B lymphocytes and up-regulated the expression of genes coding for molecules involved in leukocyte trafficking (Cabas et al., 2011). Moreover, specimens bath-exposed to EE_2 showed alterations in their capacity to appropriately respond to infection (Cabas et al., 2012). Although, EE_2 modulates tha expression pattern of immune molecules in gilthead seabream macrophages, which are known to be a key cell type in the immune-modulatory role played by E_2 in the gilthead seabream gonad (Liarte et al., 2011a; Cabas et al., 2012), little is known on the effects of EE_2 and other environmental estrogens on the gene expression of immune-relevant molecules in the gonad of fish in general.

In the gilthead seabream, EE_2 promotes an increase in the gonadal transcripts of the pro-inflammatory cytokines, *il1b* and *tnfa*, and the anti-inflammatory cytokine, *tgfb1*, although the response differs between pre-SG and SG specimens. These increases could be correlated with the decrease in androgen production and suggest, as occurs in mammals (Lui et al., 2003), that these cytokines are involved in testicular steroidogenesis; however, further studies are needed to confirm this observation. A similar conclusion was reached for the goldfish testis, in which a heterologous recombinant IL1β, murine IL1β, inhibited basal and human chorionic gonadotrophin-stimulated T production (Lister et al., 2002). *Mmp9* and *mmp13* gene expression in the testis of gilthead seabream suggests a pivotal role for them in the regulation of the testicular physiology and, in particular, in the organization of the cysts during spermatogenesis and post-spawning, as well as in AG infiltration (Chaves-Pozo et al., 2088c). EE_2 dietary intake promotes the transcription of *mmp9* and *mmp13* genes in the gonad of both pre-SG and SG specimens, which concords with the induction of the post-spawning stage and AG infiltration upon estrogen (E_2 or EE_2) exposure (Chaves-Pozo et al., 2007, 2008c; Cabas et al., 2011). TLRs play important roles in the innate immunity of the male mammalian reproductive tract (Palladino et al., 2007). Although several *tlr* gene sequences have been reported in gilthead seabream, *tlr9* is the only one

which is expressed in the gonad (Chaves-Pozo et al., 2008a). Our data show that the dietary intake of EE_2 increases the expression of *tlr9* and *mhc1a* genes in the gonad of both pre-SG and SG fish, suggesting that this estrogenic pollutant stimulates the ability of the gonad to recognise and respond to pathogens. This is important when we consider that there are some pathogens that use the gonads to be transmitted to the next generations or to other animals (Chaves-Pozo et al., 2010b). However, further studies are needed to clearly determine the ability of the gonad to respond to gonad invasive pathogens under estrogenic pollutant conditions.

Finally and concerning the expression of genes that code for molecules involved in leukocyte recruitment (AGs and lymphocytes) into the gonad (Roca et al., 2008; Chaves et al., 2011), and for B lymphocyte markers (IgM and IgT), higher expression levels were observed in pre-SG than in SG specimens (Cabas et al., 2011), in agreement with previous results obtained in gilthead seabream gonad upon E_2 exposure (Chaves-Pozo et al., 2007). Probably, these differences in the level of gene expression observed between pre-SG and SG specimens resulted in differences in the leukocyte influx into the gonad in response to EE_2.

In conclusion, our data demonstrate that the dietary intake of EE_2 promotes an estrogenic response and modifies the expression pattern of steroidogenic molecules, cytokines and other immune-related molecules involved in different processes of testicular physiology, ranging from steroidogenesis to pathogen recognition. Interestingly, a developmental adjustment of the sensitivity to EE_2 in the gilthead seabream gonad was observed, pointing the need for further studies to clearly determine the most susceptible life stages to estrogenic pollutants in fish.

CHAPER III

17α-ETHYNYLESTRADIOL ALTERS THE IMMUNE RESPONSE OF THE TELEOST GILTHEAD SEABREAM (*Sparus aurata* L.) BOTH IN VIVO AND IN VITRO

ABSTRACT

There is increasing public attention concerning the effect of endocrine disruptor chemicals (EDCs) on the immune system. One important group belonging to EDCs are the environmental estrogens. Commonly found in the effluents in wastewater treatment plants, 17α-ethynylestradiol (EE_2) which is used in contraceptive pills, is an endocrine disruptor with strong estrogenic effects. This study aims to investigate the capacity of EE_2 to modulate *in vivo* and *in vitro* the innate immune response of the gilthead seabream (*Sparus aurata* L.), a teleost species of great commercial value. For this purpose, adult specimens were bath-exposed to EE_2 (0, 5 and 50 ng/L) and then immunized with hemocyanin in the presence of the adjuvant aluminum. The results indicate that, after 15 days of EE_2-exposure, the disruptor was able to inhibit in a dose-dependent manner the induction of interleukin-1β (IL-1β) gene expression, but did not significantly alter the specific antibody titer. To shed light on the role played by EE_2 in to seabream immune response, leukocytes were exposed *in vitro* to several concentrations of EE_2 (0, 0.5, 5, 50 and 500 ng/ml) for 3, 16 and 48 hours and the production of reactive oxygen intermediates, the phagocytic activity and the gene expression profile of these cells were analyzed. EE_2 was seen to inhibit both cellular activities and to alter the immune gene expression profile in primary macrophages. Thus, low concentrations of EE_2 increase the mRNA levels of IL-1β, IL-6, tumour necrosis factor α and tumour growth factor β in non-activated macrophages. In contrast, EE_2 treatment of activated macrophages resulted in the decreased expression of pro-inflammatory genes and the increased expression of genes encoding anti-inflammatory and tissue remodelling/repair enzymes. Taken together, our results suggest that EE_2 might alter the capacity of fish to appropriately respond to infection although it does not behave as an immunosuppressor.

1. INTRODUCTION

The impact of endocrine disruptor chemicals (EDCs) in the environment and their potential effects on wildlife species and humans has received increasing public attention (Colborn et al., 1993; Sumpter and Johnson, 2005). EDCs are able to interfere with endocrine pathways at multiple levels, altering physiological functions in wildlife and humans (Tyler et al., 1998; Vos et al., 2000). One important group belonging to EDCs, found in the aquatic environment, are the environmental estrogens, such as the natural estrogens 17β-estradiol (E_2), estrone and estriol, and the synthetic estrogen 17α-ethynylestradiol (EE_2), among others. These estrogens have been found in sewage effluent in the ng/L range (Desbrow et al., 1998; Snyder et al., 1999; Baronti et al., 2000). EE_2, a pharmacological compound widely used as oral contraceptive and in hormone replacement therapy, has a widespread presence in the aquatic environment (Ternes et al., 1999; Johnson et al., 2005; Hinteman et al., 2006; García-Reyero et al., 2011), where it is present in sewage treatment plant effluents at concentrations ranging from 1 to 10 ng/L, although levels as high as 42 ng/L have also been documented (Desbrow et al., 1998; Ternes et al., 1999). The effects of EE_2 on the reproductive performance of animals have been widely studied (Lai et al., 2002a; Peters et al., 2007; Lange et al., 2008; Xu et al., 2008; Hashimoto et al., 2009; Hogan et al., 2010; Kaptaner and Unal, 2010; Marlatt et al., 2010; Cabas et al., 2011). More recently, the estrogenic actions of EE_2 have been described at transcriptomic levels in the liver and testis of the fathead minnow (García-Reyero et al., 2011), at transcriptomic and proteomic levels in zebrafish liver (De Wit et al., 2010) and at transcriptomic level in zebrafish liver and telencephalon (Martyniuk et al., 2007).

It is well known that EE_2 possesses several characteristics that make it one of the most potent estrogenic compounds. For example, it has been described that EE_2 shows a low degree of susceptibility to metabolic breakdown, a high persistence (Lai et al., 2002b), a high tendency to bioconcentrate in organism (Larsson et al., 1999), a long half-life (Ying et al., 2003) and a strong affinity for estrogen receptors (ER) (Tilton et al., 2005). In fish, several reports have shown that EE_2 can be 10–50-fold more potent *in vivo* than E_2 (Thorpe et al., 2003; Nash et al., 2004) and even 100-fold more potent than E_2 in luciferase reporter zebrafish (Legler et al., 2002). This potent estrogenic activity of EE_2 has recently been illustrated by Kidd et al. (2007), who showed that contaminant EE_2 may result in the near collapse of wild fish populations.

In mammals, the interaction between endocrine disrupters and the immune system has been studied (Ahmed, 2000) and it is a well-known that estrogens have an immune-modulatory role (Straub, 2007). Nevertheless, there are few studies on the effects of estrogens on the fish immune response (Iwanowicz and Ottinger, 2009; Milla et al., 2011). For example, it has been found that *in vitro* treatment with high doses of E_2 and EE_2 reduces the phagocytic activity of tilapia head kidney leukocytes (Law et al., 2001). The addition of EE_2 to weakly estrogenic effluents results in lymphopenia in the fathead minnows (Filby et al., 2007) while EE_2 treatment during early developmental stages reduces the number and size of the splenic melanomacrophages center and impairs the expression of IGF-I and IGF-II in immune organs in fathead minnows (Shved et al., 2009) and increases tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) mRNA levels in zebrafish (Jin et al., 2010).

The gilthead seabream (*Sparus aurata* L.) is a seasonally breeding, marine, protandrous hermaphrodite teleost with a bisexual gonad. The gilthead seabream specimens are males, at least, during the first two reproductive cycles in the Western Mediterranean area and in the rearing conditions of the Centro Oceanográfico of Murcia (Instituto Español de Oceanografía), although their gonads possess a non-developed ovarian area separated from the testicular area by connective tissue (Chaves-Pozo et al., 2005a; Liarte et al., 2007).

This species has a great commercial value and, therefore, the impact of EDCs on its immune system is an important concern. Several observations in this species suggest that estrogens interact with the immune system. The exogenous administration of E_2 or EE_2 induces the infiltration of acidophilic granulocytes (AGs) from the head kidney, the main hematopoietic organ in fish, into the gonad (Chaves-Pozo et al., 2007; Cabas et al., 2011). Curiously, AGs do not express any of the three known ER, namely ERα, ERβ1 and ERβ2 (Pinto et al., 2006; Liarte et al., 2011a), while macrophages (MΦ) and lymphocytes (L) constitutively express ERα (Liarte et al., 2011a). Moreover, E_2 has been reported to be able to regulate MΦ activation (Liarte et al., 2011a, b) and to profoundly alter the gene expression profile of these cells, especially gene ontology category immune-related processes and pathways (Liarte et al., 2011b). To our knowledge, a combined *in vivo* and *in vitro* approach to studying the impact of EE_2 on the innate and adaptive immune responses has not been carried out to date. The aim of this study was, therefore, to evaluate the ability of realistic EE_2 *in vivo* exposure on

innate and adaptive immunity of gilthead seabream and to assess the effects of EE_2 *in vitro* treatment on leukocyte activities and macrophage gene expression patterns.

2. MATERIAL AND METHODS

2.1. Animals, EE_2 in vivo treatment and sample collection

Healthy specimens of gilthead seabream (*Sparus aurata* L., Perciformes, Sparidae) were maintained at the Centro Oceanográfico of Murcia, Instituto Español de Oceanografía (IEO) (Mazarrón, Murcia). They were kept in running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/h) at 23˚C under natural photoperiod, and fed twice a day with a commercial pellet diet (Skretting, Spain) at a feeding rate of 1.5% of fish biomass.

EE_2 *in vivo* treatment was carried out in July (resting stage) with male specimens of 350 g mean weight kept in 2 m^3 aquaria. For this purpose, EE_2 (98% purity; Sigma) was administred 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. Duplicate aquaria for each EE_2 concentration were arranged in a closed circuit in order to maintain the EE_2 concentration, and connected with a sand filter (600 mm diameter, 14 m^3/h), a biological filter and an aeration pump (30 m^3/h). The seawater in the aquaria was completely renewed and fresh EE_2 added, when the nitrite concentration reached 1 ppm (every 3 days, approximately). In order to evaluate the effect of EE_2 on an induced adaptive immune response, the animals were intraperitoneally injected with phosphate buffered saline (PBS) (control fish) or hemocyanin (300 μg/fish, Sigma) and Imject Alum Adjuvant (4 mg/fish, Thermo Scientific) (immunized/vaccinated fish) after 14 (priming), 28 (first booster) and 43 (second booster) days of EE_2 exposure. The samplings were carried out on day 15 (1 day post-priming), 29 (1 day after first boost), 43 (14 days after first boost) and 58 (15 days after second boost) (Figure 1). Specimens (n=6 fish/group and time) were anesthetized with 40 ppm of clove oil, decapitated, weighed and the head kidneys, livers and gonads were removed. The gonads were weighed, while serum samples from trunk blood were obtained by centrifugation and immediately frozen and stored at -80ºC until use. The head kidneys and livers were processed for light microscopy and gene analysis, as described below.

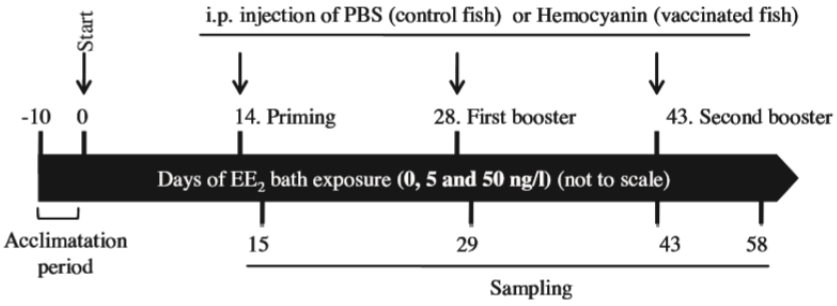

Fig. 1. Schematic drawing of the experimental design for gilthead seabream under EE_2 bath exposure and immunization schedule. Fish were exposed to several EE_2 concentrations (0, 5 and 50 ng/L) for 2 months. After 14, 28 and 43 days of exposure, fish were i.p. injected with PBS (control fish) or vaccinated with hemocyanin plus Alum adjuvant (stimulated fish). Sampling was carried out on days 15, 29, 43 and 58. More details are provided in the Materials and methods section.

For the *in vitro* experiments, male specimens (650 g mean weight kept in 14 m^3 aquaria) were sampled in spring and summer (postspawning and testicular inversion stages) 2009. In this period, the sex hormone levels remain constant (Chaves-Pozo et al., 2008). Fish were decapitated, and the head kidneys were removed and cell suspensions obtained as described elsewhere (Chaves-Pozo et al., 2003, 2005b).

The experiments described comply with the Guidelines of the European Union Council (86/609/EU), the Bioethical Committees of the University of Murcia (Spain) and the IEO for the use of laboratory animals.

2.2. Gonadosomatic index

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

2.3. Determination of hormone levels

To test the effect of *in vivo* EE_2 treatment, serum levels of E_2 and testosterone (T) were quantified by ELISA analysis following the method described by Rodríguez et al. (2000). Steroids were extracted from 20 μl of serum in 0.6 ml methanol (Panreac). E_2 and T standards were purchased from Sigma-Aldrich. Mouse anti-rabbit IgG monoclonal antibody (mAb), and specific anti-steroid antibodies and enzymatic tracers (steroid acetylcholinesterase conjugates) were obtained from Cayman Chemical. Microtiter plates (MaxiSorp) were purchased from Nunc. A standard curve from 6.13x

10^{-4} to 2.5ng/ml (0.03-125 pg/well) was established in all the assays. Standards and extracted serum samples were run in duplicate. The lower limit of detection for all the assays was 12.21pg/ml. The intra-assay coefficients of variation (calculated from sample duplicates) were 4.7 ± 0.4% for E_2 and 6.3 ± 1.7% for T. Details on cross-reactivity for specific antibodies were provided by the supplier (0.1% of anti-E_2 reacts with T).

2.4. Determination of IgM specific titer

The hemocyanin specific IgM titer was determined by an ELISA kit (Aquatic Diagnostic, Ltd.) following the manufacturer's instructions. In short, doubling-dilutions of serum from control or hemocyanin-immunized fish were added to hemocyanin pre-coated 96-well ELISA plates, followed by a monoclonal antibody specific to seabream IgM and, finally, an anti-rabbit IgG (whole molecule)-peroxidase antibody produced in goat (Sigma). Finally, the chromogen tetramethylbenzidine (TMB) was added and the absorbance read at 450 nm using a FLUOstart luminometer (BGM, LabTechnologies).

2.5. Cell culture and EE_2 in vitro treatments

Seabream head kidney leukocytes were maintained in sRPMI [RPMI-1640 culture medium (Gibco) adjusted with 0.35% NaCl to gilthead seabream serum osmolarity] medium containing 100 i.u./ml penicillin and 100 μg/ml streptomycin (Biochrom). Aliquots of head kidney cell suspension ($0.5x10^6$) were incubated for 3, 16 or 48 h in sRPMI medium supplemented with 5% charcoal/dextran-treated hormone-free fetal bovine serum (hf-FBS, Hyclone) alone (control) or containing 0.5, 5, 50 or 500 ng/ml EE_2 in the presence or absence of 50 μg/ml *Vibrio anguillarum* genomic DNA (VaDNA). After incubation, reactive oxygen intermediate (ROI) production and phagocytosis assays were performed. MΦ monolayers were obtained as adherent cells from head kidney cell suspensions by overnight incubation in serum-free sRPMI medium (Roca et al., 2006). The identity of the monolayer was confirmed by the expression of the macrophage colony stimulating factor receptor (*mcsfr*) (Roca et al., 2006). MΦ were then incubated in sRPMI medium supplemented with 5% hf-FBS alone (control) or containing 0.5, 5 or 50 ng/ml EE_2 in the presence or absence of 50 μg/ml VaDNA for 3 and 16 h. After the stimulation, MΦ cell pellets were processed for gene expression analysis.

2.6. ROI production assay

ROI production was measured as the luminol-dependent chemiluminescence produced by $0.5x10^6$ head kidney cell suspensions pre-incubated with EE_2, as indicated above (Mulero et al., 2001). This was achieved by adding 100µM luminol (Sigma) and 1µg/ml phorbol myristate acetate (PMA, Sigma), while the chemiluminescence was recorded every 127s for 1h in a FLUOstart luminometer (BGM, LabTechnologies). The values reported are the average of triple readings from 6 different samples, expressed as the slope of the reaction curve from 127 to 1016s, from which the apparatus background was subtracted.

2.7. Phagocytosis assay

Head kidney cell suspensions (aliquots of $0.5x10^6$) pre-incubated with EE_2 were challenged with FITC-labelled *V. anguillarum* (strain R-82, serotype 01) for 60 min as described by Esteban et al. (1998). The number and the intensity of green fluorescent cells were analyzed using flow cytometry and the phagocytic ability (% green fluorescent cells) and capacity (mean fluorescent intensity) were calculated.

2.8. Viability assay

Aliquots of $0.5x10^6$ of head kidney cell suspensions pre-incubated with EE_2 were diluted in 200µl PBS containing 40µg/ml propidium iodide. The number of red fluorescent cells (dead cells) from duplicated samples was analyzed by flow cytometry.

2.9. Analysis of gene expression

Total RNA was extracted from head kidney, liver and MΦ monolayers (n=6) with TRIzol Reagent (Invitrogen), following the manufacturer's instructions, and quantified with a spectrophotometer (NanoDrop, ND-1000). RNA was then treated with DNase I (amplification grade, 1unit/µg RNA, Invitrogen), and SuperScript III RNase H- Reverse Transcriptase (Invitrogen) was used to synthesize first strand cDNA with oligo $(dT)_{18}$ primer from 1µg of total RNA, at 50°C for 50 min. The *mcsfr* and β-actin (*actb*) genes were analyzed by semi-quantitative PCR performed with an Eppendorf 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 for each gene, 1 min at 72°C, and finally 10 min at 72°C. For the analysis of the MΦ gene expression profile, the expression of genes coding for the following was analysed: (i) pro-inflammatory molecules IL-1β, IL-6, TNFα and cyclooxygenase 2 (Cox-2); (ii) anti-inflammatory molecule transforming growth factor β1 (TGFβ1); (iii) CXC

chemokine IL-8; and (iv) matrix metalloproteinases (MMP) 9 and MMP13. In addition, the mRNA levels of the genes encoding IL-1β and vitellogenin (VTG) were analyzed in the head kidney and liver, respectively, of animals exposed to EE_2 by bath immersion. Real-time PCR were performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10min at 95ºC, followed by 40 cycles of 15 s at 95ºC, 1min at 60ºC, and finally 15s at 95ºC, 1min 60ºC and 15s at 95ºC. For each mRNA, gene expression was corrected by the ribosomal protein S18 gene (*rps18*) content in each sample. The gilthead seabream specific primers used are shown in Table 1. In all cases, each PCR was performed with triplicate samples and repeated at least twice. Less than 2% variation in the *rps18* gene expression was observed between samples.

Gene	Accesion number	Name	Sequence (5′–3′)	Used
actb	X89920	F3	ATCGTGGGGCGCCCCAGGCACC	PCR
		R3	CTCCTTAATGTCACGCACGATTTC	
mcsfr	AM050293	F3	CTGCCCTACAATGACAAG	PCR
		R4	TCAGACATCAGAGCTTCC	
illb	AJ277166	F2	GGGCTGAACAACAGCACTCTC	Real-time RCR
		R3	TTAACACTCTCCACCCTCCA	
il8	AM765841	F2	GCCACTCTGAAGAGGACAGG	Real-time RCR
		R2	TTTGGTTGTCTTTGGTCGAA	
il6	AM749958	F	AGGCAGGAGTTTGAAGCTGA	Real-time RCR
		R	ATGCTGAAGTTGGTGGAAGG	
tnfa	AJ413189	FE1	TCGTTCAGAGTCTCCTGCAG	Real-time RCR
		RE3	CATGGACTCTGAGTAGCGCGA	
Ptgs2(Cox-2)	AM296029	F2	CATCTTTGGGGAAACAATGG	Real-time RCR
		R2	AGGCAGTGTTGATGATGTCG	
tgfb1	AF424703	F	AGAGACGGGCAGTGAAAGAA	Real-time RCR
		R	GCCTGAGGAGACTCTGTTGG	
mmp9	AM905938	F1	GGGGTACCCTCTGTCGATTT	Real-time RCR
		R1	CCTCCCCAGCAATATTCAGA	
mmp13	AM905935	F	CGGTGATTCCTACCCATTTG	Real-time RCR
		R	TGAGCGGAAAGTGAAGGTCT	
vtg	S4589	F1	CTGCTGAAGAGGGACCAGAC	Real-time RCR
		R1	TTGCCTGCAGGATGATGATA	
era	AF136979	F1	GCTTGCCGTCTTAGGAAGTG	Real-time RCR
		R1	TGCTGCTGATGTGTTTCCTC	
rps18	AM490061	F1	AGGGTGTTGGCAGACGTTAC	Real-time RCR
		R1	CTTCTGCCTGTTGAGGAACC	

Table 1. Gene accession numbers and primer sequences used for gene expression analysis. The gene symbols followed the Zebra fish Nomenclature Guidelines (http://zfin.org/zf info/nomen.html)

2.10. Statistical analysis

Data were analyzed by analysis of variance (ANOVA) and a Tukey multiple range test to determine differences between groups ($P \leq 0.05$).

