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Sex Steroids Modulate Fish Immune Response

Elena Chaves-Pozo[1], Isabel Cabas[2] and Alfonsa García-Ayala[2]
[1]*Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (IEO), Carretera de la Azohía s/n. Puerto de Mazarrón, Murcia*
[2]*Department of Cell Biology and Histology, Faculty of Biology, University of Murcia, Murcia*
Spain

1. Introduction

For some time behavioural and ecological studies have suggested that sex steroid hormones regulate several immune processes in fish. For example, the immunocompetence handicap hypothesis relates the heritability of parasite resistance with secondary sexual ornaments, which are determined and maintained by androgens. Such ornaments are probably a good indicator to potential mates of genetic resistance to infections (Dijkstra et al., 2007; Roberts et al., 2004). Among vertebrates, the prevalence and intensity of parasitic infections is higher in males than females (Klein, 2004). Some fish species show altered sex steroid hormones levels upon parasite infection. The main alterations recorded upon infection are decreases in androgen, estrogen and vitelogenin serum levels (Hecker & Karbe, 2005). For example, during an infective period of vibriosis, silver seabream showed gradually increasing testosterone serum levels, whereas serum estradiol levels significantly decreased at an early stage of infection and remained low until death. This process coincided with increasing macrophages phagocytic activity (Deane et al., 2001). Such field studies prompted immunologists to try to establish how sex steroid hormones are able to alter the functions of the circulating leukocytes. In fish, most existing information on reproductive-immune interactions deals with the modulation of immune responses by circulating hormones, including cortisol, growth hormone, prolactin and reproductive hormones and some proopiomelanocortin-derived peptides (Engelsma et al., 2002; Harris & Bird, 2000). Although the exact effect of these endocrine mediators depends on the species, in general, they are known to modulate immune responses by integrating the activities of all the systems. In this way they help to adapt the organism to its environment (Lutton & Callard, 2006).

From a reproductive biology point of view, the leukocytes located in mammalian gonads orchestrate important reproductive physiology processes, including gametogenesis and steroidogenesis. A long time has passed since leukocytes were first described in the gonad of teleosts. Since them, several types of leukocytes have been described in the testis of different teleost species using light and electron microscopy. Moreover, differences in the number and localization of leukocytes within the testis have also been observed during the different stages of the reproductive cycle (Besseau & Faliex, 1994; Billard, 1983; Bruslé-Sicard & Fourcault, 1997; Lo Nostro, 2004; Scott & Sumpter, 1989). 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; Loir et al., 1995; Scott & Sumpter, 1989; Shrestha & Khanna, 1976). Although macrophages, granulocytes and lymphocytes have been described in the testis of some sparid fish, only macrophages have been shown to be phagocytic cells (Besseau & Faliex, 1994; Bruslé-Sicard & Fourcault, 1997; Micale et al., 1987).

The gilthead seabream (*Sparus aurata* L.) is a protandrous hermaphrodite seasonal breeding teleost with a bisexual gonad (Figure 1) 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. The specimens undergo sex change during the second or third year of life, depending on the natural environment of the populations studied (Lasserre, 1972). Our previous studies performed in the western Mediterranean area demonstrated that gilthead seabream are males during the first and second reproductive cycles 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). The reproductive cycle of males is divided into four gonad stages: gametogenic activity, spawning, post-spawning and resting. 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).