3. RESULTS

3.1. EE_2 in vivo treatment does not affect fish survival but drastically alters sex hormone levels and vitellogenin expression

The survival of animals exposed to EE_2 was 100% during the trial. The GSI slightly decreased in EE_2-treated groups compared with non-treated animals but the differences were not statistically significant (Figure 2a). In addition, the fish exposed to EE_2 for 15 days showed no significant changes in E_2 serum levels (Figure 2b) but a significant decrease in T levels compared with non-treated specimens was observed (Figure 2c). Unexpectedly, the lower dose of EE_2 (5 ng/L) resulted in a significant increase in T levels (Figure 2c).

VTG is a known endocrine disruptor marker that is strongly induced after *in vivo* EE_2 exposure (Sumpter and Jobling, 1995). No significant changes in hepatic VTG expression were observed after 15 days of EE_2 exposure to the lower dose (5 ng/L), while 650-fold increase in VTG gene expression was observed after 29 days of EE_2 exposure (Figure 2d). Moreover, the VTG mRNA levels in animals exposed to the highest EE_2 concentration (50 ng/L) increase approximately 40,000-fold by days 15 and 29 (Figure 2d).

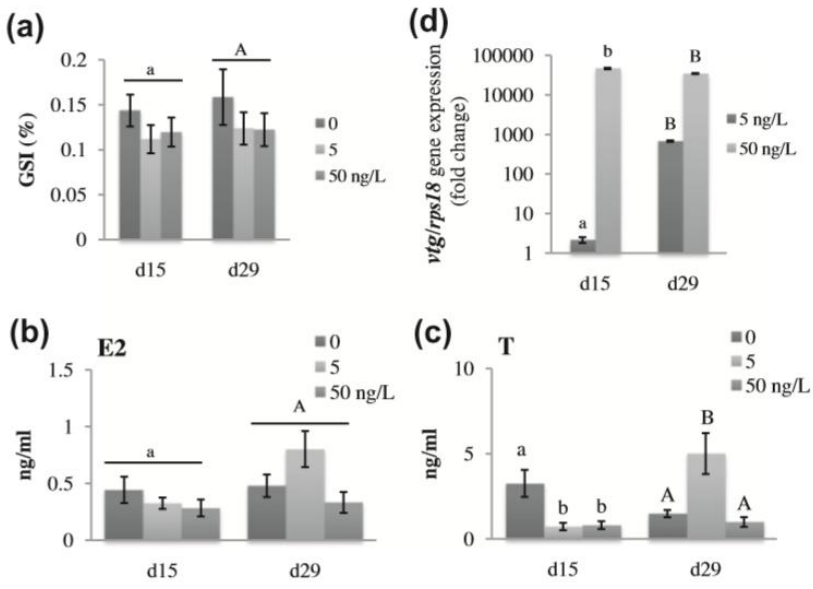

Fig. 2. Analysis of endocrine disrupter markers for *in vivo* EE_2 bath exposure. The gonadosomatic index (GSI) (a), the serum levels of E_2 (b) and T (c), and the hepatic mRNA levels of vitellogenin (*vtg*) (d) were analyzed after 15 and 29 days of EE_2 exposure in non-vaccinated fish. In all the analyses, the sample size was $n = 6$ fish/group/time. The GSI was calculated as $100*[MG/MB]$ (%), where MG is gonad mass (in grams) and MB is body mass (in grams). The serum levels (ng/ml) of E_2 and T were analyzed by ELISA

and data represent means ± SE in duplicate. The hepatic mRNA levels of *vtg* were analyzed by real-time PCR. Data for *vtg* expression represent means ± SE in triplicate. Gene expression levels were normalized to *rps18* mRNA levels and are expressed as relative fold change compared with the control fish (not exposed to EE_2). Different letters denote statistically significant differences among the groups according to a Tukey test ($P \leq 0.05$). The groups labeled with a and A do not show statistically significant differences from control fish at 15 and 29 days, respectively.

3.2. EE_2 in vivo treatment inhibits cytokine expression after immunization but hardly alters the antibody response

The expression of IL-1β, a pivotal pro-inflammatory molecule, was evaluated after 15 (Figure 3a) and 29 days (Figure 3b) of EE_2 exposure in control and immunized fish. In control fish, EE_2 exposure did not significantly alter the mRNA levels of IL-1β at any of the sampling times. Interestingly, however, EE_2 was able to decrease in a dose-dependent manner the induction of IL-1β observed in the head kidney of primed fish. Unexpectedly, EE_2 slightly increased the mRNA levels of IL-1β in immunized fish after boosting (Figure 3b).

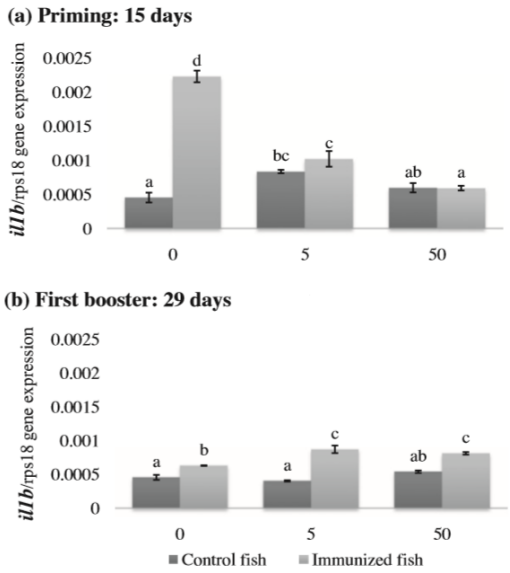

Fig. 3. Modulation of IL-1β gene expression in the head kidney of EE2-exposed fish. The mRNA levels of il1b were analyzed by real-time PCR on day 15 (a; 1 day after priming) and day 29 (b; 1 day after first booster). Gene expression levels were normalized to rps18 mRNA levels and data represent means ± SE in triplicate. Different letters denote statistically significant differences among the groups according to a Tukey test ($P \leq 0.05$). The groups labeled with "a" do not show statistically significant differences from control cells.

The impact of EE_2 exposure on the adaptive immune response was assayed as the presence of hemocyanin-specific IgM in the serum of immunized fish after 43 (Figure 4a) and 58 days (Figure 4b) of EE_2 exposure; that is, 15 days after the first and second booster, respectively. As expected, immunized animals showed a strong antibody response to the antigen on both days. Moreover, although the higher dose of EE_2 slightly decreased the antibody titer of immunized fish, no differences were observed in the serum IgM levels between EE_2-exposed and control groups after the second booster.

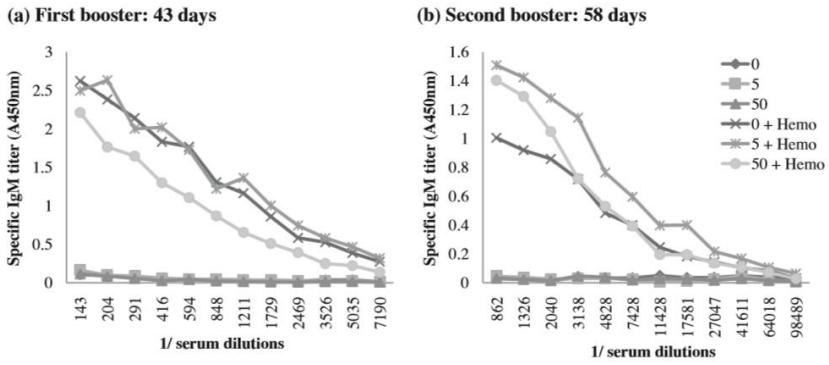

Fig. 4. Modulation of the humoral adaptive immune response by EE2. Hemocyanin-induced specific IgM levels were determined by ELISA 43 (a) and 58 (b) days after EE2 exposure (15 days after first and second booster, respectively). The absorbance was read at 450 nm using a FLUO start luminometer and the data represent the mean ± SE in triplicate pooled sera from 10 fish at all serum dilution levels used.

3.3. Both EE_2 exposure and immunization increase the expression of the gene encoding ERα

The expression of ERα gene, which is constitutively expressed by seabream MΦ and lymphocytes (Ly) (Liarte et al., 2011a), was analyzed in control and immunized fish after 15 (Figure 5a) and 29 days (Figure 5b) of EE_2 exposure. In control fish, both doses of EE_2 were able to significantly increase the mRNA levels of ERα in the head kidney. Strikingly, immunization also resulted in a significant increase in ERα transcript levels in this organ. However, the combination of immunization and the highest dose of EE_2 significantly reduced the ERα transcript levels at 15 and 29 days. Unfortunately, we were unable to analyze the expression of ERβ1 and ERβ2 genes, since no primer pairs that fulfill the real-time PCR requirements were obtained due to the high degree of similarity between the three ER genes.

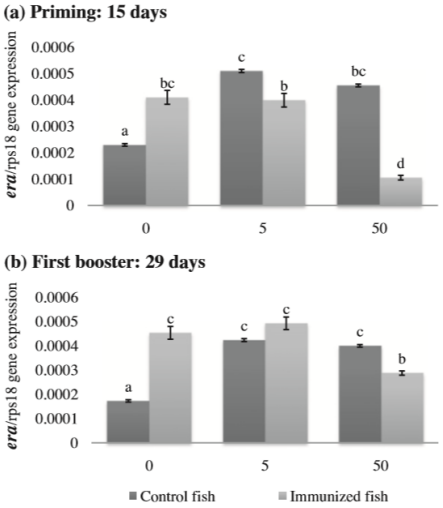

Fig. 5. Modulation of ERα gene expression in head kidney by EE2 bath exposure. The mRNA levels of ERα were analyzed by real-time PCR on day 15 (a; 1 day after priming) and day 29 (b; 1 day after first booster). Gene expression levels were normalized to rps18 mRNA levels and data represent means ± SE in triplicate. Different letters denote statistically significant differences among the groups according to a Tukey test (?0.05). The groups labeled with "a" do not show statistically significant differences from control cells.

3.4. EE_2 modulates the respiratory burst and phagocytic activity of leukocytes in vitro

None of the EE_2 doses used in this study significantly altered leukocyte viability. After 48 h of EE_2 treatment, the longest time used in this study, viability ranged from 90 ± 1.2% in control cells to 87.6 ± 1.5% in cells treated with 500 ng of EE_2/ml.

EE_2 alone was unable to significantly alter the production of ROI by non-stimulated head kidney phagocytes after 3 and 16 h of incubation (Figure 6a). Nevertheless, when the leukocytes were stimulated with VaDNA, the highest concentrations of EE_2 (50 and 500 ng/ml) were able to inhibit the ROI production induced by VaDNA after 16 h of incubation (Figure 6b). Similarly, EE_2 was also able to decrease in a dose-dependent manner the phagocytic capacity of leukocytes after 16 h of incubation (Figure 7a). However, 500 ng/ml EE_2 slightly, but significantly, increased the percentage of phagocytic cells at the two incubation times assayed (Figure 7b).

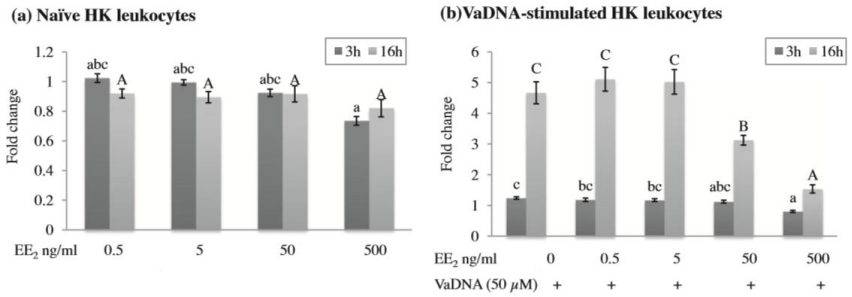

Fig. 6. ROI production of head kidney leukocytes treated with EE2. Head kidney leukocytes were incubated with 0, 0.5, 5, 50 and 500 ng/ml EE2 alone (a) or in the presence of 50 μg/ml VaDNA (b) for 3 h (light grey bars) or 16 h (dark grey bars). The results are presented as mean ± SE of triplicates from six independent samples. Different letters denote statistically significant differences among the groups according to a Tukey test (P ≤ 0.05). The groups labeled with "a" and "A" do not show statistically significant differences from control cells incubated with medium alone for 3 and 16 h, respectively.

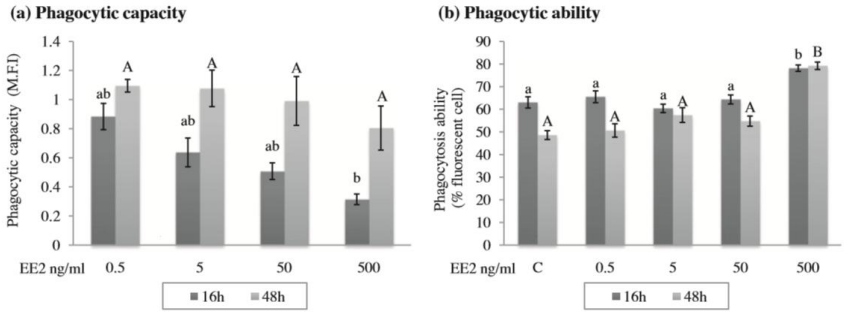

Fig. 7. Phagocytosis of head kidney leukocytes treated with EE2. Head kidney leukocytes were incubated for 16 h (light grey bars) or 48 h (dark grey bars) with the indicated treatments, and then the phagocytosis of FITC-labeled V. anguillarum was assayed by flow cytometry. The results represent mean ± SE in triplicate from 6 independent samples. Different letters denote statistically significant differences among the groups according to a Tukey test (P ≤ 0.05).

3.5. EE_2 modulates the gene expression profile of primary macrophages

We examined the gene expression profile of gilthead seabream MΦ in response to EE_2 since these cells express ERα and are able to respond to E_2 (Chaves-Pozo et al., 2007; Liarte et al., 2011a). The results demonstrated the capacity of EE_2 to alter the gene expression profile of several immune-relevant genes, the effect being complex and time- and dose-dependent. As regards pro-inflammatory genes, EE_2 was able to slightly increase the mRNA levels of IL-1β, IL-6 and TNFα in naïve MΦ, whereas it did not affect the levels of Cox-2 (Figure 8a-d). In stimulated MΦ, however, EE_2 was able to

significantly decrease the mRNA levels of IL-1β and Cox-2, while it increased the levels TNFα (Figure 8e-h). On the other hand, EE_2 was able to increase the mRNA levels of the anti-inflammatory molecule TGFβ1, the chemokine IL-8 and the extracellular matrix remodeling enzymes MMP9 and MMP13 in VaDNA-stimulated MΦ (Figure 9e-h) and, to some extent, in non-stimulated MΦ (Figure 9a-d).

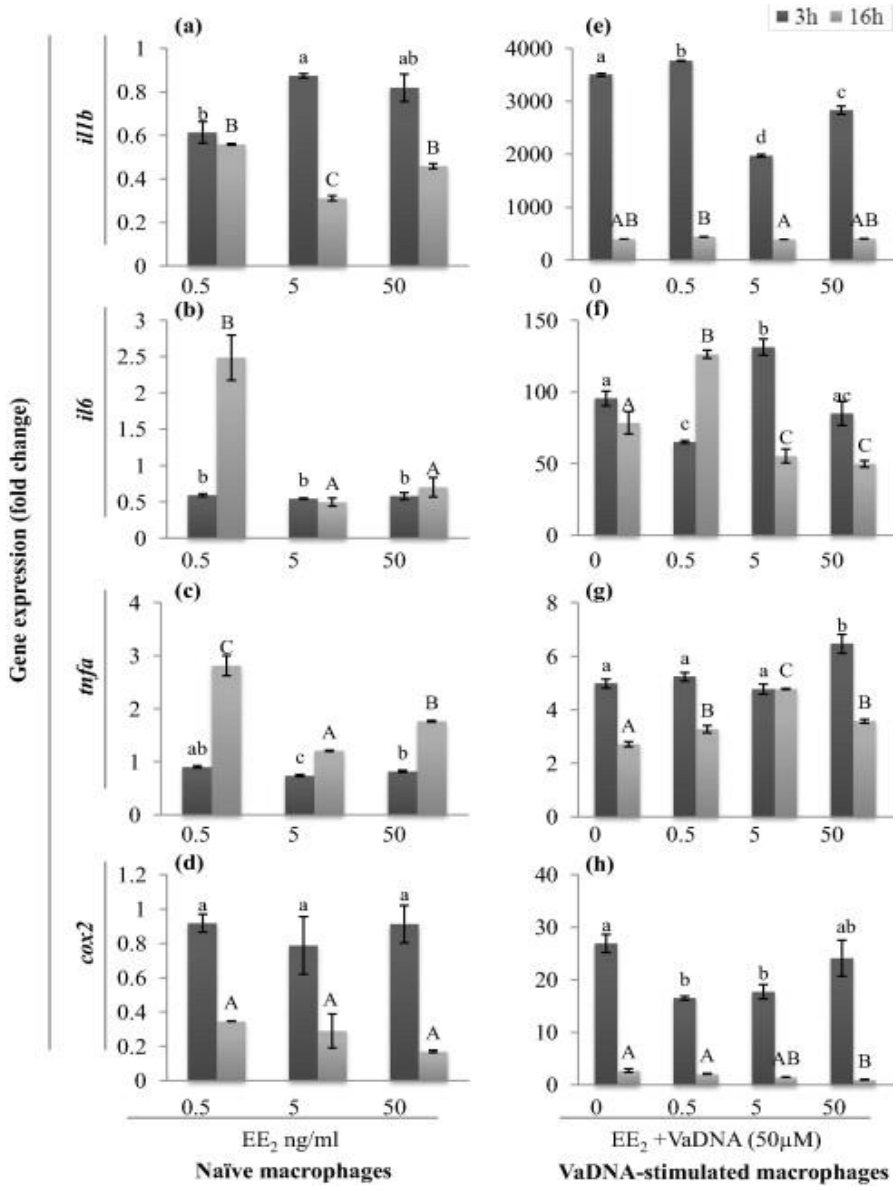

Fig. 8. EE_2 alters the gene expression profile of head kidney macrophages. The RNA levels of*il1b, il6, tnfa* and *ptgs2* (Cox-2) (pro-inflammatory genes) were studied by real-time PCR in MΦ monolayer incubated for 3 h (dark grey bars) or 16 h (light grey bars) with 0, 0.5, 5 and 50 ng/ml EE_2 alone (left panel) or in the presence of 50 µg/ml VaDNA (right panel). Gene expression levels were normalized to *rps18* mRNA levels and are expressed as relative fold change (means ± SE) compared with the control

cells incubated with medium alone. Different letters denote statistically significant differences among the groups according to a Tukey test ($P \leq 0.05$). The groups labeled with "a" and "A" do not show statistically significant differences from control MΦ incubated with medium alone for 3 and 16 h, respectively.

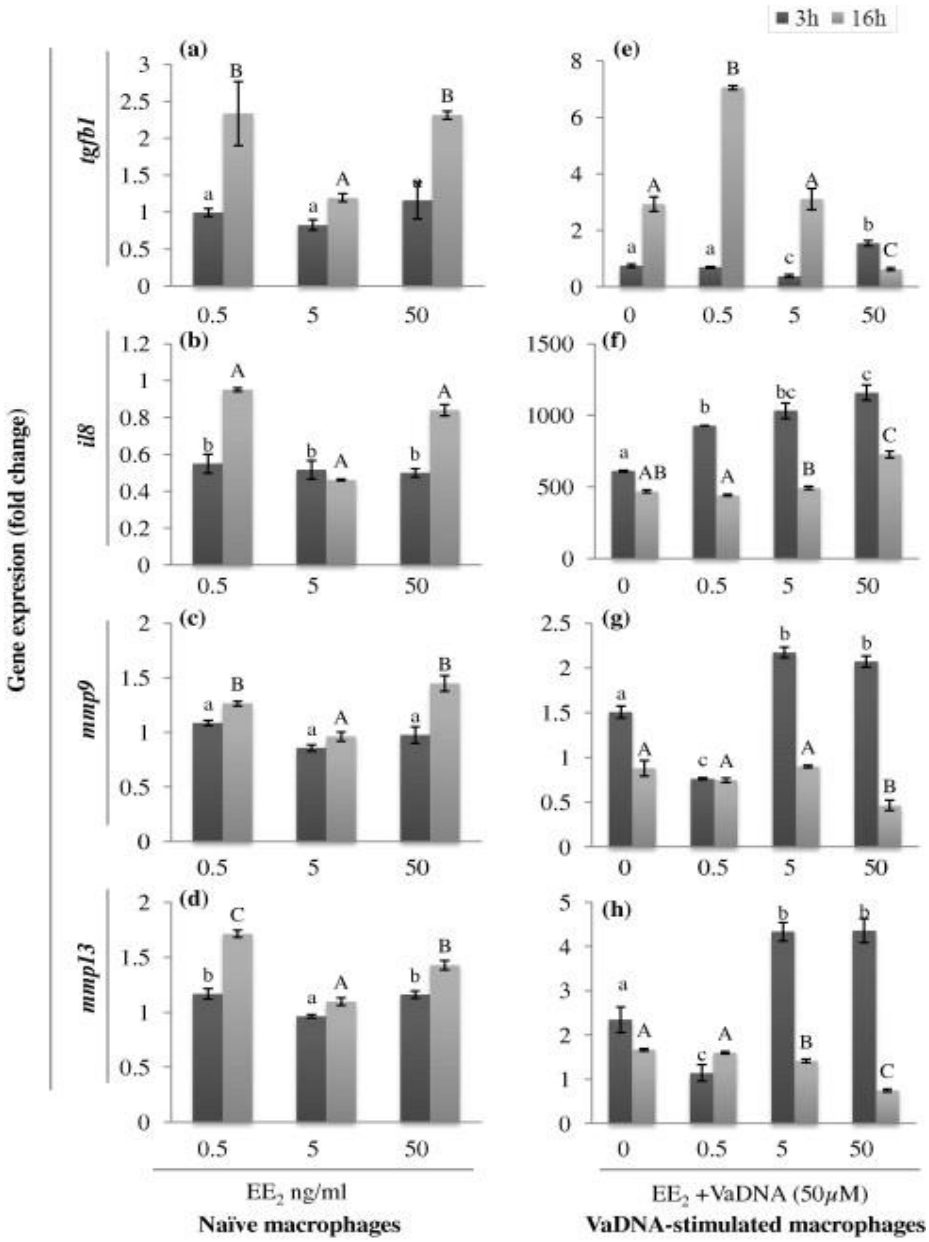

Fig. 9. EE_2 alters the gene expression profile of head kidney macrophages. The RNA levels of *il8, tgfb1, mmp9* and *mmp13* (anti-inflammatory, chemokines, and metalloproteases genes) were studied by real-time PCR in MΦ monolayer incubated for 3 h (dark grey bars) or 16 h (light grey bars) with 0, 0.5, 5 and 50 ng/ml EE_2 alone (left panel) or in the presence of 50 μg/ml VaDNA (right panel). Gene expression levels were normalized to *rps18* mRNA levels and are expressed as relative fold change (means ± SE) compared with the control cells incubated with medium alone. Different letters denote statistically significant differences among the groups according to a Tukey test ($P \leq 0.05$). The groups labeled with

"a" and "A" do not show statistically significant differences from control MΦ incubated with medium alone for 3 h and 16 h, respectively.

4. DISCUSSION AND CONCLUSIONS

There is an increasing debate on the effect of EDCs on the fish immune system (Bols et al., 2001; Milla et al., 2011). Fish represent the animal group most affected by EDC exposure since they are continuously and directly exposed to these contaminants. In the present study we evaluate, for the first time, the effects of EE_2 exposure on both *in vivo* and *in vitro* immunity in gilthead seabream, a teleost species with great commercial value. The survival of EE_2-exposed fish was 100% during the experiment, in contrast to the high mortality (38%) observed when gilthead seabream were exposed to a dietary EE_2 treatment (Cabas et al., 2011). A slight reduction in GSI and altered hormone levels were observed after EE_2 exposure, as is usual in studies performed using bath exposure (Saaristo et al., 2009; Hogan et al., 2010). Similar effects in GSI and hormone levels were also observed in gilthead seabream exposed to EE_2 by dietary administration (Cabas et al., 2011), although dietary administration caused more pronounced effects. This agrees with studies in mice where the mode of EE_2 administration determines its effects (Brummer, 2007). The induction of VTG, a yolk precursor protein produced by hepatocytes, is considered as an endocrine disruptor marker for estrogenic exposure in male fish (Sumpter and Jobling, 1995). We found that VTG expression in the liver is the most sensitive marker of EE_2 exposure in the gilthead seabream, since it showed a 40,000-fold increase in 50 ng/l-EE_2 exposed animals after both 15 and 29 days.

Since the innate immune response is the first line of defense against infections, any effect of EE_2 on this response could alter the susceptibility of fish to infection. In mammals, it is known that the susceptibility to infection changes during the reproductive cycle. In fish, knowledge of the interaction between estrogen exposure and susceptibility to infections is very limited. For example, E_2-treated goldfish are more susceptible to infection with *Trypanosoma danilewskyi* than control fish (Wang and Belosevic, 1994), while E_2 long-term exposure decreases the survival of rainbow trout infected with *Yersinia ruckeri* (Wenger et al., 2011). In the present work, we immunized the seabream with the widely used antigen hemocyanin in the presence of Alum adjuvant in order to precisely evaluate the impact of EE_2 exposure on the adaptive immune response of this species. To the best of our knowledge, no studies have

evaluated the effect of EE_2 on antibody production in fish. We observed that EE_2 exposure suppressed in a dose-dependent manner the induction of IL-1β gene expression observed in immunized fish after priming, and slightly increased the expression of this gene after boosting. Our results agree with those obtained in mammals, in which the complex role of estrogens on immunity is accepted (Straub, 2007). While estrogens play an immunesuppressive role in trauma and sepsis, it may exert proinflammatory effects in some chronic autoimmune diseases in humans (Straub, 2007). Strikingly, the modulation of IL-1β by EE_2 did not result in the impaired production of specific IgM in immunized fish. This result contrasts with the data reported in rainbow trout, in which plasma IgM levels decrease after *in vivo* E_2 exposure (Hou et al., 1999). These differences might be explained by the immune stimulus and subsequent antigen-specific immune responses (cellular vs. humoral immune responses), the cell types involved during different phases of the disease and their ER expression profile, or the concentration, timing and type of estrogen administrated, among other reasons (Straub, 2007). Whatever the outcome, our results demonstrate that short exposures (<2 month) of seabream to EE_2 concentrations of <50 ng/ml do not significantly impair the systemic antibody response.

The gilthead seabream has three ER, namely ERα, ERβ1 and ERβ2 (Pinto et al., 2006) which are differentlly expressed in the three leukocyte cell populations of the head kidney, the main hematopoietic organ in fish (Liarte et al., 2011a). We analyzed the expression of ERα in the head kidney of control and immunized fish exposed to EE_2 and found that EE_2 and antigen challenge were both able to significantly increase the mRNA levels of ERα. This suggests that EE_2 might increase estrogen signaling in leukocytes and that head kidney leukocytes might be able to increase their sensitivity to estrogens during the course of adaptive immune responses. Although the expression of ER following fish immunization has not been examined to date, EE_2 is known to be able to increase hepatic ERα expression in the estuarine killifish (Hogan et al., 2010). Furthermore, we have previously observed that *in vitro* E_2 treatment up-regulates the expression of the genes encoding ERα, ERβ1 and ERβ2 in gilthead seabream MΦ (Liarte et al., 2011a), further suggesting the complexity of the interactions between the immune and the endocrine systems in fish.

The production of ROI has been proposed as a useful marker of endocrine disruption in fish, since this activity is altered by many EDCs (Bols et al., 2001). We

found that *in vitro* EE_2 alters the respiratory bust and the phagocytic activity of seabream leukocytes, with a clearly dose-dependent inhibition of the phagocytic capacity of these cells but a less powerful effect on the ROI production, which was only inhibited at the highest doses used. Similarly, the effects of EE_2 on fish phagocytes have usually been reported as immunesuppressive. Thus, high doses of E_2 and EE_2 inhibit phagocytosis in tilapia (Law et al., 2001), carp (Yamaguchi et al., 2001) and seabream (Liarte et al., 2011a) leukocytes.

In mammals, the strongest action mechanism of estrogens in the monocyte-macrophage system is the transcriptional repression of cytokine genes (Härkönen and Väänänen, 2006). Similarly, in the gilthead seabream, E_2 was able to alter the immune-related gene expression profile in primary MΦ, one of the main targets being the master immune/inflammation transcription factor NF-κB (Liarte et al., 2011b). It also promoted the up-regulation of genes encoding chemokines, pro-inflammatory cytokines and MMPs (Liarte et al., 2011a). In contrast, the present study found that EE_2 was able to promote an anti-inflammatory stage in MΦ, since it decreased the expression of IL-1β, IL-6, TNFα and Cox-2 and simultaneously increased that of TGF-β. Moreover, EE_2 might be involved in the mobilization of phagocytes and tissue remodeling, since it increased the expression of IL-8, MMP9 and MMP13. These results are not surprising, since the exogenous administration of either E_2 or EE_2 induces the mobilization of leukocyte from the head kidney to the gonad (Chaves-Pozo et al., 2007; Cabas et al., 2011).

To conclude, this manuscript demonstrates that although EE_2 alters *in vivo* the immune response induced by an antigenic challenge as well as the immune activities and gene expression profile of head kidney phagocytes *in vitro*, no evidence of immunesuppression is observed using environmentally relevant concentrations for period of less than two months. Our results show that the clearest effects of EE_2 on immune activities or gene expression are observed in challenged fish and activated cells, in agreement with Köllner's view that only in the challenge state will the effect of environmental factors be noticeable (Köllner et al., 2002). Further studies are needed to determine the complex mechanistic of action of EE_2 and to clarify its effect on the capacity of fish to respond to infection.

CHAPER IV

ESTROGEN SIGNALLING THROUGH THE G PROTEIN-COUPLED ESTROGEN RECEPTOR, GPER, REGULATES GRANULOCYTE ACTIVATION IN FISH

ABSTRACT

Neutrophils are major participants in innate host responses. Is a well-known that estrogens have an immune-modulatory role and some evidence exists that neutrophil physiology can be altered by these molecules. Traditionally, estrogens act via classical nuclear estrogen receptors but the identification of a G-protein estrogen receptor, GPER, a membrane estrogen receptor that binds estradiol and other estrogens, has opened up the possibility of exploring the estrogen-mediated effects. However, information on the importance of GPER for immunity, especially, in neutrophils is scant. In this study, we report that gilthead seabream (*Sparus aurata* L.) acidophilic granulocytes (AGs), which are the functional equivalent of mammalian neutrophils, express GPER at both mRNA and protein levels. By using a GPER selective agonist, G1, it was found that GPER 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. In addition, GPER signaling *in vivo* hardly modulated adaptive immunity. Finally, a cAMP analog mimicked the effects of G1 in the induction of the gene coding for prostaglandin-endoperoxide synthase 2 (PTGS2) and in the induction of CREB phosphorylation, while pharmacological inhibition of protein kinase A super-induced PTGS2. Taken together, our results demonstrate for the first time that estrogens are able to modulate vertebrate granulocyte functions through a GPER/cAMP/PKA/CREB signalling pathway and could establish therapeutic targets for several immune disorders where estrogens play a prominent role.

1. INTRODUCTION

It is a well known that estrogens have an immune-modulatory role (Straub, 2007). Traditionally, estrogens act via classical nuclear estrogen receptors (ER), ERα and ERβ, in a process involving ligand binding to receptors, dimerization and binding to regulatory response elements (ERE) in the promoter regions of target genes (Deroo and Korach, 2006), being the primary mode of action of these receptors as transcriptional regulators. Nevertheless, there are physiological responses to estrogens that cannot be explained by the activation of classical nuclear ERs. In 2005, an orphan G protein-coupled receptor (GPCR), GPR30, now officially designated GPER (Alexander et al., 2011), was identified as an estrogen-binding intracellular membrane GPCR (Revankar et al., 2005; Thomas et al., 2005). It was later shown that GPER is activated by estradiol (E_2) (Filardo et al., 2002; Funakoshi et al., 2006; Filardo et al., 2007). The mechanisms through which GPER is activated include the rapid activation of MAPKs, ERK-1 and ERK-2 activation through the transactivation of epidermal grown factor receptor (EGFR) (Filardo et al., 2000), PI3K signaling activation (Revankar et al., 2005), cAMP activation (Filardo et al., 2002) and intracellular calcium mobilization (Revankar et al., 2005), most of which were reviewed in (Prossnitz and Barton, 2009; Prossnitz and Maggiolini, 2009). Moreover, rapid signaling events initiated by GPER have been shown to regulate gene expression (Prossnitz and Maggiolini, 2009). Thus, physiological responses to estrogens are often categorized as rapid/non-genomic or genomic, although is much evidence that these artificially defined categories are connected (Moriarty et al., 2006; Ma and Pei, 2007). Thus, the activation of GPER by G1, a GPER selective agonist (Bologa et al., 2006), does not trigger ERE-mediated activation but up-regulates CFOS by means of a non-genomic mechanism similar to E_2 (Albanito et al., 2007).

The identification of G1 has allowed the involvement of GPER in several physiological functions to be investigated. For example, GPER has been linked with nervous, reproductive, cardiovascular and immune systems, metabolism and obesity, cancer and cell growth, and inflammatory vascular diseases reviewed in (Prossnitz and Barton, 2009). However, information concerning the relevance of GPER for the immune system is scant. However, it is known that GPER contributes to estrogen-induced thymic atrophy (Wang et al., 2008) and the impaired production of T cells in the thymus (Isensee et al., 2009). In the context of experimental autoimmune

encephalomyelitis (EAE), GPER knockout mice show impaired estrogen-mediated protection against EAE (Wang et al., 2009), while G1 has a beneficial role in multiple sclerosis (Blasko et al., 2009). In addition, G1 is able to promote a suppressive phenotype of CD4 regulatory T cells (Brunsing et al., 2013) and is able to induce IL-10 in Th17 effector populations (Brunsing and Prossnitz, 2011). Although the information on the role of GPER in innate immunity is very limited, it has been described that G1 is able to decrease the expression of TLR4 in murine macrophages, limiting the sensitivity of these cells to LPS (Rettew et al., 2010). Finally, it has been described that both the affinity and the signaling pathway of GPER are conserved in both mammals and fish (Thomas et al., 2010).

Neutrophils play a key role in the innate host response: they are the most abundant type of white blood cells and often the first to migrate to inflammatory lesions. In response to specific stimuli, neutrophils can synthesize an array of factors such as anti-microbial proteins, extracellular matrix proteins, cytokines and chemokine, all of which play a major role in early stages of the inflammatory response (Cassatella, 1995). In the bony fish gilthead seabream (*Sparus aurata* L.), acidophilic granulocytes (AGs) are the major cell type participating in innate host responses, while the head kidney (HK), the main hematopoietic organ in fish comparable to the bone marrow of mammals (Sepulcre et al., 2002), is the central immune organ that provides a source for AGs (Sepulcre et al., 2002; Mulero et al., 2007). 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 reactive oxygen species (ROS) production capabilities (Sepulcre et al., 2002; Sepulcre et al., 2007), produce cytokines in response to several immunological stimuli (Chaves-Pozo et al., 2004a; Sepulcre et al., 2007) and express a broad range of TLRs, although not TLR3 (Sepulcre et al., 2007).

In mammals, neutrophil physiology may be altered by estrogens (Nalbandian and Kovats, 2005; Stygar et al., 2007; Cutolo et al., 2010). These cells express ERα and ERβ as well as their various splice variants (Molero et al., 2002; Stygar et al., 2006). A recent study has shown that neutrophil-like HL60 cells express a functional GPER (Blesson and Sahlin, 2012). However, it is unknown whether primary neutrophils express GPER and, if this is the case, what its functional relevance in neutrophil biology is. In gilthead seabream, AG physiology is also shown to be regulated by estrogens.

Despite the massive infiltration of AGs into the testis after spawning (Chaves-Pozo et al., 2003; Chaves-Pozo et al., 2005b; Chaves-Pozo et al., 2007), they show impaired phagocytic and ROS production activities (Chaves-Pozo et al., 2005b). Moreover, exogenous administration of E_2 and 17α-ethinylestradiol (EE_2) induces AGs infiltration into the gonad (Chaves-Pozo et al., 2007; Chaves-Pozo et al., 2008c; Cabas et al., 2011). Strikingly, however, AGs do not express any of the three ERs (ERα, ERβ1, ERβ2) known to exist in the gilthead seabream (Pinto et al., 2006; Liarte et al., 2011a). In the present study, we demonstrate that AGs express a functional GPER, which modulates the gene expression profile and immune activities of these cells after *in vitro* and *in vivo* exposure. The identification of GPER as an important regulator of vertebrate neutrophil biology points to new therapeutic targets for autoimmune diseases where estrogens are involved, such as multiple sclerosis and EAE. The findings are also relevant in the context of endocrine disruption, since xeno-estrogens can activate GPER (Thomas and Dong, 2006; Watson et al., 2011).

2. MATERIALS AND METHODS

2.1. Animals, in vivo treatment and sample collection

Healthy specimens of the hermaphroditic protandrous marine fish gilthead seabream (*Sparus aurata* L., Actinopterygii, Perciformes, Sparidae) 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 volume/h) with a natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Skretting, Spain) at a feeding rate of 1.5% of fish biomass. Fish were fasted for 24 h before sampling.

In vivo G1 treatment was carried out with mature gilthead seabream males (n=150) with a body weight of 225 ± 35 g kept in 2 m^3 aquaria. Briefly, G1 was incorporated in the commercial food (44% protein, 22% lipids, Skretting, Spain) to give a concentration of 0, 2, and 20 μg/fish/day, using the ethanol evaporation method (0.3l ethanol/kg of food) as described elsewhere (Shved et al., 2007). The specimens were fed three times a day *ad libitum* with the G1-containing food for up to 50 days and fasted for 24 h before sampling. In order to evaluate the effect of G1 on adaptive immunity, the animals were injected i.p. with PBS (control fish) or hemocyanin (200 μg/fish,

Sigma-Aldrich) and Inject Alum Adjuvant (4 mg/fish, Thermo Scientific) (vaccinated fish) after 7 (priming) and 20 (booster) days of G1 exposure. The samplings were carried out on days 8 (1 day post-priming) and 21, 30 and 50 (1, 10 and 30 days post-booster, respectively). Specimens (n=6 fish/group and time) were anesthetized with 40 ppm of clove oil, decapitated, weighed and the HKs, spleens, livers and gonads were removed and then processed for gene expression analysis, as described below. The gonads were weighed, while serum samples from trunk blood were obtained by centrifugation and immediately frozen and stored at -80ºC until use. Cell suspensions from HKs were obtained as described elsewhere (Chaves-Pozo et al., 2003; Chaves-Pozo et al., 2005b). As an index of the reproductive stage and in order to evaluate the effect of the *in vivo* G1 treatment, the gonadosomatic index (GSI) was calculated as 100*[MG/MB] (%), where MG is gonad mass and MB is body mass (both in grams).

For the *in vitro* experiments, mature male gilthead seabream (300-650 g mean weight, kept in 14 m^3 aquaria) were decapitated. The HKs were removed, and cell suspensions were obtained (Chaves-Pozo et al., 2003; Chaves-Pozo et al., 2005b). The experiments described comply with the guidelines of the European Union Council (86/609/EU) and the Bioethical Committees of the University of Murcia (Spain) (approval no. #333, 2008) for the use of laboratory animals.

2.2. Isolation of AGs, cell culture and in vitro G1 treatments

AGs were obtained by MACS as described earlier (Roca et al., 2006). Briefly, HK cell suspensions were incubated with a 1:10 dilution of a mAb specific to gilthead seabream AGs (G7) (Sepulcre et al., 2002), washed twice with PBS containing 2mM EDTA (Sigma-Aldrich) and 5% FCS (Life Technologies) and then incubated with 100-200 µl per 10^8 cells of micro-magnetic-bead-conjugated anti-mouse IgG (Miltenyi Biotec). After washing, G7+ (AG) and G7- (AG-depleted) cell fractions were collected by MACS following the manufacturer´s instructions and their purity was analyzed by flow cytometry (Roca et al., 2006).

Seabream HK leukocytes or purified AGs were maintained in sRPMI [RPMI-1640 culture medium (Life Technologies) adjusted with 0.35% NaCl to gilthead seabream serum osmolarity] containing 100 i.u./ml penicillin and 100 µg/ml streptomycin (Biochrom). Depending on the experiments, aliquots of HK cell suspension ($0.5x10^6$) or purified AGs ($0.2\text{-}1x10^6$) from HK were incubated for 5, 30 min, 1, 2, 3 and 16 h in sRPMI medium supplemented with 5% charcoal/dextran-treated

hormone-free FCS (hf-FCS, Hyclone) alone (untreated cells) or containing 1 and 100 μM G1 (Sigma-Aldrich) in the presence or absence of 50 μg/ml of phenol-extracted genomic DNA from the bacterium *Vibrio anguillarum* ATCC19264 cells (VaDNA). In some experiments, purified AGs ($0.2\text{-}1x10^6$) from the HK were incubated with 0.1, 0.2 and 0.5 mM of the cell-permeable cAMP analog 2′-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (dbcAMP, Sigma-Aldrich) for 30 and 2 hours, or pre-incubated for 30 min with 10 μM of the protein kinase A (PKA) inhibitor H89 (Sigma-Aldrich) and then stimulated with G1 or VaDNA for an additional two hours. After the incubations, viability assay, reactive oxygen intermediate (ROS) production, GPER immunofluorescence staining, western blot and/or gene expression analysis were performed (see below).

2.3. Cloning of gilthead seabream GPER

A partial sequence of gilthead seabream GPER cDNA was obtained by PCR amplification using a *Taq* DNA polymerase (Biolone), testis cDNA as template and degenerated primers (Table 1), targeting conserved domains of GPER from a phylogenetically close species (*Micropogonias undulatus*, Actinopterygii, Perciformes, Sciaenidae) (European Nucleotide Archive, ENA, accession number **EU274298**). The PCR-amplified fragment was cloned into the pCRII-TOPO cloning vector (Life Technologies) and transformed into competent *Escherichia coli* DH5α cells. Positive clones were sequenced and analysed by BLAST. A partial gilthead seabream GPER sequence was deposited in the ENA with accession number **HG004163**).

2.4. Analysis of gene expression

Total RNA was extracted from tissues or cell pellets with TRIzol Reagent (Invitrogen), following the manufacturer's instructions, and quantified with a spectrophotometer (NanoDrop, ND-1000). The RNA of 3 or 6 fish per group, in the case of cell pellets or tissue, respectively, were pooled using the same amount of RNA from each specimen. The RNA was then treated with DNase I, amplification grade (1 unit/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-dT_{18} primer from 0.5-1μg of total RNA, at 50ºC for 50 min. The β-actin (*actb*) gene was analyzed by semi-quantitative PCR performed with an Eppendorf 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 GPER, CFOS, IL-1β, IL-8, IL-10, prostaglandin-endoperoxide synthase 2 (PTGS2, also known as COX2), prostaglandin D2 synthase (PTGDS) and vitellogenin (VTG), 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 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.