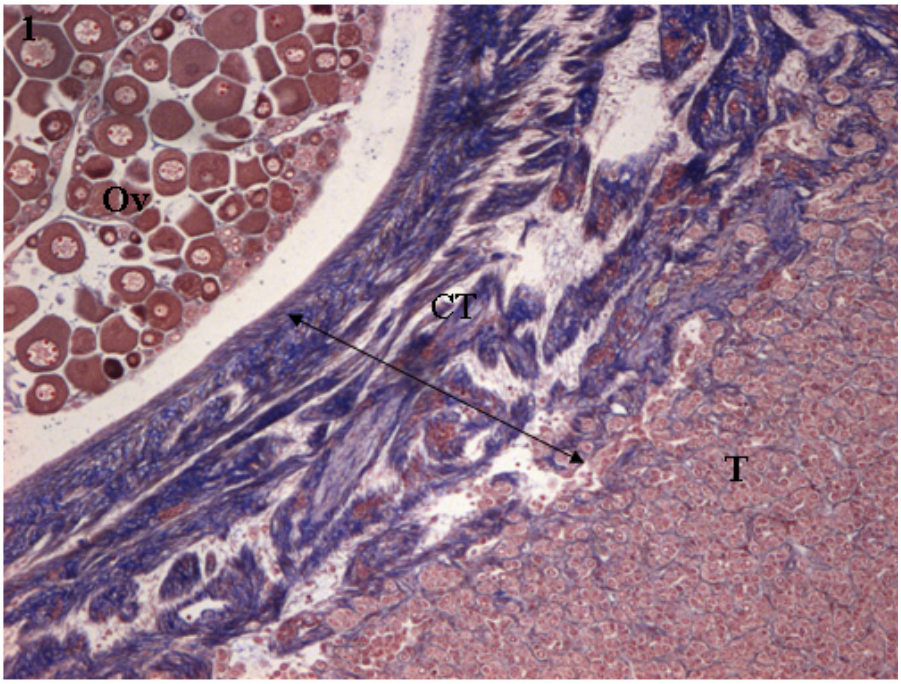

Fig. 1. Section of the gonad of gilthead seabream (*Sparus aurata* L.) during the male phase. The bisexual gonad is formed of a testis and an ovary separated by a thin layer of connective tissue. The testis is constituted by seminiferous tubules which are in the initial stage of spermatogenesis and the ovary is occupied with pre-vitellogenic ovocytes. T, testis; Ov, ovary; CT, connective tissue. (Mallory trichromic) Magnification x 10.

During the first reproductive cycle, 11-ketotestosterone and testosterone, the main androgens in fish, play different and specific roles in the testicular physiology as they peak at different stages of the reproductive cycle. Moreover, the profiles of testosterone serum levels during the second reproductive cycle demonstrated that this androgen is not essential to the testicular regression process that occurs during this cycle. In contrast, changes in 17β-estradiol serum levels suggest that this hormone orchestrates the testicular regression process during both reproductive cycles. Moreover, the data suggest that there is a threshold level of 17β-estradiol that determines the initiation of ovarian development during the second reproductive cycle without promoting complete feminization (Chaves-Pozo et al., 2008a).