2.5. GPER immunofluorescence and flow cytometry analysis

Aliquots of HK cell suspensions ($0.5x10^6$) were washed in PBS containing 2% FCS and 0.05% sodium azide (FACS buffer). Cells were fixed with 4% paraformaldehyde for 15min at room temperature. After three rinses, cells were incubated in ice-cold PBS containing 0.1% Triton X-100 (Sigma-Aldrich) at 4ºC to permeabilize the membranes. Cells were then stained with 0, 0.2, 0.4, 0.8 µg/ml (0, 1:1000, 1:500 and 1:250, respectively) of a commercial affinity purified rabbit polyclonal antibody raised against a conserved peptide mapping near the N-terminus of GPER of human origin (Santa Cruz Biotechnology), in PBS containing 2% FCS for 30min at 4ºC. For competition studies, a 10-fold excess (in molarity) of a commercial blocking peptide (Santa Cruz Biotecnology) were pre-incubated with the GPER antibody overnight at 4ºC. After washing, cells were incubated with a 1:500 dilution of a phycoerythrin (PE)-conjugated goat F (ab) anti-rabbit IgG (H+L) (Life Technologies) for 30 min at 4ºC, washed again and analyzed by flow cytometry. Data were collected in the form of forward scatter (FSC) vs. side scatter (SSC) dot plot, green (FL1) vs. red (FL2) fluorescence dot plot and red (FL2) fluorescence histograms using a flow cytometer (BD Biosciences). The percentage and the intensity of red fluorescence cell were analyzed on all regions (ungated), gate R1 (high FSC and SSC, i.e. AGs) and/or gate 2 (low FSC and SSC, i.e. mainly MΦ and lymphocytes) (Esteban et al., 1998; Sepulcre et al., 2002).

2.6. Western blot

G7+ (AG) and G7- (AG-depleted) cell fractions collected by MACS or AGs untreated or treated with 100 μM G1 or 0.2 mM of dbcAMP for 2 h, as described above, were lysed in lysis buffer (10mM Tris–HCl pH 7.4, 150mM NaCl, 1% Triton X-100, 0.5% NP-40). The protein concentrations of cell lysates were estimated by the BCA protein assay reagent (Pierce) using BSA as a standard. Cell extracts (10 or 45 μg of protein, depending on the experiments) were boiled in SDS sample buffer, resolved on 12% SDS-PAGE, and transferred for 30 min at 200 mA to nitrocellulose membranes (Bio-Rad). Blots were probed with 1 μg/ml (1:200) of the anti-GPER antibody or with a 1:1000 dilution of a commercial rabbit monoclonal antibody raised against CREB phosphorylated at serine 133 (phospho-CREB) (the epitope is fully conserved in all vertebrates and the antibody also reacts with phospo-ATF-1) (#9198, Cell Signalling Technology). Then, blots were probed with 1:5000 of an anti-rabbit-HRP antibody and developed with ECL reagents (GE Healthcare) according to the manufacturer's protocol. Membranes were then re-probed with a 1:5.000 dilution of an affinity purified rabbit polyclonal to histone H3 (#ab 1791, Abcam).

2.7. Viability assay

Aliquots of $0.2x10^6$ of purified AGs treated as describe above were diluted in 200μl PBS containing 40μg/ml propidium iodide (PI). The number of red fluorescent cells (dead cells) from duplicate cultured samples was analyzed by flow cytometry.

2.8. ROS production assay

ROS production was measured as the luminol-dependent chemiluminescence produced by $0.2\ x10^6$ AGs pre-treated or not with G1, in the presence or absence of 50 μg/ml VaDNA or by $0.5x10^6$ HK leukocytes from G1-treated fish (both control or vaccinated fish), as described elsewhere (Mulero et al., 2001). This was achieved by adding 100μM luminol (Sigma) and 1μg/ml phorbol myristate acetate (PMA, Sigma), while the chemiluminescence was recorded every 127s for 1h in a FLUOstart luminometer (BGM, LabTechnologies). The values reported are the average of triple readings from 6 different samples, expressed as the maximum and slope of the reaction curve from 127 to 1016s, from which the apparatus background was subtracted.

2.9. Determination of IgM specific titer

The hemocyanin specific IgM titer was determined by an ELISA kit (Aquatic Diagnostic, Ltd.) following the manufacturer's instructions. In short, serial dilutions of serum from control or hemocyanin-immunized fish were added to hemocyanin pre-coated 96-well ELISA plates, followed by a monoclonal antibody specific to seabream IgM and, finally, 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).

2.10. Statistical analysis

ANOVA and a Tukey multiple range tests were applied to determine differences among groups. A Student *t*-test was used 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. GPER is expressed in immune organs and is differentially expressed in HK cell populations

The mRNA levels of GPER were analyzed in several gilthead seabream tissues, such as the HK (main hematopoietic organ in fish), spleen, liver and testis. Although the GPER was expressed in immune organs, its expression was much lower than in liver and testis (Figure 1A). The mRNA levels of GPER were 4.3-fold higher in G7+ (AGs) than in G7- (AG-depleted) cell fractions (Figure 1B). Similarly, most AGs (Gate R1: cells with high FSC and SSC) (Sepulcre et al., 2002) were immunostained with the GPER Ab in a dose-dependent manner (Figures 1C-1E). This staining was found to be specific, since pre-incubation of GPER Ab with a specific blocking peptide significantly reduced this staining (Figures 1D-1F). The same analysis was performed in gate R2 (low FSC and SSC, i.e. MΦ, lymphocytes and precursor cells), for which the results showed a weaker immunostaining compared with AGs (as low as 25, 7 and 4 % of GPER positive cells with 1:250, 1:500 and 1:1000 dilutions of GPER Ab, respectively, Figure 1G). However, the immunostaning of R2 cells was also reduced by the blocking peptide (Figure 1G). These results were further confirmed by western blot, where G7+

(AGs) cell fractions showed a more robust expression of GPER protein than G7- (AG-depleted) cell fractions (Figure 1H).

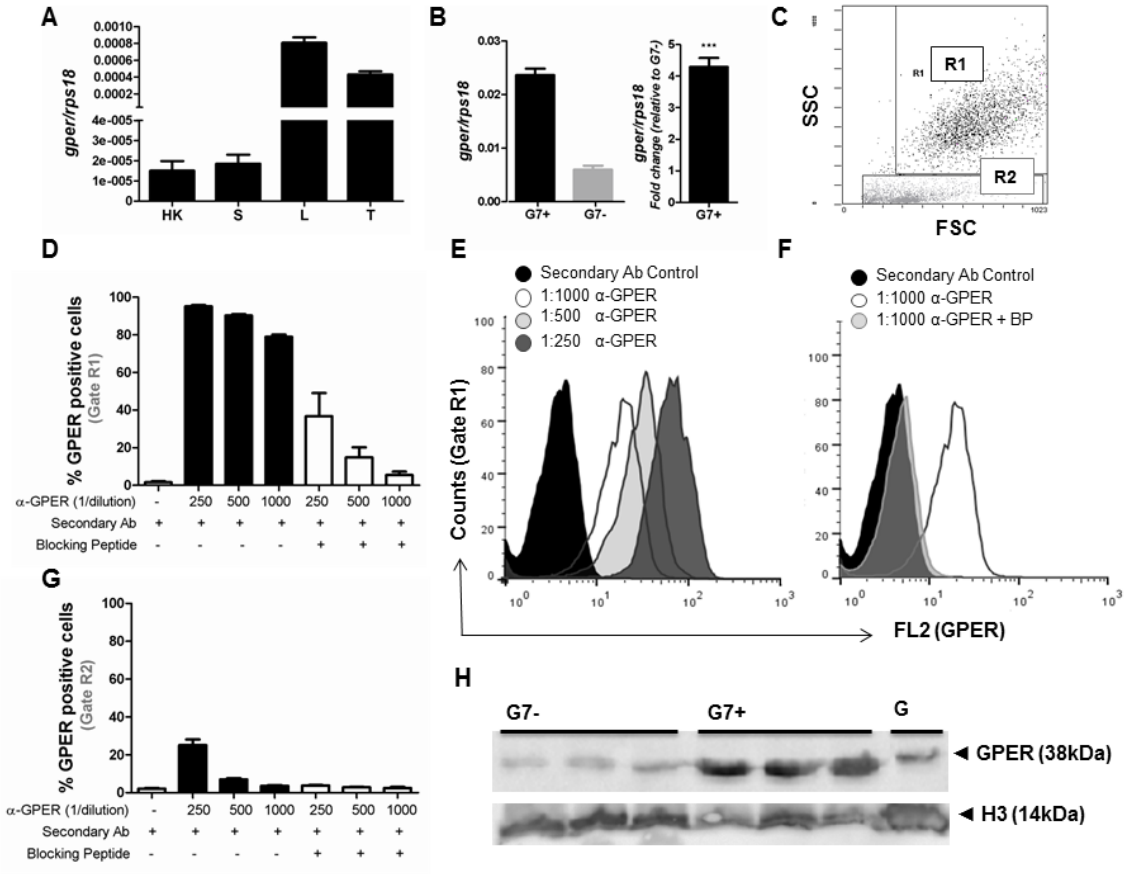

Figure 1. GPER mRNA and protein levels in HK cell populations. The mRNA levels of gper were analyzed by real-time PCR in (A) several gilthead seabream tissues such as head kidney (HK), spleen (S), liver (L) and testes (T) and in (B) HK G7+ (AGs) and G7- (AGs-depleted) cell fractions obtained by MACS. Gene expression levels were normalized to rps18 mRNA levels. Data for gper expression represent means ± SEM in triplicate. The results are representative of three independent samples and analyses. (C) Representative FSC/SSC dot plots of HK leukocytes showing two main regions: one of high FSC and SSC (AGs, gate R1) and the other of low FSC and SSC (MΦ, Ly and precursor cells, gate R2). The percentage of GPER positive cells was analyzed by flow cytometry in HK leukocytes unstained or immunostained with a 1:250, 1:500 and 1:1000 dilution of GPER antibody pre-incubated or not with the blocking peptide (BP). The analysis was made in (D) gate R1 and (G) gate R2. Values are means ± SEM of values of three specimens and are representative of multiple independent experiments. (E) The fluorescence of the HK leukocytes (gate R1) unstained or immunostained with a 1:250, 1:500 and 1:1000 dilution of GPER, and (F) the fluorescence of the HK leukocytes (gate R1) immunostained with a 1:1000 dilution of anti-GPER pre-incubated or not with the BP was analyzed by flow cytometry. The red fluorescence histograms (FL2, GPER) shown are representative of multiple independent experiments. (H)

Western blot analysis of GPER in G7+ (AGs) and G7- (AGs-depleted) cell fractions obtained by MACS. The results are representative of three independent experiments. The asterisks denote statistically significant differences among groups according to t-Student test. *p <0.05, **p <0.01 and ***p <0.001.

3.2. GPER activation fails to modulate the expression of GPER in vitro

After 16 h of G1 *in vitro* treatment, the longest time used in this study, AG viability was 88 ± 1.5% in control cells, 92 ± 1 % in VaDNA-stimulated cells and 85 ± 2% in cells treated with 100 μM G1 plus VaDNA, as assayed by PI exclusion and flow cytometry analyses (Figure 2A).

To examine the possibility that G1 is able to regulate the expression levels of GPER, the mRNA levels of GPER (Figure 2B) and the percentage (Figure 2C) and intensity (Figure 2D) of GPER positive cells were analyzed after G1 treatment of AGs *in vitro* for 16 h in the presence or absence of VaDNA. There were no significant changes after G1 treatment either at GPER mRNA or protein levels. In the case of 100 μM G1 plus VaDNA, *gper* mRNA levels were lower than in VaDNA-stimulated control cells, an effect that might be related to the fact that the cell viability was slightly lower in this condition.

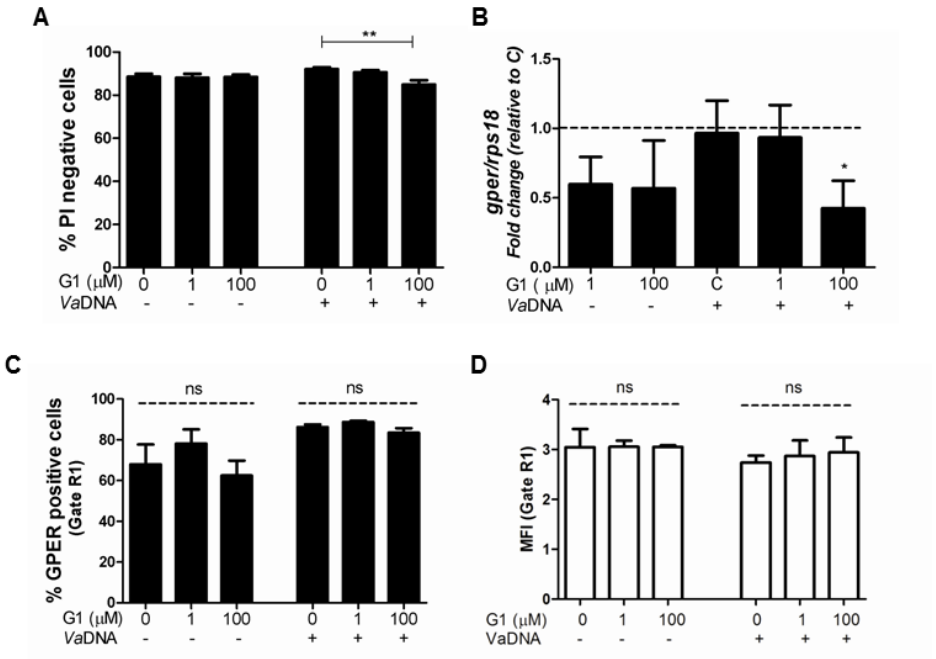

Figure 2. GPER signaling does not alter GPER mRNA and protein levels in AGs. AGs were incubated with 0, 1 and 100 μM G1 alone or in the presence of VaDNA (50 μg/ml) for 16 h. Afterwards, cell viability and GPER expression levels were analyzed. (A) The percentage of PI-negative AGs was

determined by flow cytometry. Values are means ± SEM of duplicates cultures and are representative of multiple independent experiments. (B) The mRNA levels of gper were analyzed by real-time PCR. Gene expression levels were normalized to rps18 mRNA levels and are shown as relative to the mean of nonstimulated cells (value 1; represented by the dashed line). Data for gper expression represent means ± SEM in triplicate and are representative of two independent experiments. The percentage (C) and the intensity (D) of GPER positive cells were analyzed by flow cytometry. Values are means ± SEM of duplicate cultures and are representative of at least two independent experiments. The asterisks denote statistically significant differences among groups according to one-way ANOVA and Tukey post-hoc test. *p <0.05, **p <0.01 and ***p <0.001. No symbol or "ns" means not significant.

3.3. GPER activation hardly modulates the respiratory burst of AGs

G1 (1 μM) alone or combined with VaDNA was unable to significantly alter the production of ROS by AGs after 16 h of incubation, while VaDNA, a potent activator of these cells through TLR9 (Sepulcre et al., 2007), significantly increased this activity (Figure 3A). Nevertheless, 100 μM G1 was able to slightly induce ROS production by naïve AGs after 5 min exposure but not 3 h, while 1 and 100 μM G1 slightly decreased the ROS production after 3 h (Figure 3B) However, neither dose of G1 was able to modulate ROS production in VaDNA-primed AGs (Figure 3C).

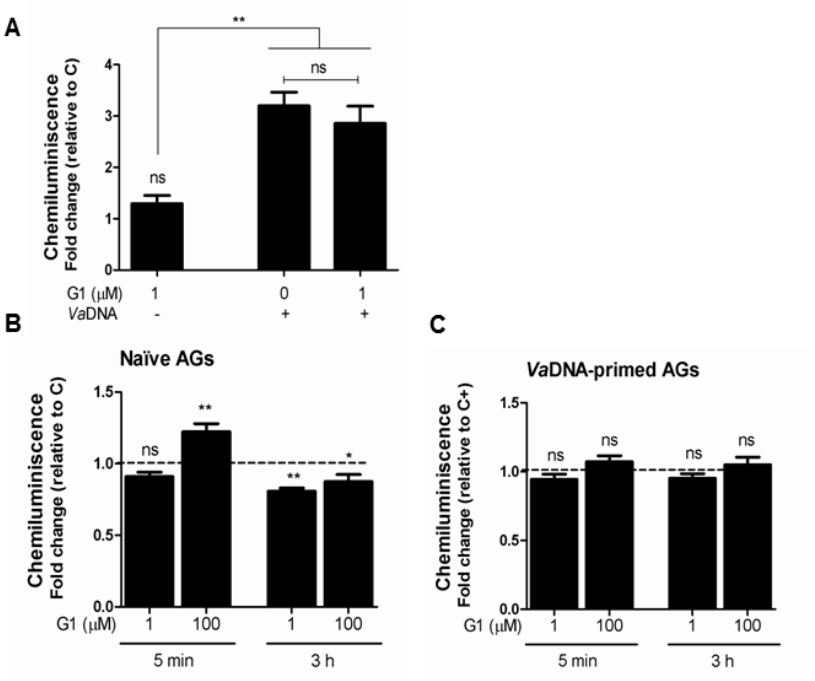

Figure 3. GPER signaling hardly modulates the respiratory burst of AGs. (A) AGs were incubated with 0 or 1 μM G1 alone or in the presence of VaDNA (50 μg/ml) for 16 h. G1 at the indicated concentrations was added and allowed to stand to (B) naïve and (C) primed AG (16 h in the presence of VaDNA) for 5

min or 3 h. Afterwards, the respiratory-burst activity by these cells triggered by PMA (1 mg/ml) was measured using a luminol-dependent chemiluminescence method. Values are normalized with respect to control cells (value 1; represented by the dashed line), cells with medium alone in naïve AGs or medium plus VaDNA in priming AGs. 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, **p <0.01 and ***p <0.001. No symbol or "ns" means not significant.

3.4. GPER signaling via the cAMP/PKA/CREB pathway regulates the gene expression profile of naïve AGs

Figures 4A and 4B show that G1 (100 μM) was able to rapidly, but transiently, induce the mRNA levels of *cfos*, a marker gene for the activation of the GPER signaling pathway (Albanito et al., 2007). When the ability of G1 to modulate the gene expression profile through GPER signaling was evaluated in naïve AGs, GPER activation was seen to induce the high mRNA levels of IL-1β very quickly (30 min) but inhibited them at later time points (Figure 4C). However, GPER engagement resulted in sustainably increased mRNA levels of PTGS2 (Figure 4D) and IL-10 (Figure 4E). In addition, GPER signaling resulted in increased mRNA levels of PTGDS after 30 min and 3 h, but lower levels after 16 h (Figure 4F). Curiously, however, GPER activation in VaDNA-primed AGs decreased the mRNA levels of IL-1β (Figure 4G) and had a negligible effect on the mRNA levels of PTGS2 (Figure 4H) and IL-10 (Figure 4I).

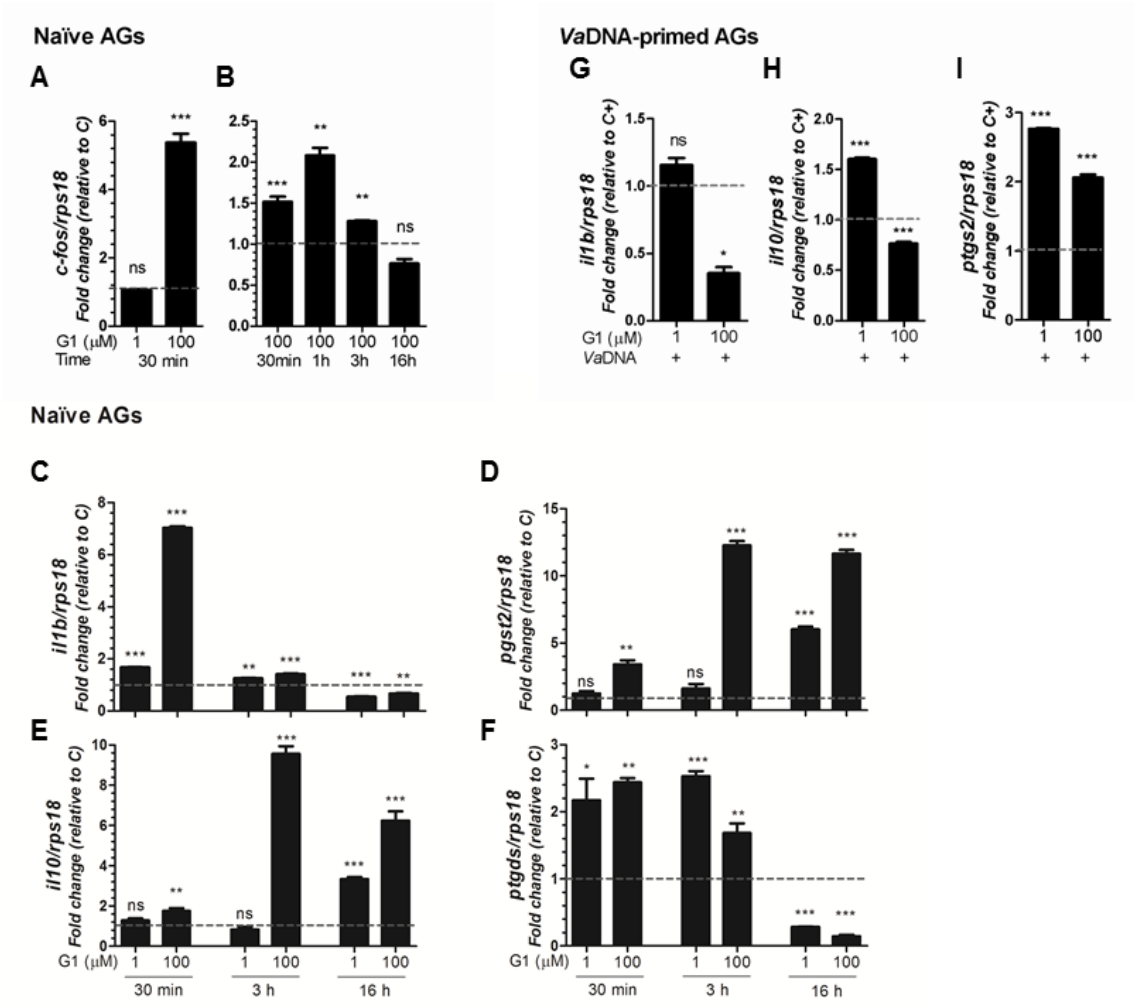

Figure 4. GPER activation regulates the gene expression profile of AGs. AGs were stimulated with 0, 1 and 100 μM G1 for 30 min, 1, 3 and 16 h or with 0, 1 and 100 μM G1 in the presence of VaDNA for 16 h. Afterwards, the mRNA levels of (A, B) c-fos, (C, G) il1b, (D, H) ptgs2, (E, I) il10 and (F) ptgds were determined by real-time RT-PCR. Gene expression is normalized against rps18 and is shown as relative to the mean of untreated cells (value 1; represented by the dashed line; either one with their corresponding time control). Each bar represents the mean ± SEM of triplicate samples. The asterisks denote statistically significant differences compared with untreated cells for each time point, according to one-way ANOVA and Tukey post-hoc test or t-Student test when comparing two treatments. *p <0.05, **p <0.01 and ***p <0.001. No symbol or "ns" means not significant.