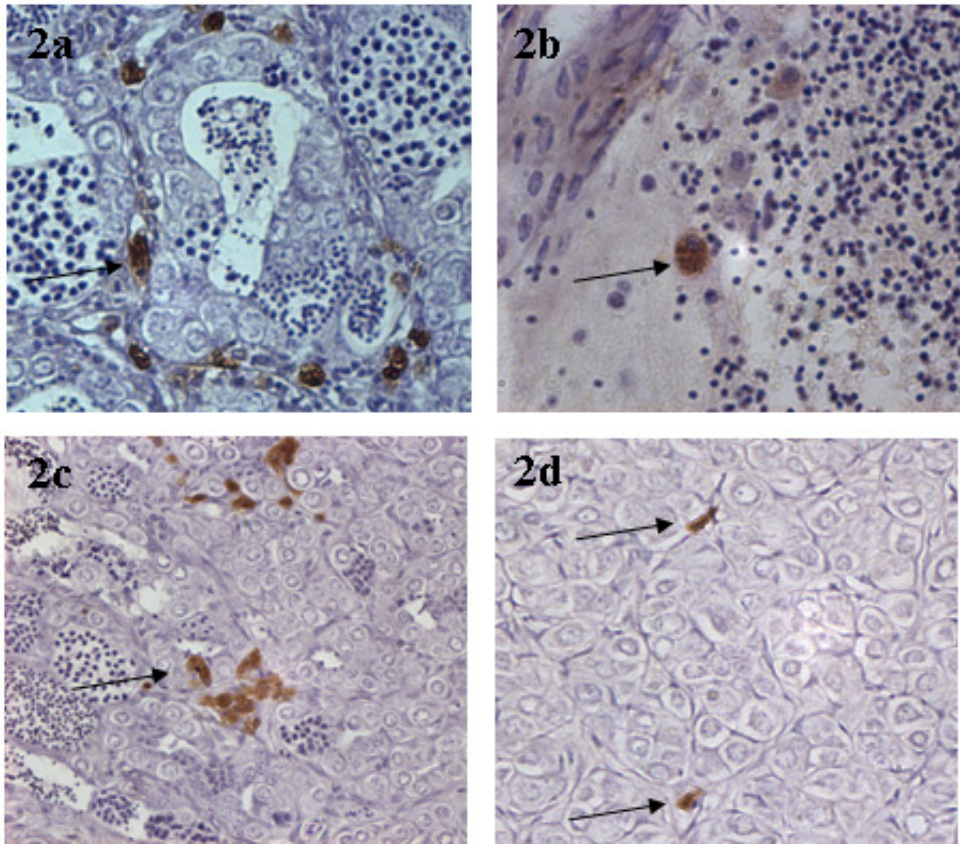

Fig. 2. Sections of the testis of gilthead seabream in the spermatogenesis (a), spawning (b), post-spawning (c) and resting (d) stages immunostained with a monoclonal antibody specific to gilthead seabream acidophilic granulocytes (Sepulcre et al., 2002). Acidophilic granulocytes (arrows) are seen in the blood vessels (a) during spermatogenesis, in the lumen of the tubules between the spermatozoa (b) and in the seminal epithelium in contact with germ cells (c) during spawning and post-spawning and in the interstitial tissue (d) during resting. Magnification x 400.

Few studies have dealt with the presence of leukocytes in the gonad of teleosts, their functions and the molecular pathways that regulate them. However, our studies in recent years have suggested that sex hormones might be key regulators of leukocyte functions in the gonad. For example, a massive infiltration of leukocytes, mainly acidophilic granulocytes (Figure 2), is orchestrated by gonadal factors including sex steroid hormones during post-spawning and testicular involution stages (Chaves-Pozo et al., 2003, 2005a, 2005b, 2007). The immune cells are produced in the head-kidney, the main haematopoietic organ in fish. However, when the acidophilic granulocytes infiltrate the testis, they show heavily impaired reactive oxygen intermediate production and phagocytic activity (hardly 1% of the testicular acidophilic granulocytes are able to phagocytise) (Figure 3) while the production of interleukin-1β (IL-1β) is sharply induced (Chaves-Pozo et al., 2003, 2005b, 2008a).

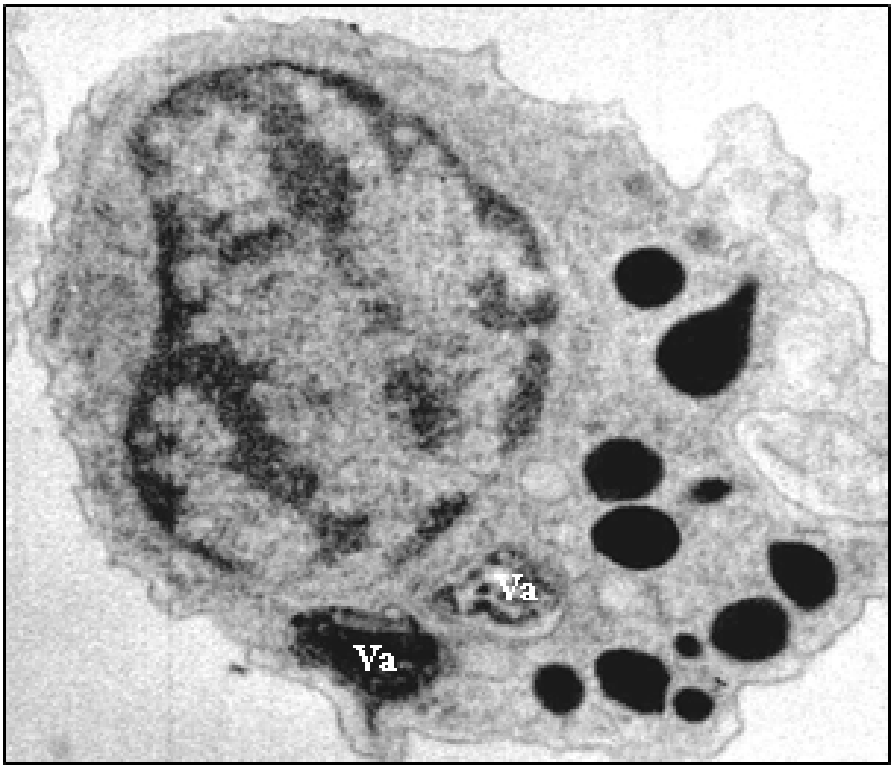

Fig. 3. An electron micrograph showing a testicular acidophilic granulocyte with the typical ultrastructure of acidophilic granulocytes and two phagosomes containing *Vibro anguillarum* cells (Va). Magnification x 5000.

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., 2005b). Moreover, 17β-estradiol and testosterone seem to be related with the infiltration of acidophilic granulocytes and probably with the magnitude of the infiltration since both hormones peak when the infiltration of these cells into the gonad occurred (Chaves-Pozo et

al., 2008a). Moreover, the infiltration of acidophilic granulocytes was correlated with an increase in the expression of gonadal aromatase, the enzyme that transforms testosterone to 17β-estradiol. Such expression was seen to remain high during the period that acidophilic granulocytes are present in the gonad (Chaves-Pozo et al., 2005a, 2008b; Liarte et al., 2007). Moreover, experimentally induced increases of 17β-estradiol serum levels in spermatogenically active males triggered the migration of acidophilic granulocytes to the gonad in a way that resembles an inflammatory process (Chaves-Pozo et al., 2007). In the adult gilthead seabream gonad, macrophages and lymphocytes have also been observed in the interstitial tissue (Chaves-Pozo et al., 2008a; Liarte et al., 2007). However, the number of testicular macrophages remains steady throughout the reproductive cycle when the specimens are males, while no data related to lymphocytes are available (Chaves-Pozo et al., 2008a). Acidophilic granulocytes and B lymphocytes (Figure 4) also infiltrated the gonad and were located in the interstitial tissue and among the spermatozoa when fish were treated with an estrogenic endocrine disruptor, 17α-ethynilestradiol. This pharmaceutical compound, used for oral contraceptives and hormone replacement therapy, has a widespread presence in the aquatic environment (Ternes et al., 1999) and may reach concentrations of 0.5 to 62 ng/l in European seawage and surface waters (Hinteman et al., 2006; Johnson et al., 2005; Kuch and Ballschmiter, 2000).