To explore the signaling pathway downstream of GPER activation in AGs, a pharmacological inhibitor of PKA (H89), a cell-permeable cAMP analog that activates PKA (dbcAMP) and a mAb against phospho-CREB were used. Pharmacological inhibition of PKA resulted in the hyperinduction of PTGS2 transcript levels in response

to G1, but had negligible effects, if any, in VaDNA-primed AGs (Figure 5A). In sharp contrast, H89 treatment had no effect on the transcript levels of IL-1β (Figure 5B). On the other hand, dbcAMP mimicked the GPER-dependent induction of PTGS2 (Figure 5C). Notably, after 30 min of exposure, G1 promoted the phosphorylation of CREB and ATF-1 in AGs, and this effect was also mimicked by dbcAMP (Figure 5D).

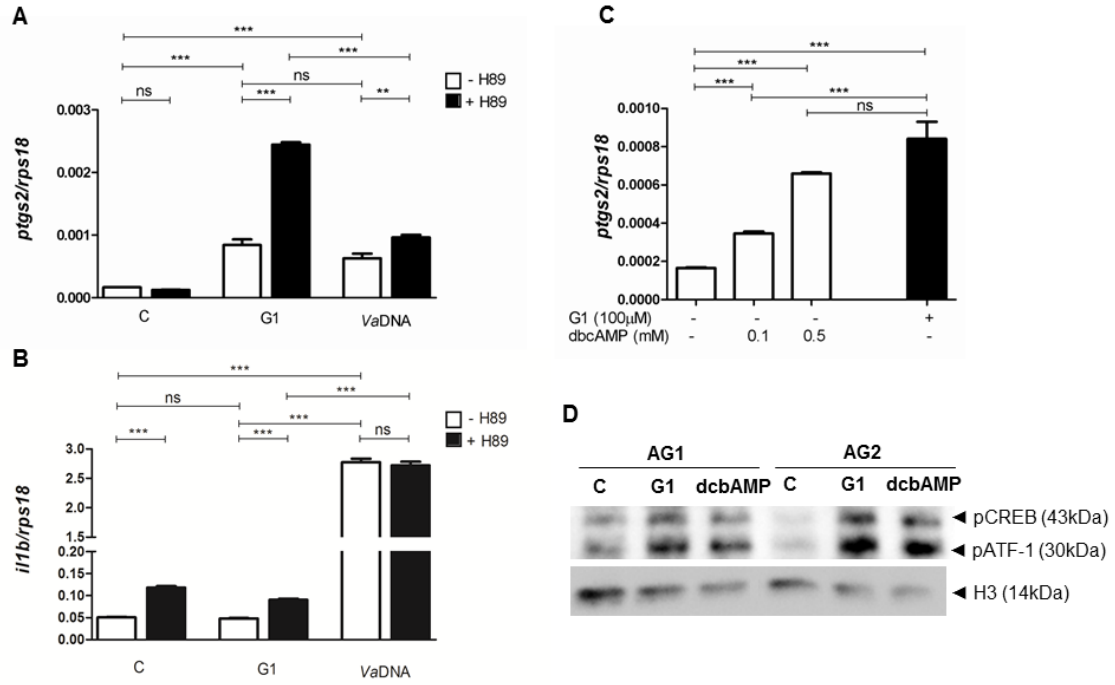

Figure 5. GPER signaling via the cAMP/PKA/CREB pathway regulates the gene expression profile of naïve AGs. (A, B) AGs were pre-incubated for 30 min with the PKA inhibitor (H-89) and further stimulated with 0 and 100 μM G1, or 75 μg/ml of VaDNA for 2 additional hours. (C) AGs were stimulated with 0, 0.1 and 0.5 mM of dbcAMP or 100 μM G1 for 2 h. Afterwards, the mRNA levels of (A, C) pgst2 and (B) il1b were determined by real-time RT-PCR. Gene expression is normalized against rps18. Data for gene expression represent means ± SE in triplicate. (H) Western blot analysis of Phospho-CREB (Ser133) in AGs untreated or treated with 100 μM G1 or 0.2 mM of dbcAMP for 30 min. The results are representative of at least two independent experiments. The asterisks denote statistically significant differences compared with untreated cells, according to one-way ANOVA and Tukey post-hoc test. *p <0.05, **p <0.01 and ***p <0.001. ns, not significant.

3.5. GPER signaling modulates innate immunity in vivo

The survival of the animals exposed to G1 was 100% during the trial. After 8 (s1), 21 (s2) and 50 (s4) days of G1-exposure the GSI was not significantly altered in non-immunized animals (Figures 6A and 6B). Liver mRNA levels of VTG, a gene induced by the activation of nuclear estrogen receptors (Sumpter and Jobling, 1995),

weakly increased in fish treated with the higher dose (20 µg) (Figure 6C). Neither G1 treatment nor vaccination was able to modulate the mRNA levels of GPER (Figures 7A-7C) or the percentage (Figure 7D and 7E) and intensity (data not shown) of GPER positive cells in the HK at any analysis time.

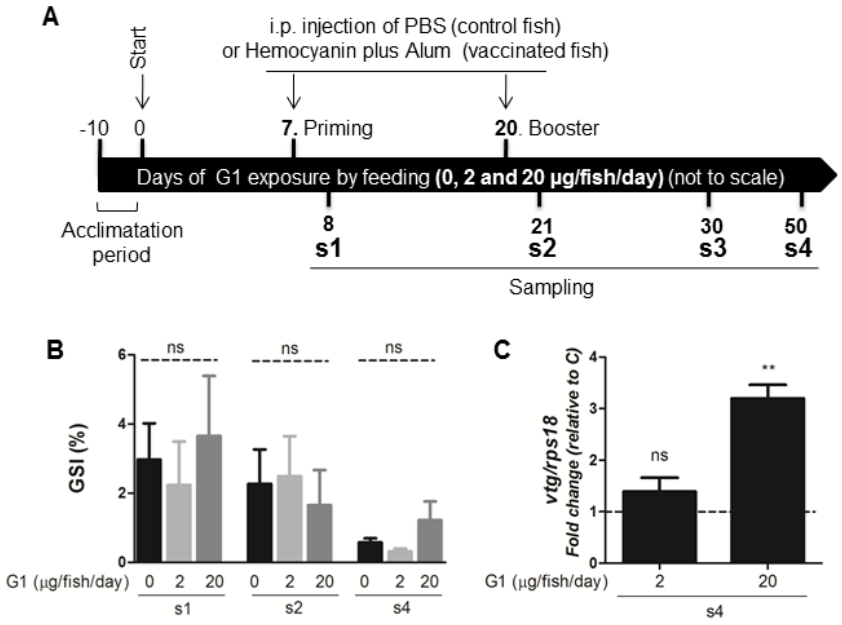

Figure 6. GPER activation in vivo does not promote an estrogenic response. (A) Schematic drawing of the experimental design for gilthead seabream under G1 dietary exposure and vaccination schedule. Fish were exposed to several G1 concentrations (0, 2 and 20 µg/fish/day) for up to 50 days. After 7 and 20 days of G1-exposure, fish were i.p. injected with PBS (control fish) or vaccinated with hemocyanin plus Alum adjuvant. Sampling was carried out on days 8, 21, 30 and 50. (B) The gonadosomatic index (GSI) and (C) the hepatic mRNA levels of vitellogenin (vtg) were analyzed after 8 (s1), 21 (s2), 30 (s3) and 50 (s4) days of G1 exposure in non-vaccinated fish. In all the analyses, the sample size was n=6 fish/group/time. The mRNA levels of vtg were analyzed by real-time PCR. Gene expression is normalized against rps18 and is shown as relative to the mean of untreated fish (value 1; represented by the dashed line). Each bar represents the mean ± SEM of triplicate samples. The asterisks denote statistically significant differences compared with untreated fish according to one-way ANOVA and Tukey post-hoc test. *p <0.05, **p <0.01 and ***p <0.001. No symbol or "ns" means not significant.

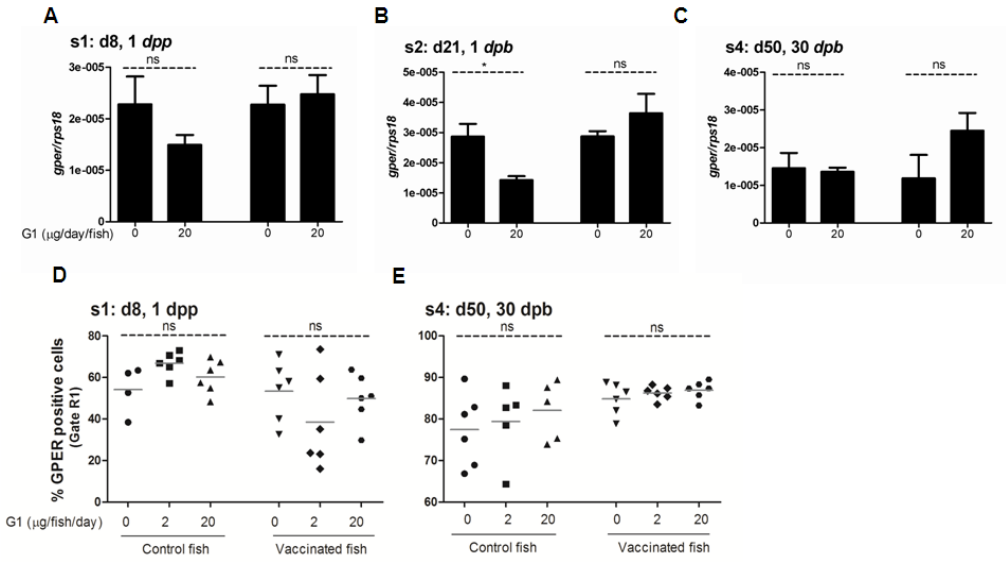

Figure 7. GPER activation in vivo does not alter GPER mRNA and protein levels in HK leukocytes. After 8 (s1; 1 day post-priming), 21 (s2; 1 day post-booster) and 50 (s4; 30 days post-booster) days of G1 exposure, HK leukocytes from G1-treated fish (both non- and vaccinated groups) were obtained to determine GPER mRNA levels. The sample size was n=6 fish/group. (A) The HK mRNA levels of gper were analyzed by real-time PCR. Gene expression levels were normalized to rps18 mRNA levels and data represent means ± SE in triplicate. (B) The percentage of GPER positive cells was analyzed by flow cytometry. The mean for each group of specimens is shown as a horizontal line. The asterisks denote statistically significant differences compared with control group (untreated control or untreated vaccinated fish) according to one-way ANOVA and Tukey post-hoc test. *p <0.05, **p <0.01 and ***p <0.001. No symbol or "ns" means not significant.

Using luminol-dependent chemiluminescence, we analyzed the production of ROS triggered by PMA in HK leukocytes from control and vaccinated fish on days 8 (s1; 1 day post-priming, dpp) (Figure 8A), 21 (s2; 1 day post-booster, dpb) (Figure 8B) and 50 (s4; 30 dpb) (Figure 8C) days after G1-exposure. Unexpectedly, the ability to produce ROS was higher in leukocytes from non-vaccinated fish compared with the vaccinated animals at all time points. Moreover, the higher dose of G1 was able to strongly reduce the production of ROS in non-vaccinated fish 1 dpp and 1 dpb, whereas had no statistically significant effect in vaccinated animals.

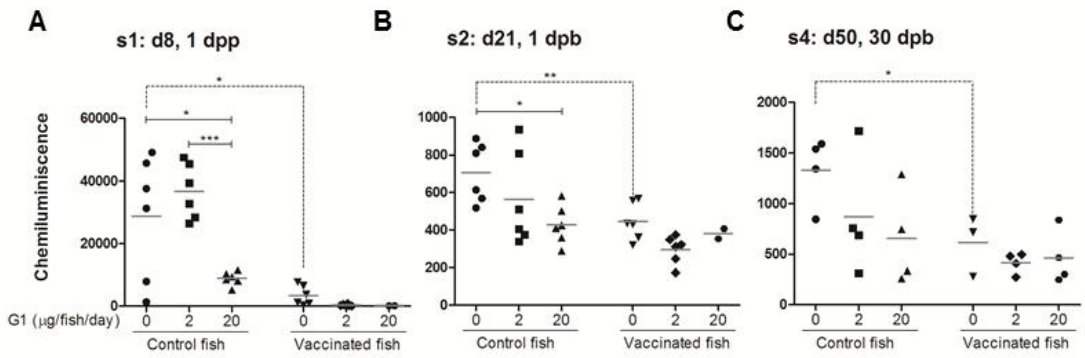

Figure 8. GPER activation in vivo slightly decreases the respiratory burst of HK leukocytes. After (A) 8 (s1; 1 day post-priming), (B) 21 (s2; 1 day post-booster) and (C) 50 (s4; 30 days post-booster) days of G1 exposure, HK leukocytes from G1-treated fish of both non-vaccinated and vaccinated fish were obtained and their respiratory burst activity triggered by PMA (1 mg/ml) was measured using a luminol-dependent chemiluminescence method. The sample size was n=6 fish/group. The mean for each group of specimens is shown as a horizontal line. The asterisks denote statistically significant differences compared with a control group (untreated control or untreated vaccinated fish) according to one-way ANOVA and Tukey post-hoc test. *p <0.05, **p <0.01 and ***p <0.001. No symbol or "ns" means not significant.

As regards the expression of genes encoding key cytokines, vaccination significantly increased the mRNA levels of IL-1β and IL-10 in the HK at all analysis times (Figures 9A-9F). Importantly, the G1 treatment had a dual effect, significantly inhibiting the mRNA levels of IL-1β and increasing those of IL-10 in non-vaccinated fish at all time points (Figures 9A-9F), while reducing IL-10 transcript levels 1 dpp and 30 dpb in vaccinated fish (Figures 9D and 9F).

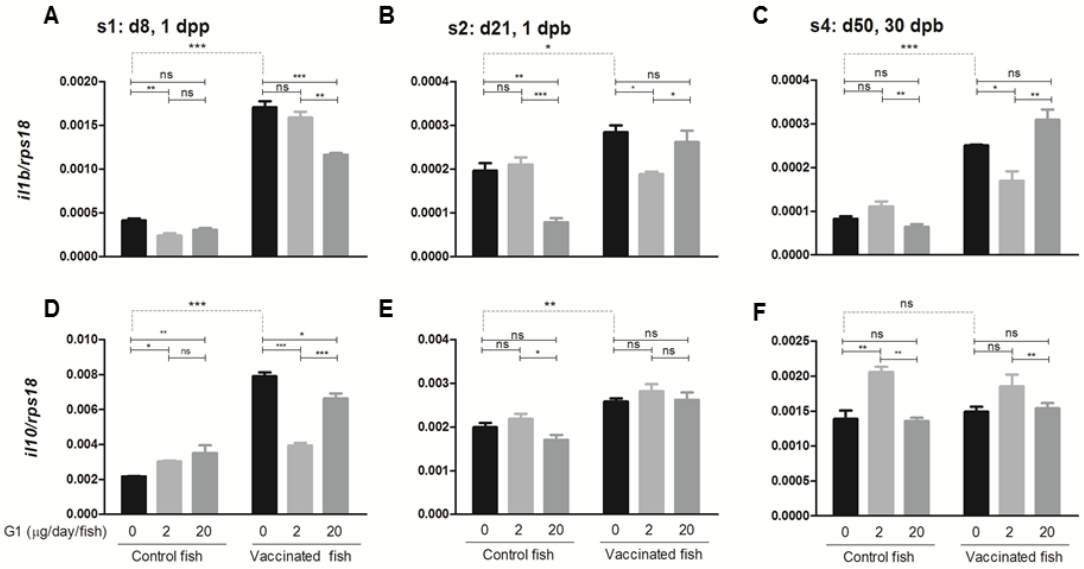

Figure 9. GPER activation in vivo regulates the expression of inflammatory genes in HK leukocytes. The mRNA levels of (A-C) il1b and (D-F) il10 were analyzed by real-time PCR on (A, D) day 8 (s1; 1 day post-priming), (B, E) day 21 (s2; 1 day post-booster) and (C, F) day 50 (s4; 30 days post-booster). Gene expression levels were normalized to rps18 mRNA levels and data represent means ± SEM in triplicate. The asterisks denote statistically significant differences compared with control group (untreated control or untreated vaccinated fish) according to one-way ANOVA and Tukey post-hoc test. *p <0.05, **p <0.01 and ***p <0.001. No symbol or "ns" means not significant.

3.6. GPER signaling in vivo hardly modulates adaptive immunity

The impact of G1 exposure on the adaptive immune response was evaluated as hemocyanin-specific IgM titers in the serum of vaccinated fish on days 30 (s3; 10 dpb) (Figure 10A) and 50 (s4; 30 dpb) (Figure 10B and 10C) after G1 exposure. As expected, vaccinated animals showed a strong antibody response to the antigen and the hemocyanin-specific IgM titers were higher at 30 dpb than at 10 dpb. More importantly, while the higher dose of G1 slightly decreased the antibody titer of vaccinated fish, the lower dose significantly increased it (Figure 10C).

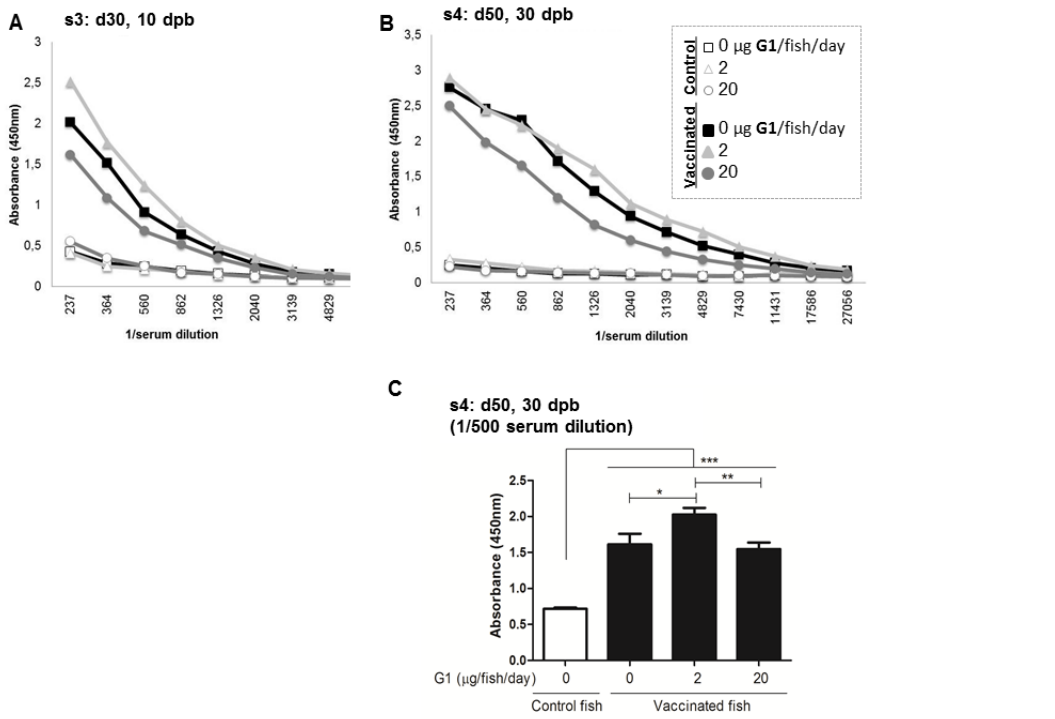

Figure 10. GPER signaling in vivo modulates adaptive immunity. Hemocyanin-induced specific IgM levels were determined by ELISA on (A, C) day 30 (s3, 10 days post-booster) and (B, C) 50 (s4; 30 days post-booster). The absorbance was read at 450 nm using a FLUOstart luminometer. The sample size was n=6 fish/group. The data represent the mean of (A, B) pooled sera from 6 fish at all indicated serum dilutions and (C, D) the mean (shown as a horizontal line) absorbance value of 6 individual fish using a 1/300 or 1/1000 serum dilution, respectively. The asterisks denote statistically significant differences compared with control group (untreated control fish) according to one-way ANOVA and Tukey post-hoc test. *p <0.05, **p <0.01 and ***p <0.001. ns, significant.

4. DISCUSSION

As many of the effects of estrogen cannot be explained by the activation of classical estrogen receptors and given the clear involvement of estrogens in several immune disorders, is important to investigate the relevance of the novel GPER in immunity in order to opening the possibility for the design of new therapeutic targets. For these reasons, there has been increasing attention to GPER in recent years. Nevertheless the knowledge on role of GPER in immune responses is very limited.