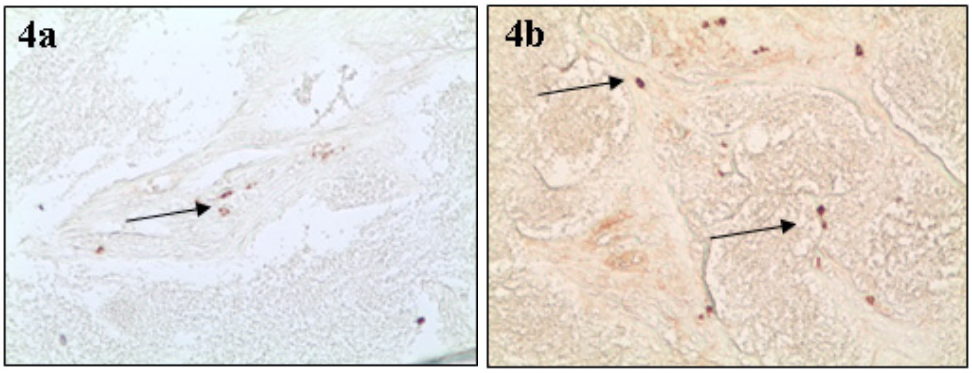

Fig. 4. Sections of the gilthead seabream testis in the spermatogenesis stage of specimens control (a) and specimens treated with 5 μg of 17α-ethynilestradiol/g food (b) immunostained with a specific anti-gilthead seabream IgM serum. B lymphocytes can be seen in the interstitial tissue of the testis, the numbers slightly increasing after 17α-ethynilestradiol treatment. Magnification x 200.

Testosterone administration *in vivo* modulates particular components of the physiological response of professional phagocytes such as respiratory burst, but does not alter their phagocytic activity. Testosterone is also able to regulate the gene expression profile of immune related molecules in head-kidney and other immune competent organs. This effect is characterized by a strong pro-inflammatory activation in the first week, after which it changes into an anti-inflammatory response (Águila et al., 2010).

These observations which, taken together, suggest that the presence of immune cells and cytokines in the gonad guarantees and modulates the reproductive functions (Figure 5),

prompted us to investigate the role of 17β-estradiol and testosterone in immune cell functions and in the regulation of the inflammatory response.
In this context, we studied the effects of estrogens and androgens on the immune system responses, bringing together the views of both immunologists and reproductive biologists. An *in vitro* approach was used to determine which types of leukocytes are able to respond to sex steroid hormones.