In mice, data on the expression of the GPR30 mRNA in tissues has not necessarily produced a consensus result. Nevertheless, the mRNA for GPR30 appears to be expressed extensively in most tissues as judged from the overall reports (reviewed in

Mizukami, 2010). We observed that, in the tissues analyzed of gilthead seabream, the higher expression of GPER expression was observed in liver and gonad. In fish, GPER has been detected in Atlantic croaker and zefrafish gonads, and its activity has been related with the maturation of oocytes (Pang et al., 2008; Liu et al., 2009; Pang and Thomas, 2009). Considerably, GPER is express in spleen and HK, the main hematopoietic organ in fish. In human, it has been described that GPER is expressed in haematopoietic stem cells but not in mature megakaryocytes (Di Vito et al., 2010).

Neutrophils play a key role in innate host immunity and their physiology can be altered by estrogens (Nalbandian and Kovats, 2005; Stygar et al., 2007; Cutolo et al., 2010). In gilthead seabream, AGs are equivalent to mammalian neutrophils (Sepulcre et al., 2002; Sepulcre et al., 2007). However, AGs do not express any of the three nuclear ERs that have been described in gilthead seabream (Pinto et al., 2006; Liarte et al., 2011a). Therefore, we hypothesized that the described effect of estrogens on AGs (Chaves-Pozo et al., 2007; Cabas et al., 2011) would primarily be mediated through GPER. We firstly found that AGs express GPER at both mRNA and protein levels and, more importantly, at much higher levels than other immune cells. To our knowledge, little is known regarding the expression of GPER in immune cells and there is only one study describing the expression of GPER in neutrophil-like H60 cells (Blesson and Sahlin, 2012). In our results, we also obtained a very low, but consistent, expression of GPER in G7- (AG-depleted) cell fractions. This fraction is mainly constituted by macrophages (MΦ), lymphocytes (Ly) and precursor cells. In mammals, the expression of GPER in MΦ and Ly, among other immune cells, has also been described (Owman et al., 1996; Blasko et al., 2009; Rettew et al., 2010; Brunsing and Prossnitz, 2011). Therefore, further studies are needed to clarify the differential expression on seabream immune cell populations and the importance of GPER in the estrogen-mediated effects on these cells, since we have previously described that the main activities and the gene expression profile of MΦ were altered by E_2 and EE_2 (Liarte et al., 2011a; Cabas et al., 2012).

To evaluate the effects of G1 on purified AGs, they were treated *in vitro* with several doses of the GPER agonist G1 in the presence or absence of VaDNA, a potent activator of AGs that acts via the NF-κB signalling pathway (Sepulcre et al., 2007). We observed slightly reduced cell viability in VaDNA-stimulated AGs treated with G1, at the highest dose and time used in this study. Similar effect in cell viability was

described by others, showing an increase in expression of the apoptotic/cell death marker Annexin V in G1-treated CD4+ T cells (Brunsing and Prossnitz, 2011). More studies are needed to determine whether these findings reflect direct or secondary effects of G1. On the other hand, neither GPER nor TLR activation *in vitro* altered the expression of GPER in AGs. In contrast, E_2 increased the expression of ERs in gilthead seabream MΦ and potentiated estrogen signaling in these cells (Liarte et al., 2011a). In contrast, GPER activation was able to slightly increase (after 5 min) and then inhibit (after 3 h) the production of ROS by naïve AGs, but not by VaDNA-primed ones. Therefore, it is tempting to speculate that the ability of natural or synthetic estrogens to inhibit the production of ROS by HK leukocytes (Liarte et al., 2011a; Cabas et al., 2012), would be mediated, at least in part, through GPER signaling. To the best of our knowledge, no studies have evaluated to date the impact of GPER signaling in the production of ROS of neutrophils. This will not be surprising, since GPER signals via adenylate cyclase (AC) and cAMP (Filardo et al., 2002), and a recent study observed that splitomicin, a cell permeable lactone, mediates the cAMP/PKA-dependent of phosphorylation of ERK to decrease superoxide anion production (Liu et al., 2012).

It has been described that GPER activation promotes the upregulation of c-FOS in an ERE-independent and ERK-dependent manner (Albanito et al., 2007; Prossnitz and Maggiolini, 2009). For this, the early expression of c-FOS has been considered has an earlier marker of estrogenic response through GPER, although it can also be induced by ERα activation (Maggiolini et al., 2004). Moreover, the induction of c-FOS has been related with the activation of the cAMP/PKA signaling pathway (Kanda and Watanabe, 2003). Similarly, we observed that GPER is functional in AGs, as shown by the significant induction on *cfos* mRNA levels after G1 treatment. Importantly, as AGs express GPER, but not nuclear ERs (Liarte et al., 2011a), GPER activation is sufficient to induce cFOS, as observed in ER-negative breast tumor cells (Albanito et al., 2008). Strikingly, GPER signaling also promotes a rapid increased expression of IL-1β, a pivotal pro-inflammatory cytokine, and a robust increased of IL-10, an anti-inflammatory cytokine, and of PTGS2, a pivotal and rate-limiting enzyme in the generation of inflammatory prostanoids. We also observed that GPER signaling rapidly induced the expression of the gene encoding PTGDS, enzyme responsible to the synthesis of the powerful anti-inflammatory PGD2 and PGJ2. In addition, at longer time points, i.e. 3 and 16 h, IL-1β expression decreased, and IL-10 and PTGS2 expression

significantly increased even with the lowest doses of G1. All these results, together with the negligible effect of GPER activation on PAMP-primed AGs, indicate that although the effects GPER on AGs are very complex, GPER signaling skews AG activation to an anti-inflammatory phenotype, and suggest a pivotal role of estrogens in the homeostasis of the immune responses through GPER signaling.

The induction in the expression of PTGS2 and IL-10 could be mediated in part by the activation of cAMP/PKA/CREB signaling pathway. In this pathway, PKA mediated the phosphorylation of CREB, a transcription factor which bind to the CRE sites presents at both IL-10 (Platzer et al., 1999) and PTGS2 promoters. It has been described that GPER activation promotes adenylate cyclase (AC) activation (Filardo et al., 2002). On the other hand, stimuli know to elevate intracellular cAMP levels, such as PGE2, adenosine and isoproterenol, positively modulate the PTGS2 expression (Pouliot et al., 2002; Cadieux et al., 2005; Avni et al., 2010). In support of this mechanism, we observed that G1 promoted the phosprorylation of CREB and that the induction of PTGS2 and the phosphorylation of CREB were both mimicked by dbcAMP, a cell-permeant cAMP analogue. Nevertheless, the PKA inhibitor, H89, not only failed to block the G1-mediated induction of PTGS2, but even superinduced it. However, it had no effect in PAMP-stimulated AGs. This has already been observed with human neutrophil and it was speculated that they are the consequence of the complex regulatory mechanisms of CREB activation/deactivation (Cadieux et al., 2005). Thus, the activation of the protein phosphatase involved in the dephosphorylation of CREB also depends on PKA activity (Beullens et al., 1993; Van Eynde et al., 1994). Although further studies are required to clarify GPER signaling in AGs, our results point to a crucial role of the cAMP/PKA/CREB pathway in the regulation of the expression of genes encoding major inflammatory molecules in these cells. It is, therefore, tempting to speculate that similar mechanisms operate in mammalian neutrophils.

GPER activation *in vivo* did not promote an evident estrogenic response, since the GSI and the expression of VTG in the liver, two markers of the estrogenic response, were not significantly altered by dietary G1 exposure. In sharp contrast, administration of EE_2, which is able to bind the nuclear estrogen receptors, decreases the GSI and super-induces the liver VTG (40.000 fold increase compared to control fish) (Cabas et al., 2012). Similarly, GPER does not mediate an estrogenic response in the reproductive

organs in mice (Otto et al., 2009). These results confirm the specificity of the G1 agonist over GPER in the gilthead seabream.

Activation of GPER *in vivo* was unable to modify the expression levels of GPER, as observed in vitro with purified AGs. Therefore, GPER activation is unable to increase the sensitivity of fish to estrogen, in contrast to EE_2 (Cabas et al., 2012). Notably, GPER activation inhibit the respiratory burst of HK leukocytes from non-vaccinated fish but had no effect in vaccinated animals, further supporting the idea of a prominent role of GPER signaling in the homeostasis of the immune response, which is largely bypassed by its activation during infection. Similarly, G1 has been reported to be able to decrease the expression of TLR4 in MΦ, limiting their ability to recognize LPS (Rettew et al., 2010). This result, together with decreased IL-1β and increased IL-10 expression in GI-treated fish, supports that GPER promotes an anti-inflammatory effect, as observed *in vitro.* Similar results has been obtained in mice, where G1 was able to induced IL-10 expression in the Th17 cells, suggesting that GPER may be involved in the estrogens ability to suppress autoimmune diseases (Brunsing and Prossnitz, 2011). Strikingly, GPER signaling fine-tunes adaptive immunity, assayed as the presence of specific IgM in the serum of vaccinated fish. To the best of our knowledge, there are no studies on the impact of GPER on antibody production. It will be worthy to investigate the relevance of GPER in mammalian adaptive immunity as well as the role of different immune cell populations on this response.

In conclusion, we have described for the first time that AGs, the functional equivalent to mammalian neutrophils, express a functional GPER and that its selective agonist G1 promotes an anti-inflammatory effect both *in vitro* and *in vivo*, and fine-tune adaptive immunity. Remarkably, most of the effects of G1 treatment were observed in naïve cells and non-vaccinated fish, in sharp contrast with the effects of other estrogens which are only apparent in a challenge state, in agreement with Köllner's postulate (Kollner et al., 2002). As estrogens play a prominent role in human autoinmmune disorders, our study also suggest that GPER could represent a therapeutic target.

CONCLUSIONS

1. Exposure to EE_2 *in vivo* promotes an evident estrogenic response characterized by decreased GSI, altered serum levels of E_2, T and 11KT, and induced hepatic expression of *vtg*. These effects are more evident when EE_2 is administered in the diet than when is administered in bath water. In addition, its effects slightly depend on the development stage of the specimens.

2. EE_2 increases the sensitivity to estrogens in the gonad and the head kidney by increasing the expression of the gene coding for ERα.

3. Exposure to EE_2 *in vivo* disrupts spermatogenesis and induces a characteristic morphology of the post-spawning in the testis. However, the seminiferous tubules were filled with sperm, causing a reduction in the volume and sperm motility. Moreover, EE_2 also generates a pro-inflammatory process in the gonad, promoting a massive infiltration of leukocytes and an increased expression of the genes encoding cytokines and molecules involved in antigen recognition and presentation.

4. EE_2 decreases the ability of specimens to respond to an immune stimulus *in vivo* by inhibiting the production of pro-inflammatory cytokines after immunization but does not behave as an immunosuppressor. Moreover, EE_2 inhibits *in vitro* the immune activities of head kidney leukocytes and direct primary macrophages towards an anti-inflammatory phenotype.

5. GPER is expressed in reproductive and immune tissues in gilthead seabream and its expression is not modulated by its activation.

6. Acidophilic granulocytes are the head kidney cells with a higher level of expression of GPER. They express a functional GPER whose activation *in vitro* leads, in general, to an anti-inflammatory phenotype. These effects are regulated, in part, by activation of the cAMP/PKA/CREB signalling pathway.

7. GPER activation *in vivo* does not promote an estrogenic response, although in general, provokes an anti-inflammatory effect and slightly modulates the adaptive immune response.

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Cabas I, Chaves-Pozo E, García Alcázar A, Meseguer J, Mulero M, García-Ayala A. Dietary intake of 17α-ethinylestradiol promotes leukocytes infiltration in the gonad of the hermaphrodite gilthead seabream. *Mol Immunol*., 2011 Sep; 48 (15-16):2079-86.

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Cabas I, Chaves-Pozo E, García Alcázar A, Meseguer J, Mulero M, García-Ayala A. The effect of 17α-ethynylestradiol on steroidogenesis and gonadal cytokine gene expression is related with the reproductive stage in marine hermaphrodite fish. *Marine Drugs*, 2013 (submitted).

Cabas I, Rodenas M.C, Abellán E, Meseguer J, Mulero V, García-Ayala A. Estrogen signalling through the g protein-coupled estrogen receptor, GPER, regulates granulocyte activation in fish. *J Immunol*., 2013 (submitted).

RESUMEN EN CASTELLANO

RESUMEN

Durante el desarrollo de la presente Tesis Doctoral se ha analizado el efecto del estrógeno sintético 17α-etinilestradiol (EE_2), componente activo de la mayoría de las píldoras anticonceptivas actuales y terapias de reemplazamiento, sobre el sistema inmunitario y reproductor de la dorada (*Sparus aurata* L.). La dorada es un pez teleósteo marino con un valor económico importante en la acuicultura Mediterránea y que está siendo utilizado como modelo para el estudio de la interacción inmuno-reproductora por su condición de pez hermafrodita protándrico de puesta estacional. Además se ha determinado la importancia del receptor de estrógenos asociado a proteína G, GPER, en la biología de los granulocitos acidófilos. El estudio ha sido desarrollado en cuatro etapas sucesivas.

En una primera aproximación evaluamos el efecto de diferentes dosis de EE_2, incorporado en la dieta, sobre determinados parámetros reproductores como el índice gonadosomático, los niveles séricos de hormonas sexuales y la espermatogénesis así como sobre algunos eventos inmunitarios que tienen lugar en la gónada. Los resultados muestran que el EE_2 interrumpe la espermatogénesis al tiempo que promueve la adquisición de una morfología testicular similar a la que se observa en la etapa de post-puesta aunque sin liberación de espermatozoides. Además promueve la infiltración de granulocitos acidófilos, un tipo celular con equivalencia funcional a neutrófilos de mamíferos, y de linfocitos B en el testículo que se correlaciona con un incremento, dosis dependiente, en la expresión de genes que codifican para marcadores de linfocitos B y de citoquinas, quimioquinas y moléculas de adhesión relacionadas con el tráfico de leucocitos.

En una segunda etapa del estudio, y como continuación de los resultados descritos previamente, evaluamos la sensibilidad a dosis bajas de EE_2, incorporado en la dieta, en ejemplares de dorada en dos etapas distintas del ciclo reproductor, espermatogénesis (SG) y una etapa previa a la espermatogénesis (pre-SG). Los resultados muestran que el EE_2 provoca una respuesta estrogénica ya que produce una inducción importante en los niveles de expresión de vitelogenina y una alteración en los niveles séricos de hormonas sexuales, este último hecho correlacionado con una modulación significativa en el perfil de expresión de varias enzimas implicadas en la síntesis de esteroides. Además, el EE_2 es capaz de modificar la expresión de genes relevantes en la respuesta inmunitaria

implicados en la fisiología del testículo, encontrándose algunas diferencias interesantes en relación con la etapa del ciclo reproductor.

A continuación evaluamos la capacidad del EE_2 de modular *in vivo* e *in vitro* la respuesta inmunitaria innata. Para ello concentraciones realistas de EE_2 fueron incorporadas en el agua del baño de los ejemplares y, a continuación, fueron inmunizados con hemocianina en presencia de aluminio como adjuvante. Los resultados indican que el EE_2 es capaz de inhibir de manera dosis dependiente la inducción de la expresión génica de interleukina 1β provocada por la inmunización, mientras que no produce una alteración significativa en el título específico de anticuerpos. Además se observa que el EE_2 *in vitro* es capaz de inhibir actividades inmunitarias de los leucocitos de riñón cefálico, como la producción de radicales libres de oxígeno y la fagocitosis, y de alterar el perfil de expresión de genes inmunitarios en macrófagos en cultivo primario. Así, el EE_2 provoca un descenso en la expresión de genes pro-inflamatorios y un incremento en genes que codifican para moléculas anti-inflamatorias y enzimas implicadas en la remodelación y reparación tisular en macrófagos previamente activados. Estos resultados sugieren que el EE_2 podría alterar la capacidad de estos ejemplares para responder de manera apropiada a infecciones aunque este estrógeno sintético no se comporte como inmunosupresor.

Finalmente realizamos una caracterización funcional del receptor de estrógenos asociado a proteína G, GPER, que se ha identificado recientemente. En un primer paso se obtuvo una secuencia parcial de GPER, lo que nos permitió analizar el perfil de expresión de GPER en diferentes tejidos y poblaciones celulares de riñón cefálico. Así observamos que el GPER se expresa en tejidos reproductores e inmunitarios de dorada y que su expresión no es modulada por la activación específica del mismo. Por otra parte comprobamos que los granulocitos acidófilos son las células del riñón cefálico que tienen un mayor nivel de expresión de GPER y que lo expresan de manera funcional. Así la activación de dicho receptor *in vitro* reduce ligeramente la explosión respiratoria de estas células y, de manera drástica, altera el perfil de expresión de varios genes que codifican para los principales mediadores pro- y anti-inflamatorios. Además la señalización *in vivo* a través de GPER modula la respuesta adaptativa. Por otro lado encontramos que los efectos de un agonista específico de GPER (G1) en la inducción del gen que codifica para la prostaglandina-endoperóxido sintasa 2, pueden ser debidos, en parte, a la activación de la ruta de señalización cAMP/PKA/CREB, ya que

observamos que un análogo de cAMP es capaz de mimetizar dichos efectos mientras que la inhibición farmacológica de proteína quinasa A induce dicha expresión. Además, G1 es capaz de promover la fosforilación de CREB, efecto también mimetizado por el análogo de cAMP. En conjunto, nuestros resultados muestran por primera vez que los estrógenos son capaces de modular funciones de los granulocitos de vertebrados a través de la señalización por GPER y podría establecer dianas terapéuticas para varios desordenes inmunitarios donde los estrógenos juegan un papel importante.

1. INTRODUCCIÓN

En un mundo poblado por, aproximadamente, 7000 millones de personas, la demanda de alimentos de origen marino, en concreto pescado, ha elevado la presión pesquera sobre los caladeros hasta el punto de poner en peligro su capacidad de renovación. La pesca extractiva, difícilmente, cubre el 70% del volumen pesquero total anual (FAO 2006), situación ante la cual la acuicultura se perfila como la única posibilidad de cubrir la demanda en un futuro próximo. La práctica de la acuicultura supone el confinamiento de un gran número de ejemplares en espacios limitados y su sometimiento a una manipulación más o menos periódica, desencadenándose situaciones de estrés en los animales que afectan tanto a los ejemplares adultos coma a larvas y a juveniles. Estas condiciones de cultivo favorecen el desarrollo de enfermedades infecciosas que son responsables de cuantiosas pérdidas económicas. Por ello, el conocimiento del sistema inmunitario de los peces, en general, y de las especies objeto de cultivo, en particular, se ha convertido en uno de los objetivos prioritarios en la investigación acuícola.

Los peces tienen una posición filogenética clave representando el primer grupo animal que posee un sistema inmunitario innato, constituido por barreras físicas y químicas, efectores celulares (leucocitos) y humorales, y un sistema inmunitario adaptativo, constituido por linfocitos y los anticuerpos que producen, bien estructurados como tales, por lo que el estudio de la inmunología de este grupo de vertebrados tiene un interés científico básico. Así, el sistema inmunitario de vertebrados presenta un patrón común aunque esto no excluye la existencia de diferencias importantes entre ejemplares de una misma especie o entre especies diferentes de vertebrados. Quizás la diferencia más importante sea el desarrollo y preponderancia de los mecanismos de defensa innatos de peces, en contraste con la respuesta adaptativa potente y bien desarrollada de vertebrados superiores (Anderson, 1992). Otro punto a destacar es la ausencia de médula ósea en los peces, siendo el riñón cefálico, un órgano primario, el órgano hematopoyético por excelencia. En la dorada, *Sparus aurata* L., modelo animal usado en este estudio y especie de interés económico importante para la acuicultura Mediterránea, el riñón cefálico está constituido por células precursoras y por tres poblaciones de leucocitos, macrófagos (MΦ), linfocitos (Ly) y granulocitos acidófilos (GAs), siendo estos últimos las principales células que participan en la respuesta inmunitaria innata.

Por otra parte, los peces son el grupo de vertebrados con mayor diversidad de estrategias reproductivas. Así hay especies gonocoristas que desarrollan un único sexo funcional y especies hermafroditas que desarrollan ambos sexos funcionales a lo largo de su vida. Las especies hermafroditas pueden ser, a su vez, protándricas o protogínicas en función del sexo que desarrollen antes, el masculino o el femenino, respectivamente, o bien ambisexuales si desarrollan ambas gónadas simultáneamente (Devlin y Nagahama, 2002). La dorada es un pez teleósteo marino, hermafrodita protándrico de puesta etacional que presenta una gónada bisexual compuesta por un área ovárica, mediodorsal, y un área testicular, lateroventral, ambas separadas por tejido conjuntivo. Los ejemplares de dorada se desarrollan, funcionalmente, como machos durante los dos primeros ciclos reproductivos, pudiendo posteriormente pasar a hembras (D'Ancona 1941; Pascuali 1941; Zohar y col. 1978; Liarte y col. 2007) Como se ha descrito en esta especie, el primer ciclo reproductivo consta de cuatro etapas: gametogénesis, puesta, post-puesta y quiescencia. Durante las etapas de post-puesta y quiescencia, la gónada de la dorada sufre importantes cambios morfológicos, fruto de una remodelación tisular, conducente a la eliminación de los espermatozoides remanentes del ciclo anterior y a la reorganización del tejido germinal (Chaves-Pozo y col. 2005c). Estos procesos se correlacionan con un marcado aumento en los niveles séricos de 17β-estradiol (E_2) y de la disminución de los andrógenos, testosterona (T) y 11-cetotestosterona (11-KT), principal andrógeno en esta especie (Chaves-Pozo y col. 2008b). Al igual que en mamíferos, en peces, la síntesis de estas hormonas esteroideas tiene lugar principalmente en la gónada, en un proceso llamado esteroidogénesis. Este proceso implica una señal de transducción intracelular (provocada por las hormonas hipofisiarias, FSH y LH), producción de colesterol y transporte de éste al interior de la mitocondria (gracias a la proteína esteroidogénica reguladora de la fase aguada, StAR) y un set de múltiples conversiones enzimáticas desde el colesterol hasta hormonas productos finales como T, dihidro-T, E_2 and estrona. EL E_2 ha sido considerado como la principal hormona sexual femenina en peces, sin embargo, estudios recientes sugieren que los estrógenos son esenciales para el desarrollo normal de la reproducción en machos (Miura y col. 1999; Miura y col. 2001; Miura y col. 2002; Hess, 2003; Miura y col. 2003). Durante el segundo ciclo reproductor, los ejemplares pasan de forma similar a lo que ocurre en el primero, por las etapas de espermatogénesis, puesta y post-puesta mientras que la etapa de quiescencia es sustituida por una etapa de involución testicular, previa al cambio de sexo (Liarte y col. 2007).

De manera tradicional se considera que los estrógenos actúan a través de los receptores de estrógenos (REs) nucleares clásicos lo que implica la unión del ligando al receptor, dimerización y unión a elementos de respuesta a estrógenos presentes en las regiones promotoras de genes diana (Deroo and Korach, 2006). Estos REs actúan como reguladores transcipcionales, como modo de acción principal, y constituyen las acciones genómicas de los estrógenos. Sin embargo se han observado ciertos efectos de los estrógenos que no pueden ser explicados por esta señalización clásica, por lo que la importancia de REs de membrana asociados con una señalización rápida y no-genómica, está siendo tenida en cuenta, recientemente. Así, en 2005, un receptor huérfano asociado a proteína G, GPER, fue identificado como un receptor de membrana intracelular para estrógenos (Revankar y col. 2005; Thomas y col. 2005) y, posteriormente, se demostró que GPER era activado por E_2 (Filardo y col. 2002; Funakoshi y col. 2006; Filardo y col. 2007). Hasta ahora, los mecanismos descritos relacionados con la activación de GPER incluyen (i) una rápida activación de MAPK, ERK-1 y ERK-2 a través de la trans-activación del EGFR (Filardo y col. 2000), (ii) activación de la señalización de PI3K (Revankar y col. 2005), (iii) activación de adenilato ciclasa y posterior activación de PKA y producción de cAMP (Filardo y col. 2002) y (iv) movilización de calcio intracelular (Revankar y col. 2005).

La expresión de los REs se ha descrito en órganos reproductores pero también está ampliamente distribuida en células del sistema inmunitario (Straub, 2007). En dorada se han identificado tres REs, REα, REβ1 and REβ2, los cuáles se expresan en tejidos reproductores y no reproductores (Pinto y col. 2006). Además, los REs se expresan, de manera diferencial, en las poblaciones de leucocitos de riñón cefálico. Así se observa que los MΦ expresan de manera constitutiva el gen que codifica el REα y la expresión de los tres REs es inducida por la estimulación con DNA genómico de *Vibrio anguillarum* (VaDNA) mientras que los linfocitos sólo expresan el gen para el REα. Sin embargo, los GAs no expresan ninguno de los tres REs (Liarte y col. 2011a). Aunque el interés por el GPER ha aumentado en los últimos años, no hay mucha información respecto a su perfil de expresión. Hasta el momento, la expresión de GPER en órganos sexuales no ha sido analizada en detalle. Por otra parte, el ARNm del GPER parece que se expresa en una amplia variedad de tejidos (Mizukami, 2010). Aunque son escasos, hay ciertos estudios que muestran que las células del sistema inmunitario expresan

GPER (Owman y col. 1996; Blasko y col. 2009; Rettew y col. 2010; Brunsing y Prossnitz, 2011).

La presencia de REs en células del sistema inmunitario hace posible que los estrógenos tengan un papel inmuno-modulador en el sistema inmunitario (Straub, 2007). Así se sabe que existe una interacción entre el sistema reproductor e inmunitario, pudiendo darse en ambos sentidos. Hay células del sistema inmunitario, y los mediadores inmunitarios que producen, presentes en la gónada capaces de modular la fisiología de la misma (Hedger, 1997; Nes y col. 2000) y, por otro lado, hormonas esteroideas son capaces de regular las actividades de las células inmunitarias. Sin embargo, el papel inmuno-modulador de los estrógenos sobre la células del sistema inmunitario es controvertido debido al hecho de que estos efectos pueden variar según el estímulo inmunitario, las células implicadas, el órgano diana y su microambiente, la concentración de estrógenos y la variabilidad en la expresión de los REs (Straub, 2007). En relación con este papel de los estrógenos en la respuesta inmunitaria existen estudios epidemiológicos que han relacionado los niveles plasmáticos de estrógenos con la producción de IL-1b y desórdenes autoinmunes asociados a varias enfermedades (Cutolo y col. 2006). Además se conoce que el E_2 tiene un efecto inhibidor en la reabsorción del hueso y en la supresión de la inflamación en varios modelos animales de enfermedades con inflamación crónica (Straub, 2007). Debido a la relevante implicación de los estrógenos en estos desórdenes es importante considerar, no solo la señalización a través de los REs clásicos, sino también por GPER, para así poder diseñar posibles dianas terapeúticas.

La dorada es una especie interesante para estudios de interacción inmuno-reproductora debido a las características descritas anteriormente. Por ello, estudios previos han sugerido que los estrógenos son reguladores clave en las funciones de leucocitos en la gónada de dorada. Así, la infiltración de GAs en el testículo, orquestada por factores producidos en la gónada entre los que se incluyen las hormonas esteroideas, ha sido relacionada con (i) épocas de post-puesta e involución testicular, (ii) picos en los niveles séricos de las hormonas E_2 y T, (iii) incremento en la expresión de la aromatasa, enzima responsable de la conversión de T a E_2 y (iv) con la inducción experimental de los niveles séricos de E_2. Sin embargo, las funciones de estos GAs se alteran cuando infiltran el testículo (Chaves-Pozo y col., 2003, 2005a, 2005b, 2007, 2008a). Además, MΦ y linfocitos, también, se observan en el tejido intersticial del testículo, sin embargo

el número de MΦ permanece constante a lo largo del ciclo reproductor (Chaves-Pozo y col. 2008a; Liarte y col. 2007). Todas estas observaciones sugieren que la presencia de células inmunitarias en la gónada garantiza y modula las funciones reproductivas. Por otro lado, se ha descrito que, el E_2 es capaz de regular la activación de macrófagos primarios de dorada (Liarte y col. 2011a, b) y de alterar el perfil de expresión de estas células clave en la repuesta inmunitaria innata (Liarte y col. 2011b).

Así, los estrógenos, además de su implicación en el sistema reproductor, tienen un papel modulador del sistema inmunitario, por lo que cualquier sustancia capaz de mimetizar o antagonizar la acción de estas hormonas, podría provocar un efecto no deseado, no solo en el sistema reproductor, sino también en aquellos procesos en los que estén implicadas. En la actualidad hay una conciencia creciente sobre el impacto que algunos contaminantes químicos, llamados "disruptores endocrinos" (DEs), son capaces de provocar en el sistema endocrino y, por tanto, en procesos regulados por hormonas. La OMS define a los DEs como *"sustancias exógenas o mezcla de ellas que alteran el sistema endocrino y, consecuentemente, causan efectos adversos en la salud en un organismo intacto o sobre sus progenie o (sub)poblaciones"* (WHO/International, 2012). Estas sustancias actúan mimetizando o antagonizando a las hormonas naturales y, por tanto, son capaces de alterar los sistemas en los que estas hormonas están implicadas, incluido el sistema inmunitario. Por tanto, la evaluación del impacto de estos compuesto sobre la inmunología de peces es una cuestión importante, en particular, en especies objeto de cultivo. Uno de los principales grupos de DEs son los estrógenos ambientales o xenoestrógenos que pueden ser de origen biológico (E_2, estrona o fitoestrógenos) o antrópico (dietilestilbestrol, bisfenol A, 17α-etinilestradiol, EE_2). Estos DEs son capaces de mimetizar la acción de los estrógenos generando una respuesta estrogénica definida, como la inducción del gen de la vitelogenina, considerado un marcador de disrupción estrogénica, en peces y aves (Sumpter y Jobling, 1995). Se ha descrito que estas sustancias son capaces de afectar el crecimiento, incrementar la incidencia de problemas relacionados con el tracto reproductor masculino, reducir la fertilidad, generar pérdida de eficacia de apareamiento, promover alteraciones en la conducta, desórdenes metabólicos, feminización, incremento en la incidencia de varios tipos de cáncer y de desórdenes en el sistema inmunitario (Colborn y col. 1993).

Dentro de los xenoestrógenos de origen antrópico se encuentra el EE_2, estrógeno sintético con homología estructural a la hormona sexual femenina E_2, diferenciándose de ésta en la presencia de un grupo etinil en el carbono 17. Es un compuesto farmacológico utilizado como el componente activo de la mayoría de las píldoras anticonceptivas modernas y es, también, usado ampliamente en las terapias de reemplazamiendo hormonal y tratamiento de cáncer de mama. Las píldoras anticonceptivas son usadas actualmente por más de 100 millones de mujeres. Una vez ingerido es degradado por hidroxilación aromática en el hígado, donde se forman gran cantidad de metabolitos hidroxilados y metilados, libres o conjugados como glucurónidos o sulfatos. Estos metabolitos que son excretados en la orina y las heces (Orme y col. 1983) pueden sufrir procesos de desconjugación en las estaciones depuradoras durante los tratamientos de las aguas residuales (Desbrow y col. 1998) por lo que se libera en su forma activa a los efluentes de dichas estaciones, siendo éstas la fuente mayor de contaminación de estrógenos en los ambientes acuáticos (Ingerslev y col. 2003). Así, el EE_2 ha sido detectado en ambientes acuáticos en concentraciones de 1-10 ng/L aunque se ha llegado a detectar en concentraciones de 47 ng/L (Desbrow y col. 1998; Ternes y col. 1999; Johnson y col. 2005; Hinteman y col. 2006; García-Reyero y col. 2011). Debido a que el EE_2 posee una serie de características particulares como una susceptibilidad baja a la degradación metabólica (Lai y col. 2002a), una tendencia alta a bioconcentrarse (Larsson y col. 1999), una potencia 10-50 veces superior, *in vivo*, al E_2 (Thorpe y col. 2003; Nash y col. 2004) y una afinidad grande por los REs (Tilton y col. 2005), es considerado uno de los estrógenos sintéticos más potentes. Por todo ello, el interés por el efecto provocado por el EE_2, no solo en el sistema reproductor sino en el sistema inmunitario, ha aumentado en los últimos años, sin que haya muchos datos referentes a éste último. Por otra parte, los peces son un grupo animal expuesto, de manera crónica y continuada, a concentraciones bajas, pero biológicamente activas de EE_2. Finalmente, teniendo en cuenta que tejidos y células del sistema inmunitario expresan REs clásicos y de membrana, y que ciertos DEs pueden unirse a ambos tipos de receptores (Thomas y Dong, 2006; Watson y col. 2011), el efecto de estrógenos ambientales sobre la inmunidad de peces no debería ser subestimado.

Situada en este marco de conocimiento, la presente Tesis Doctoral se centra en determinar si el EE_2 provoca una respuesta estrogénica en ejemplares macho de una

especie hermafrodita, como la dorada, y si tiene algún efecto sobre la espermatogénesis y la respuesta inmunitaria local en la gónada de la dorada. También nos ha interesado conocer si estos efectos varían con el estado de desarrollo de los ejemplares. Por otro lado hemos explorado el efecto del EE_2 sobre la capacidad que tienen los peces de responder de manera apropiada a una respuesta inmunitaria inducida, alterando las actividades de los leucocitos. Finalmente hemos estudiado la importancia de un RE de membrana en la regulación de la biología de los granulocitos.

2. OBJETIVOS

Este trabajo tiene los siguientes objetivos específicos:

1. Evaluar la capacidad del EE_2 de provocar una respuesta estrogénica *en vivo* en ejemplares macho de dorada

2. Evaluar la capacidad del EE_2 de alterar la fisiología y la respuesta inmunitaria local de la gónada

3. Evaluar la habilidad del EE_2 de alterar la respuesta inmunitaria sistémica, analizando actividades inmunitarias de leucocitos de riñón cefálico, el perfil de expresión de macrófagos y la capacidad e*n vivo* de responder a un reto inmunológico.

4. Realizar una caracterización funcional del receptor de estrógenos asociado a proteína G tanto *in vitro* como *in vivo.*

3. PRINCIPALES RESULTADOS Y DISCUSIÓN

3.1. El EE_2, incorporado en la dieta, promueve una infiltración de leucocitos en el testículo de ejemplares macho de dorada

En el primer capítulo estudiamos como el EE_2, administrado en la dieta (0, 5, 50, 125 y 200 μg/g de comida), es capaz de alterar la fisiología de la gónada, de ejemplares macho de dorada en la etapa de espermatogénesis, centrándonos en los eventos inmunitarios que tienen lugar en ella.

Podemos observar que concentraciones elevadas de EE_2 provocan la mortalidad. Con el fin de evaluar si el EE_2 provoca una respuesta estrogénica en los ejemplares macho de dorada se evaluó el índice gonadomático (IGS) y se observó, al igual que ocurre en la mayoría de los experimentos en los que usan una exposición a compuestos estrogénicos mediante agua de baño (Hogan y col. 2010; Saaristo y col. 2009), que al final del tratamiento, éste es inferior en todos los grupos tratados con EE_2. Además se observa que el tratamiento *in vivo* con EE_2 modula los niveles séricos de los esteroides sexuales como ha sido descrito, ampliamente, para otros compuestos estrogénicos (Peters y col. 2007; Hogan y col. 2010; Marlatt y col. 2010). Así, los peces alimentados con EE_2 muestran un incremento en los niveles de E_2 en todos los grupos analizados aunque el efecto es particularmente evidente con altas concentraciones de EE_2. Este efecto es claramente diferente al observado en especies de peces gonocoristas (Peters y col. 2007; Swapna y Senthilkumaran, 2009; Hogan y col. 2010). Sin embargo, los niveles de T disminuyen en todos los grupos tratados después de 14 días de exposición y los de 11KT disminuyen en todas las condiciones evaluadas, excepto a tiempos cortos y a bajas dosis de EE_2.

De manera significativa también se observa que, aunque algunas células germinales proliferan, la espermatogénesis de los ejemplares expuestos a EE_2, se interrumpe. En general se observa un epitelio germinal reducido formado por células germinales pre-meióticas, un incremento en la cantidad de espermatozoides libres en el interior y un aumento del intersticio y de células eosinófilas, como ocurre tras la puesta, aunque sin liberación de espermatozoides. De manera similar a lo que ocurre cuando los ejemplares de dorada son tratados con E_2 (Chaves-Pozo y col. 2007), el EE_2 no promueve la inducción de la puesta.

A continuación, y conocida la relación inmuno-reproductora en esta especie descrita en previos trabajo por el grupo de investigación, nos dispusimos a analizar los eventos inmunitarios que tienen lugar en la gónada de los ejemplares expuestos a EE_2. Así observamos que los GAs infiltran el testículo a los 14 días de exposición con dosis altas de EE_2 y a tiempos largos de exposición con todas las dosis utilizadas. Estos GAs ocupan la misma localización en el testículo que los presentes, de manera fisiológica, en la post-puesta (Chaves Pozo y col. 2008a). Sin embargo, la localización y la abundancia de MΦ, un tipo celular clave en el papel inmuno-regulador de los estrógenos en dorada (Liarte y col. 2011a), no se alteraron por la exposición a EE_2. Además, el EE_2 promueve el reclutamiento de Ly B (células IgM positivas) desde el día 7 de tratamiento. La cuantificación de células G7 positivas (GAs) e IgM positivas en la gónada revela un incremento significativo y dosis dependiente en el número de estas células inmunitarias al final de los tratamientos.

Con el fin de poder interpretar esta infiltración de leucocitos se analizó la expresión de genes que codifican para marcadores de Ly B y genes implicados en el tráfico de leucocitos como citoquinas, quimioquinas y moléculas de adhesión. Encontramos que la expresión de los genes que codifican para IgM e IgT, los cuáles son expresados en dos subpoblaciones de Ly B, es generalmente inducida de manera dosis dependiente por el EE_2. Además, y correlacionado con la infiltración de leucocitos descrita anteriormente, observamos que los niveles de RNAm de los genes que codifican para TNFα, CCL4, IL8 y SELE, son significativamente mayores después de 7, 14 y 21 días de la exposición en los grupos tratados comparados con el control. La mayoría de ellos son inducidos de manera dosis-dependiente tras la exposición con EE_2, alcanzando las mayores inducciones (10-16 veces) en los peces tratados con 200 μg EE_2/g de comida después de 14 días de tratamiento y con 125 μg EE_2/g de comida después de 21 días de exposición (10-20 veces).

Sin embargo, los GAs no expresan ninguno de los tres REs identificados en dorada (Pinto y col. 2006, Liarte y col. 2011a), por lo que nuestros datos sugieren que independientemente de la expresión de los REs en los diferentes tipos celulares, la inducción en la expresión de TNFa, TNFα, CCL4, IL8 y SELE está involucrada en la migración dentro de la gónada en respuesta a agentes estrogénicos. No podemos descartar que otras quimioquinas y molélulas de adhesión descritas en mamíferos, pero no en dorada, estén implicadas en esta infiltración de leucocitos.

En conjunto, el EE_2 promueve un proceso inflamatorio en la gónada de la dorada, marcado por una infiltración de leucocitos pudiendo estar orquestada por señales del tejido dañado y del proceso degenerativo que tiene lugar, correlacionado con un incremento en la expresión de moléculas inmunitarias relacionadas con el tráfico de leucocitos.

3.2. El efecto del EE_2 sobre la esteroidogénesis y la expresión génica de citoquinas en la gónada es, ligeramente, dependiente del estado reproductor de los ejemplares

En este capítulo, y como continuación del anterior, evaluamos la capacidad de dosis bajas de EE_2 de modular la esteroidogensis y la expresión de citoquinas en la gónada a tiempo corto, 7 días, y a tiempo largo, 28 días, de exposición, y la posible diferencia entre ejemplares en SG y en un estado previo, pre-SG.

En especies de peces gonocoristas, diferentes compuestos estrogénicos provocan la modificación de la calidad del esperma, de los niveles de hormonas sexuales androgénicas y de la producción VTG hepática (Sumpter y Jobling, 1995; Hogan y col. 2010) Sin embargo, poco se sabe acerca de estos efectos en las especies de peces hermafroditas. En concordancia con la interrupción de la espermatogénesis descrita anteriormente, comprobamos que el EE_2 disminuye el volumen y la motilidad del esperma sin alterar la concentración del mismo.

Como hemos descrito previamente, el EE_2 modula los niveles séricos de las hormonas sexuales en ejemplares en SG, incrementando los de E_2 a día 7 y disminuyendo los de los andrógenos T y 11KT al final del tratamiento. Sin embargo, en ejemplares en pre-SG, el EE_2 no modula de manera significativa los niveles de E_2 mientras que disminuye los de T y 11KT al final del experimento aunque de manera menos pronunciada de lo que lo hace en ejemplares en SG (Cabas y col. 2011). Esta alteración en los niveles hormonales está en concordancia con la regulación de la expresión génica de las moléculas esteroidogénicas más relevantes que intervienen en su producción. Así, el EE_2 inhibe la expresión de *star, cyp11a1, hsd3b, cyp11b1* y *sdr5a* (involucradas en la producción de andrógenos) en la gónada de ejemplares en pre-SG y SG mientras que los niveles de expresión de *cyp19a1a* (involucrada en la producción

de estrógenos) se regula de manera negativa o positiva en la gónada de ejemplares en pre-SG o en SG, respectivamente.

La expresión del gen de la *vtg,* un marcador de disrupción estrogénica (Sumper y Jobling, 1995), fue inducida significativamente por el EE_2 en ambos grupos de ejemplares y en todos los tiempos de exposición analizados, como ha sido descrito en numerosos estudios (Hogan y col. 2010).

Con el fin de determinar si algún otro parámetro reproductivo podría ser utilizado como marcador de disrupción endocrina en dorada, hemos analizado los niveles de expresión génica de la proteína específica de testículo, DMRT1, y algunos genes de receptores de hormonas en la gónada de ejemplares en pre-SG y en SG. Como era de esperar, la expresión de *dmrt1,* fue mayor en ejemplares en SG. Además, el EE_2 disminuyó la expresión de *dmrt1* en ejemplares en SG solo a día 7 de tratamiento, desapareciendo dicho efecto al final del tratamiento. Sin embargo, en ejemplares en pre-SG, el EE_2 no modula de manera significativa la expresión de *dmrt1*.

Aunque la expresión de *era* es inducida en todos los casos analizados, como también ha sido observado durante la exposición a EE_2 mediante agua de baño (Cabas y col. 2012), y por tanto podría ser usada como un biomarcador de disrupción estrógenica, como ya ha sido considerado por otros autores (Carnevali y col. 2011), la magnitud de este efecto no es comparable con el observado en la expresión de *vtg.* Este efecto sugiere cambios en la sensibilidad a estrógenos durante la maduración sexual.

Por otra parte, el EE_2 disminuye la expresión de *fshr* y *lhr* excepto en la gónada de ejemplares en SG tratados durante 28 días, en la que también se observa un incremento en la expresión de *dmrt1*, como hemos descrito anteriormente. Estos resultados, conjuntamente, están en concordancia con la interrupción de la espermatogénesis y el proceso de recrudecimiento de la zona testicular de la gónada y la no inducción del cambio de sexo observado previamente en la gónada de ejemplares en SG (Cabas y col. 2011).