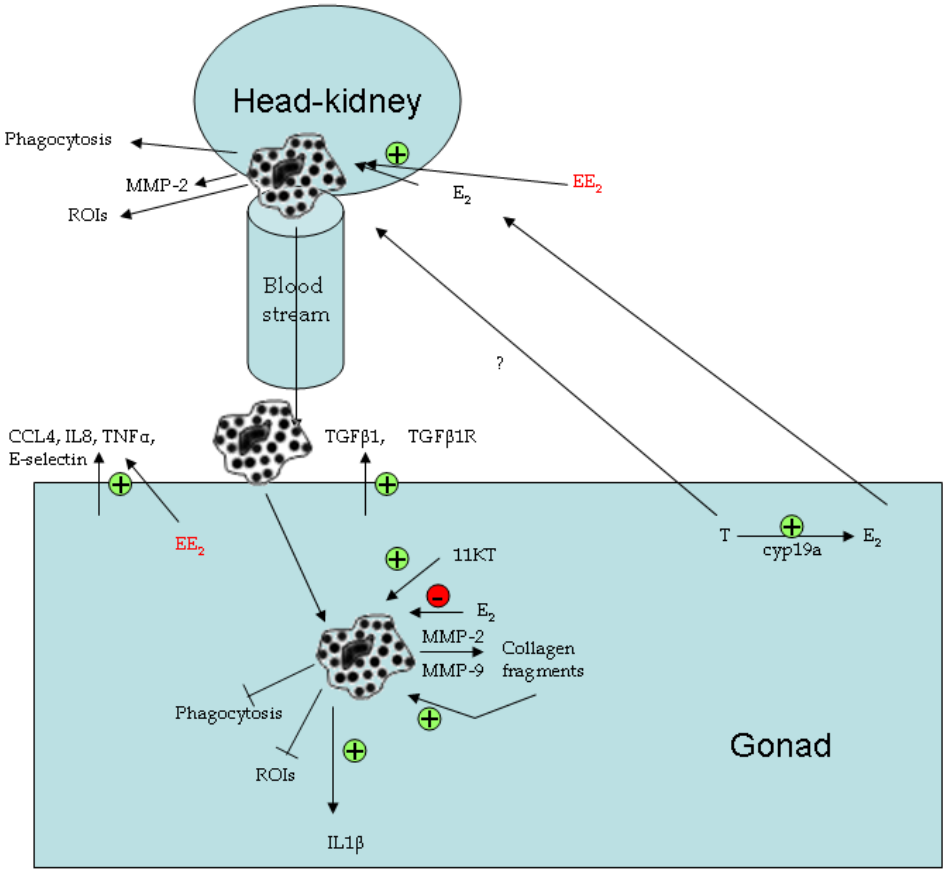

Fig. 5. Molecules involved in the mobilisation of acidophilic granulocytes from the head-kidney to the testis, as deduced from our *in vivo* and *in vitro* data (Chaves-Pozo et al., 2003, 2005 a,b, 2007, 2008a,b,c; Cabas et al., 2010). Although further studies are needed, the data clearly identify estrogens (17β-estradiol and 17α-ethynilestradiol) as key modulators of this process. MMP, matrix metalloproteinase; ROIs, reactive oxygen intermediates; E_2, 17β-estradiol; EE_2, 17α-ethynilestradiol; CCL4, CC chemokine-like 4 ; IL, interleukin; TNFα, tumour necrosis factor α; E-selectin, leukocyte adhesion molecule E-selectin; TGFβ1, transforming growth factor β1; TGFβ1R, transforming growth factor β1 receptor; 11KT, 11-ketotestosterone; cyp19a, P450 aromatase.

2. Sex steroid hormones as regulators of the immune response

In mammals, androgens and estrogens exert their main long-term effects on cell growth, cell differentiation and cell functions through intracellular androgen receptors (AR) and estrogen receptors (ER), ERα and ERβ, respectively, all of which belong to the nuclear receptor superfamily (Evans & Bergeron, 1988). These AR and ER are ligand-inducible transcription factors that cause the activation or repression of genes (Beato & Klug, 2000; Kumar & Tindall, 1998). 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 17β-estradiol on the immune system (Straub, 2007). The main effect 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) and stimulating the secretion of tumor necrosis factor (TNF) (Janele et al., 2006). Furthermore, using ER knock-out mice, researchers have shown that ER participates in the stimulation of interleukin (IL)-10 and immunoglobulin (Ig) M 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, 17β-estradiol also has an inhibitory effect on bone resorption and the suppression of inflammation in several animal models of chronic inflammatory diseases (Straub, 2007). Unlike estrogens, androgens are thought to be exclusively immunosuppressive in mammals. For example, androgens have a negative effect on the expression of inflammatory cytokines, increase apoptosis in human monocytes/macrophages, and inhibit lymphocyte proliferation (Cutolo & Straub, 2009; Cutolo et al., 2005; Lehmann et al., 1988).

In teleosts, the large number of different species and the genome duplications that have occurred during their phylogeny make it very difficult to assess the number of AR and ER existing in each specie. Depending on the species studied, three or four different ER 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 (Iwanowicz & Ottinger, 2009; Nagler et al., 2007). In order to determine whether immune tissues are potential targets for estrogens, several studies have looked at the expression of ER 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). In the gilthead seabream, *in vitro* long term treatment of head-kidney leukocytes with 17β-estradiol revealed a suppressive effect on the production of reactive oxygen intermediates and the *Vibrio anguillarum* DNA (VaDNA)-stimulated production of IL-1β (Chaves-Pozo et al., 2003). However, short term treatment with higher concentrations of 17β-estradiol inhibited the phagocytic capability, while the percentage of phagocytic cells and the VaDNA-stimulated production of reactive oxygen intermediates and cell migration activity remained steady (Liarte et al., 2011b). In the case of AR, most vertebrates are believed to have one active form of nuclear AR with high specificity for the androgen 5α-dihydrotestosterone, whereas there appear to be two subtypes of AR in some teleosts, ARα and ARβ. These are differentially expressed in tissues and show high affinity for both testosterone and 11β-hidroxytestosterone (review in Rempel & Schlenk, 2008). However, little is known about the expression of these AR in fish immune tissues, although in mammalian models AR are present in liver, spleen and thymus (Butts et al., 2011).

There is increasing evidence supporting the transcription-independent non-genomic actions of steroid hormones, including testosterone and 17β-estradiol (Christ et al., 1997; Falkenstein et al., 2000). For example, mammalian mast cells, T and B cells and macrophages shows membrane AR and membrane ER (Benten et al., 1998, 2001, 2002; Zaitsu et al., 2007). A membrane ER (Pang et al., 2008) and a membrane AR (review in Thomas et al., 2006) have recently been cloned and characterized in atlantic croaker, although nothing is known about membrane AR in fish immune tissues. The complexity of the way in which sex steroid hormones act in fish through membrane and intracellular receptors, as well as the complexity of the systemic and gonadal immune responses and the several cell types involved, prompted researchers to characterize sex steroid hormone receptors and the effects of their ligands in purified immune cells and cell lines. However, since each cell type has its own response pattern these issues will be dealt with separately.

2.1 Macrophages

Macrophages are ubiquitous cells that play a central role in the innate immune response through the secretion of inflammatory cytokines, such as IL-1β and TNFα, the production of cytotoxic reactive oxygen intermediates, and the secretion of leukostatic factors and other regulatory molecules. They are also important accessory cells for many other immune responses. In addition, during development, these cells are thought to have a trophic role through their remodelling capabilities and ability to produce cytokines. Interestingly, whereas a similar pattern of functioning has been demonstrated for macrophages in different tissues (Guillemin & Brew, 2004; Laskin et al., 2001; Stout & Suttles, 2004), testicular macrophages and their functions are largely determined by the local environment (Hedger, 1997, 2002), including not only cytokines and chemokines, but also steroid hormones. In mammals, it has been known for many years that ER are expressed in monocytes (Cunningham & Gilkeson). However, their response to estrogens and the predominance of ERα or ERβ expression appear to be dependent on their stage of differentiation. For example, Mor et al. (2003) demonstrated that monocytes express more ERβ and macrophages express more ERα. The behaviour of 17β-estradiol functions in mammalian macrophages has been described as double-edge-sword (depending on 17β-estradiol concentration). Thus, lower 17β-estradiol concentrations stimulated IL-1β production, whereas higher concentrations inhibited lipopolysaccharide (LPS)-induced TNFα production. This dichotomous effect of 17β-estradiol on IL-1β and TNFα at high and low concentrations is most probably due to inhibition of NF-κB at high concentrations (review in Straub, 2007).

Gilthead seabream macrophages constitutively express only the ERα gene, although stimulation with VaDNA drastically up-regulates the expression of ERα, ERβ1 and ERβ2 genes, suggesting that the immune system is able to increase its sensitivity to 17β-estradiol during development of the immune response (Liarte et al., 2011b). In long-term leukocyte cell lines of monocytes/macrophages from channel catfish, the expression of both ERα and ERβ has been described (Iwanowicz & Ottinger, 2009). Although evidence conclusively demonstrates that fish leukocytes express ER genes, the literature in this respect does not deal with the possible differential roles of the two ERβ genes (ERβ1 and ERβ2) present in fish. Our data in the gilthead seabream demonstrate for the first time that ERβ1 and ERβ2 are differentially regulated in macrophages. Thus, ERβ1 gene expression is only induced by VaDNA and its VaDNA-induced expression is slightly increased by 17β-estradiol, in

contrast to ERβ2 gene whose expression is induced by both stimuli, which, moreover, have a synergic effect on ERβ2 gene expression (Liarte et al., 2011b).