Por otro lado se ha descrito que algunas moléculas inmunitarias ejercen un papel relevante en la regulación de la espermatogénesis y/o la esteroidogénesis en vertebrados, incluidos los peces (Chaves-Pozo y col. 2008a; O'Bryan y Hedger, 2009). Para explorar el efecto del EE_2 sobre la regulación inmunitaria local que ocurre en la gónada analizamos la expresión de genes que codifican para varias citoquinas pro- y

anti-inflamatorias, metaloproteasas (MMPs) y moléculas relacionas con el reconocimiento de patógenos, presentación de antígenos y reclutamiento de leucocitos y marcadores de linfocitos B. Es de destacar que la expresión de la mayoría de estos genes fue mayor en ejemplares en pre-GS que en SG. Observamos, además, que el EE_2 promueve un aumento en la expresión de citoquinas pro-inflamatorias, IL1β y TNFα, y la citoquina anti-inflamatoria, TGFB1, aunque la respuesta es diferente en los ejemplares en pre-SG y en SG. Estos incrementos en la expresión podrían estar correlacionados con la disminución de la producción de andrógenos y sugiere, como ocurre en mamíferos (Lui y col. 2003) y en algunas especies de peces (Lister y Van Der Kraak, 2002), que estas citoquinas están implicadas en la esteroidogénesis testicular. La administración de EE_2 también promueve un incremento en la expresión de genes que codifican para la MMP9 y la MMP13, lo que concuerda con la inducción de un estado de post-puesta e infiltración masiva de AGs (Cabas y col. 2011) y en respuesta a la administración exógena de E_2 (Chaves-Pozo y col. 2007). Nuestros datos muestran que el EE_2 aumenta la expresión de *tlr9* y *mhc1a* en ejemplares en pre-SG y SG, lo que sugiere que este contaminante estrogénico estimula la capacidad de la gónada para reconocer y responder a patógenos. En el capítulo I se describe que el EE_2 es capaz de promover una regulación positiva en la gónada de los genes que codifican para *ccl4*, *il8* y *sele* y para los marcadores de Ly B, *ighm* e *ight*, lo que se correlaciona con una infiltración de leucocitos en la gónada (Cabas y col. 2011). En este capítulo, estudiamos la regulación por el EE_2 en la expresión de estos genes en ejemplares en pre-SG y SG, observándose una modulación diferente de los niveles de expresión de dichos genes en pre-SG. Probablemente, estas diferencias resultaron en diferencias en la afluencia de leucocitos en la gónada en respuesta a EE_2.

Como conclusión a este capítulo, nuestros datos demuestran que la administración de EE_2 promueve una respuesta estrogénica y modifica el patrón de expresión de moléculas esteroidogénicas, citoquinas y otras moléculas relacionadas con la inmunidad que participan en diferentes procesos de la fisiología testicular que van desde la esteroidogénesis hasta el reconocimiento de patógenos. Curiosamente se ha observado una modificación en el desarrollo de la sensibilidad a EE_2 en la gónada de dorada y apunta a la necesidad de realizar más estudios para determinar con claridad las etapas de la vida de las especies de peces más susceptibles a los contaminantes estrogénicos.

3.3. El EE_2 altera la respuesta inmunitaria innata de la dorada tanto in vivo como in vitro

El principal objetivo de este estudio fue investigar la capacidad del EE_2 de modular *in vivo* e *in vitro* la respuesta inmunitaria innata en la dorada.

La exposición *in vivo* a EE_2 (0, 5 y 50 ng/L), incorporado en el agua de baño, no afecta a la supervivencia de los ejemplares, siendo ésta del 100% durante toda la experiencia. Con el fin de evaluar la respuesta estrogénica promovida por el EE_2 se analizó el IGS, el perfil de hormonas sexuales en suero y la expresión de *vtg* en hígado, marcador de disrupción estrogénica, como se ha comentado anteriormente. Así, de manera similar a otros estudios con exposición por baño a compuestos estrogénicos (Saaristo y col. 2009; Hogan y col. 2010), se observó que el IGS disminuye en los grupos tratados con EE_2 comparados con el control aunque estas diferencias no son estadísticamente significativas. Además, los peces expuestos a EE_2 durante 15 días no muestran cambios significativos en los niveles séricos de E_2 mientras que muestran un descenso en los niveles de T comparados con el grupo control. Estos efectos fueron similares a los observados previamente por la exposición de EE_2 mediante la dieta aunque en este último caso los efectos fueron más pronunciados. La expresión hepática de *vtg* es inducida drásticamente en los peces expuestos a EE_2. Así, observamos una inducción de 650 veces con dosis bajas de EE_2 y tiempos largos de exposición y de aproximadamente 40.000 veces con dosis altas de EE_2 a ambos tiempos de exposición, 15 y 29 días.

Debido a que la respuesta inmunitaria innata es la primera línea de defensa frente a infecciones, cualquier efecto del EE_2 sobre ella podría alterar la susceptibilidad de los peces frente a la infección. En peces hay pocos estudios a este respecto y, por ejemplo, se ha observado que ejemplares de pez dorado expuestos a E_2 son más susceptibles a la infección por *Tripanosoma danilewskyi* (Wang y Belosevic, 1994). En este trabajo, ejemplares de dorada fueron simultáneamente tratados con EE_2 e inyectados con hemocianina, como antígeno, y con aluminio, como adjuvante, con el fin de precisar el impacto de la exposición a EE_2 en la respuesta inmunitaria de esta especie. De manera significativa se observa que el EE_2, tras 15 días de exposición, es capaz de inhibir de manera dosis dependiente la inducción de IL-1β observada en los

peces vacunados. Sin embargo, el EE_2 incrementa, ligeramente, dichos niveles de expresión después de la re-inmunización. Estos resultados están en consonancia con los efectos atribuidos a los estrógenos pudiendo mostrar efectos opuestos (Straub, 2007).

El impacto del EE_2 en la respuesta adaptativa fue evaluado analizando la presencia de IgM específica en el suero de los peces vacunados tras 15 días de una primera y una segunda inmunización. Como cabe esperar, los animales inmunizados mostraron unos mayores niveles de IgM específica frente al antígeno usado. Además, extrañamente, la modulación de IL-1β no resulta en una alterada producción de IgM específica en peces inmunizados. Este efecto es contrario al publicado en trucha arcoíris donde los niveles de IgM disminuyen tras una exposición *in vivo* con E_2 (Hou y col. 1999).

De manera interesante, se observa que ambos, el EE_2 y la inmunización, incrementan de manera significativa los niveles de RNAm de REα. Esto sugiere que el EE_2 podría aumentar la señalización de estrógenos en los leucocitos y que los leucocitos de riñón cefálico podrían incrementar su sensibilidad a los estrógenos durante el curso de la respuesta inmunitaria adaptativa. En consonancia con estos resultados, hemos descrito previamente que el tratamiento *in vitro* con E_2 induce la expresión de los genes que codifican para REα, REβ1 y REβ2 (Liarte y col. 2011a).

Ensayos *in vitro* han mostrado que el EE_2 modula algunas actividades inmunitarias de los leucocitos de riñón cefálico sin afectar por ello la viabilidad celular de las células tratadas. Se ha observado que el EE_2, por sí solo, no es capaz de modular la producción de ROS en leucocitos no estimulados mientras que tras la estimulación de estas células con VaDNA, concentraciones altas de EE_2 son capaces de inhibir la producción de ROS inducida por este PAMP, tras 16 horas de incubación. De manera similar, el EE_2 fue capaz de inhibir, de manera dosis dependiente, la capacidad fagocítica de los leucocitos tras 16 h de incubación. Efectos similares han sido encontrados previamente, ya que altas dosis de E_2 y EE_2 inhiben la fagocitosis en leucocitos de tilapia (Law y col. 2001), carpa (Yamaguchi y col. 2001) y dorada (Liarte y col. 2011a).

A continuación se analizó la capacidad del EE_2 de alterar el perfil de expresión de varios genes relevantes en el sistema inmunitario en MΦ, células clave en la regulación por estrógenos en la dorada, los cuáles expresan de manera constitutiva ERα

y son capaces de responder a E_2 (Chaves y col. 2007; Liarte y col. 2011a). Se observa que el EE_2 promueve principalmente un efecto anti-inflamatorio en MΦ de dorada, de manera contraria a lo observado con tratamiento *in vitro* con E_2 (Liarte y col. 2011a). Así, referente a los genes pro-inflamatorios analizados, el EE_2 fue capaz de incrementar, ligeramente, los niveles de RNAm de IL-1β, IL-6 y TNFα en MΦ no estimulados. En MΦ estimulados (activados con VaDNA), el EE_2 fue capaz de inhibir, de manera significativa, los niveles de RNAm de IL-1β y Cox-2 mientras que incrementa los de TNFα. Por otro lado, el EE_2 fue capaz de incrementar los niveles de expresión de la molécula anti-inflamatoria TGFβ1, la quimioquina IL-8 y de las enzimas implicadas en la renovación de la matriz extracelular, MMP9 y MMP3, en MΦ previamente estimulados. Estos datos sugieren que el EE_2 podría modular la movilización de fagocitos y la renovación tisular, resultado no sorprendente ya que administración exógena de E_2 (Chaves y col. 2007) y de EE_2 (Cabas y col. 2011) son capaces de inducir la movilización de la gónada desde el riñón cefálico hacia la gónada.

Para concluir, este capítulo demuestra que aunque el EE_2 altera *in vivo* la respuesta inmunitaria inducida por un reto inmunológico y también las actividades inmunitarias y el perfil de expresión de fagocitos de riñón cefálico *in vitro*, no hay evidencias de inmunosupresión usando concentraciones ambientales relevantes de EE_2 en un periodo de tiempo inferior a 2 meses. Además, y de acuerdo con la opinión de Kollner (Kollner, 2002), los efectos más claros de EE_2 sobre las actividades inmunitarias o la expresión de genes se observaron en los peces vacunados y células activadas.

3.4. La señalización por estrógenos, a través de un receptor de estrógenos asociado a proteína G, regula la activación de granulocitos en la dorada

En este último capítulo nos centramos en la caracterización funcional del receptor de estrógeno asociado a proteína G, GPER, identificado recientemente. Nuestro estudio se ha centrado en la importancia de este receptor en la inmunidad y, en concreto, en la biología de los GAs, equivalentes a los neutrófilos humanos (como ha sido previamente mencionado). Debido a que muchos de los efectos de los estrógenos no pueden ser explicados por la activación de los REs clásicos y a la clara implicación de los estrógenos en ciertos desórdenes inmunitarios, es importante investigar la relevancia de

GPER en la inmunidad en el sentido de abrir la posibilidad de diseñar nuevas dianas terapeúticas.

Los datos de expresión del GPER son muy escasos y solo en algunos trabajos la describen en tejidos reproductores y no reproductores (Mizukami y col. 2010). Nosotros observamos que, aunque el GPER es expresado en tejidos inmunitarios como el riñón cefálico y el bazo, la expresión es mucho más baja que la encontrada en hígado y testículo. Además, los niveles de RNAm del GPER fueron 4.3 veces superiores en fracciones celulares G7+ (enriquecidas en GAs) que en fracciones celulares G7- (fracciones carentes de GAs). De manera similar, la mayoría de GAs (región R1; alto tamaño y granularidad) se marcaron con un anticuerpo contra el GPER de manera dosis dependiente. Esta tinción fue específica ya que la pre-incubación del anticuerpo GPER con un péptido de bloqueo específico reduce este marcaje. El mismo análisis se realizó en la región R2 (células con bajo tamaño y granularidad, es decir, MO, Ly y células precursoras), y los resultados muestran un marcaje débil de estas células comparado con el observado en GAs, siendo también reducido por el péptido de bloqueo. Estos resultados fueron confirmados por western blot, en el que las células G7+ muestran una expresión más robusta de la proteína GPER que en las células G7-. Hasta donde conocemos, se sabe muy poco acerca de la expresión del GPER en células inmunitarias y hay solo un estudio que describe la expresión de GPER en una línea celular similar a neutrófilos, H60 (Blesson y Sahlin, 2012).

Tras la activación *in vitro* del GPER con el agonista específico, G1, no se producen cambios significativos en la expresión del GPER, ni de RNAm ni de proteína. En contraste con estudios donde el E_2 aumenta la expresión de REs en MΦ de dorada (Liarte y col. 2011a). Por otro lado observamos que la activación del GPER difícilmente modula la explosión respiratoria de los GAs pero si la disminuye tras 3 h de incubación. Por lo tanto, es tentador especular que la capacidad del E_2 (Liarte y col. 2011a) y del EE_2 (capítulo III de la presente tesis) de inhibir la producción de ROS de leucocitos de riñón cefálico, podría estar mediada, al menos en parte, a través de la señalización por el GPER.

A continuación evaluamos como la activación del GPER es capaz de alterar el perfil de expresión génica en GAs no estimulados. En primer lugar comprobamos que el GPER es funcional en GAs, como muestra la inducción significantiva en los niveles de

RNAm de *cfos*, un marcador de la activación de la ruta de señalización del GPER (Albanito y col. 2007), después del tratamiento con G1. Es de destacar, que como los GAs expresan GPER, pero no los REs nucleares, la activación del GPER es suficiente para inducir cFOS, como ha sido observado en células de tumor de mama negativas para REs (Albanito y col. 2008). Además observamos que la activación del GPER: (i) induce niveles altos de RNAm de IL-1β de manera muy rápida, los cuales son inhibidos a tiempos más largos de incubación, (ii) produce un incremento sustancial de los niveles de RNAm de PTGS2 e IL-10 y (iii) induce un incremento en los niveles de RNAm de PTGD2 tras 30 min y 3 h, pero disminuye los niveles de expresión tras 16 h. Curiosamente, aunque los efectos observados son similares a los observados en GAs no estimulados, la activación del GPER en GAs activados con VaDNA produce efectos insignificantes en la expresión de estos genes. Como se ha mencionado los GAs no expresan ninguno de los tres REs conocidos en dorada (Pinto y col. 2006; Liarte y col. 2011a), por lo que, nuestros datos sugieren que los efectos descritos en estas células principalmente serían mediados a través del GPER. Además, estos datos sugieren que, aunque los efectos de la activación del GPER en GAs son muy complejos, la señalización a través del GPER dirige la activación de los GAs a un fenotipo anti-inflamatorio y sugiere un papel importante de los estrógenos en la homeostasis de la respuesta inmunitaria a través de la señalización por el GPER.

Para explorar la ruta de señalización aguas debajo de la activación de GPER usamos un inhibidor farmacológico de PKA (H-89), un análogo de AMPc (dbcAMP) que activa PKA y un anticuerpo monoclonal contra la forma fosforilada (Ser133) del factor de transcripción CREB. La inhibición farmacológica de PKA resulta en la super-inducción de los niveles transcripcionales de PTGS2 en respuesta a G1, efecto que ha sido observado previamente con neutrófilos humanos y atribuido al complejo mecanismo de activación/desactivación de CREB (Cadieux y col. 2005), pero tuvo efectos insignificantes, si es el caso, en GAs activados con VaDNA. En contraste, el tratamiento con H89 no tuvo efectos evidentes sobre los niveles transcripcionales de IL-1β. Por otro lado, dbcAMP imitó la inducción de PTGS2 dependiente de la activación del GPER. Además, la activación de GPER promueve la fosforilación de CREB y de ATF-1 en GAs, efecto que también es imitado por dbcAMP. En conjunto, nuestros resultados sugieren que la inducción en la expresión de PTGS2 e IL10 podría estar mediada en parte por la activación de la ruta de señalización AMPc/PKA/CREB.

Nos dispusimos a evaluar la importancia del GPER en la respuesta inmunitaria innata *in vivo* exponiendo a los ejemplares de dorada a G1 *y* simultáneamente inmunizando con hemocianina y aluminio como adjuvante. Comprobamos que la activación del GPER *in vivo* no promueve una respuesta estrogénica evidente ya que ni el IGS ni la expresión de *vtg* se alteraron significativamente por la exposición a G1, en contraste con lo observado por la exposición *in vivo* a EE_2 mediante la dieta o agua de baño, donde si se produce una respuesta estrogénica significativa (Cabas y col. 2012; capítulo II y III de la presente tesis). Estos resultados confirman la especificidad del agonista G1 sobre el GPER en dorada. Además, como ocurre *in vitro*, ni el tratamiento con G1 ni la inmunización fueron capaces de modular *in vivo* la expresión del GPER, ni de alterar el porcentaje, ni la intensidad de células GPER positivas en el riñón cefálico en ninguno de los tiempos analizados, no incrementando por ello la sensibilidad a estrógenos, como si lo hizo el EE_2 (capítulo III de la presente tesis).

De manera importante, la activación del GPER *in vivo* inhibe la explosión respiratoria de leucocitos de riñón cefálico en peces no vacunados pero no tuvo efecto en los animales vacunados, apoyando aún más la idea de un papel prominente de la señalización del GPER en la homeostasis de la respuesta inmunitaria, que es en gran parte al margen de su activación durante la infección. De manera similar ha sido publicado que G1 es capaz de inhibir la expresión de TLR4 en MΦ limitando así su capacidad para reconocer LPS (Rettew y col. 2010). Este resultado, junto con la disminución en la expresión de IL-1β y el aumento de IL-10 en los peces expuestos a G1, apoya que el GPER promueve un efecto anti-inflamatorio, como se ha observado *in vitro.* Resultados similares se han obtenido en ratones, en los que G1 fue capaz de inducir la expresión de IL-10 en las células Th17, lo que sugiere que el GPER puede estar implicado en la capacidad de los estrógenos para suprimir enfermedades autoinmunes (Brunsing y col. 2011). Sorprendentemente, la señalización por GPER modula, ligeramente, la inmunidad adaptativa. Por ello será interesante investigar la relevancia del GPER en la inmunidad adaptativa de mamíferos así como el papel de las diferentes poblaciones de células inmunitarias en esta respuesta.

Como conclusión a este capítulo decir que hemos descrito por primera vez que los GAs, equivalentes funcionales a los neutrófilos humanos, expresan un GPER funcional y que su agonista selectivo, G1, promueve un efecto anti-inflamatorio tanto *in vitro* como *in vivo*, y modula aunque débilmente la inmunidad adaptativa. De manera

interesante, todos los efectos descritos los hemos encontrado en células en estado no activado y en peces no vacunados en contraste con los efectos de otros estrógenos ya que son solo aparentes en un estado activado, de acuerdo con el postulado de Kollner (Kollner, 2002). Además ya que los estrógenos juegan un papel relevante en desordenes autoinmunes, nuestro estudio sugiere que el GPER podría representar una diana terapéutica.

4. CONCLUSIONES

Los resultados de este trabajo conducen a las siguientes conclusiones:

1. La exposición *in vivo* a EE_2 promueve una respuesta estrogénica evidente caracterizada por un descenso en el IGS, alteración en los niveles séricos de E_2, T y 11KT y una inducción en la expresión hepática de *vtg*. Estos efectos son más evidentes cuando el EE_2 se administra en la dieta que cuando se administra en el agua del baño. Además, estos efectos dependen, ligeramente, del estado de desarrollo de los ejemplares.

2. El EE_2 incrementa la sensibilidad a estrógenos en gónada y en riñón cefálico ya que aumenta la expresión del REα.

3. La exposición *in vivo* a EE_2 interrumpe la espermatogénesis e induce en el testículo la morfología característica de la etapa de post-puesta. Aunque los túbulos seminíferos continúan llenos de espermatozoides se observa una reducción en el volumen y motilidad de los espermatozoides. Además, el EE_2 genera un proceso pro-inflamatorio en la gónada, promoviendo una infiltración masiva de leucocitos y un incremento en la expresión de citoquinas y moléculas implicadas en el reconocimiento y presentación de antígenos.

4. El EE_2 disminuye la capacidad de los ejemplares de responder a un estímulo inmunológico *in vivo* ya que inhibe la producción de citoquinas pro-inflamatorias tras una inmunización aunque no se comporta como inmunosupresor. Además, el EE_2 inhibe *in vitro* las actividades inmunitarias de leucocitos de riñón cefálico y dirige hacia un fenotipo anti-inflamatorio en macrófagos.

5. EL GPER se expresa en tejidos reproductores e inmunitarios de dorada y su expresión no es modulada por su activación.

6. Los granulocitos acidófilos son las células del riñón cefálico que tienen un mayor nivel de expresión de GPER. Éstos expresan un GPER funcional cuya activación *in vitro* dirige, de manera general, hacia un fenotipo anti-inflamatorio. Dichos efectos

son regulados, en parte, por la activación de la ruta de señalización AMPc/PKA/CREB.

7. La activación de GPER no promueve una respuesta estrogénica aunque provoca, en general, un efecto anti-inflamatorio y modula ligeramente la respuesta inmunitaria adaptativa.

ANEXXES

1. Other publications/collaborations related to the thesis

Liarte S**, Cabas I**, Chaves-Pozo E, Arizcun M, Meseguer J, Mulero V, García-Ayala A. Natural and synthetic estrogens modulate the inflammatory response in the gilthead seabream (*Sparus aurata* L.) through the activation of endothelial cells. *Mol Immunol*. 2011 Sep; 48(15-16):1917-25.

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2. Participation in (inter) national conferences

Cabas I, Liarte S, Meseguer J, Mulero V, García-Ayala A. **17α-ethinylestradiol modulates activiation of the professional phagocytes of the bony fish gilthead seabream.** XIII Congreso de la Sociedad Española de Biología Celular. Comunicación oral. Murcia, España. 16 al 18 de diciembre de 2009.

Cabas I, Liarte S, Meseguer J, Mulero V, García-Ayala A. **17 alpha-ethynylestradiol *in vivo* treatment alters inflammatory gene expression profile in the gilthead seabream (Sparus aurata L.).** 9th International congress on the biology of fish. Póster. Barcelona, España. 5 al 9 de julio de 2010.

Cabas I, Liarte S, García-Alcázaz A, Meseguer J, Mulero V, García-Ayala A. **17α-ethinylestradiol alters *in vivo* and *in vitro* the cytokine and chemokine expression profile of professional phagocytes from the bony fish gilthead seabream**. 8º Congreso de la Asociación Ibérica de Endocrinología Comparada. Póster. Madrid, España. 5 al 7 de septiembre de 2011.

Cabas I, Liarte S, Chaves-Pozo E, García-Alcáraz A, Meseguer J, Mulero V, García-Ayala A. International Symposium on Genomics in Aquaculture 2011. **Dietary intake of 17α-ethinylestradiol disrupts spermatogenesis on males of the hermaphrodite bony fish gilthead seabream.** Póster. Heraklion, Creta, Grecia. 14 al 17 de septiembre de 2011.

Cabas I, Rodenas M.C, Arizcun M, Meseguer J, Mulero V, García-Ayala A. **G1, a selective GPR30 agonist, alters *in vivo* the immune response of gilthead seabream.** 26th Conference of European Comparative Endocrinologists. Póster (Tercer premio). Zurich, Suiza. Del 21 al 25 de agosto de 2012