The biological effect of 17β-estradiol on fish head-kidney macrophages is mainly anti-inflammatory, although controversial data were observed depending on whether the studies were *in vivo* or *in vitro*. Intra-peritoneal injections of 17β-estradiol in common carp inhibit phagocytosis and the production of reactive oxygen intermediates and reactive nitrogen intermediates by head-kidney macrophages in a dose-dependent manner (Watanuki et al., 2002). However, upon *in vitro* treatment, these head-kidney macrophages only showed impaired phagocytic capability (Yamaguchi et al., 2001) and, in goldfish macrophages, 17β-estradiol inhibited the percentage of phagocytic cells (Wang & Belosevic, 1995). In the European flounder, microarray studies have revealed that 17β-estradiol suppresses immune system-related transcripts in liver (Williams et al., 2007). In rainbow trout, 17β-estradiol repressed the acute phase immune response genes (Tilton et al., 2006), as occurs in mammalian macrophages (Kramer & Wray, 2002). However, in gilthead seabream macrophages, 17β-estradiol up-regulates some genes coding for key immune molecules, including inflammatory and anti-inflammatory molecules, innate immune receptors, molecules related to leukocyte infiltration, matrix metalloproteinases (MMP) and the anti-viral molecule *Myxovirus* (*influenza*) resistance protein (Mx). Moreover, the soluble factors produced by those 17β-estradiol-stimulated macrophages modify the immune functions of head-kidney leukocytes (Liarte et al., 2011b), suggesting that the soluble factors produced by testicular macrophages in response to 17β-estradiol contribute by blocking the phagocytic activity of testicular acidophilic granulocytes (Chaves-Pozo et al., 2005b). A suppression subtractive library was constructed to isolate and identify mRNA species up-regulated by a supra-physiological dose of 17β-estradiol (50 ng/ml) to macrophages. Interestingly, this showed that 4% of up-regulated genes are related with the immune response, 6% with the stimulus response and 0.5% with physiological interactions between different organism categories, all of them probably involved in the interaction of immune cells with the immune stimulus. Although the number of identified genes within these categories was relatively low, other well-represented subcategories such as these related with biological regulation could contain genes whose functions may influence the behaviour of macrophages and thus affect their ability to respond to an immunological challenge upon exposure to estrogens (Liarte et al., 2011a; Xia & Yue, 2010).

Although less data are available for AR than for ER in mammalian species, several studies have demonstrated that testosterone alters macrophage functions in a complex manner, since it has both pro-inflammatory and anti-inflammatory effects. For example, wound healing is impaired in males, especially the elderly, which has been directly linked to a pro-inflammatory action of testosterone on tissue macrophages in the skin (Ashcroft & Mills, 2002). Moreover, castration increased macrophage-mediated damage at sites of injury in the skin, suggesting an anti-inflammatory role for testosterone (Ashcroft & Mills, 2002). Testosterone also inhibits inducible nitric oxide synthase and nitric oxide production in a mouse macrophage cell line (Friedl et al., 2000). The expression of AR in microglia, the brain macrophage, increases after injury and indicates that the innate immune cells of the brain may be modulated by androgens (García-Ovejero et al., 2002). Other data indicate that 5α-dihydrotestosterone acts as an anti-inflammatory agent and depresses both nitric oxide and TNFα production in a dose-dependent fashion. However, testosterone treatment of microglia and peritoneal macrophages increased supernatant nitrite levels, suggesting a pro-inflammatory effect (Brown & Angel, 2005).

In fish, the data obtained show that androgens are also able to modulate the immune system responses. In common carp, intraperitoneal injections of 11-ketotestosterone inhibit phagocytosis and the production of reactive oxygen intermediates and reactive nitrogen intermediates by head-kidney macrophages in a dose-dependent manner (Watanuki et al., 2002). However, *in vitro* studies with head-kidney macrophages have demonstrated that this hormone inhibits phagocytosis and the production of reactive nitrogen intermediates and has no effect on the production of reactive oxygen intermediates (Yamaguchi et al., 2001). Interestingly, although gilthead seabream macrophages do not express the AR at a level detectable by real time polymerase chain reaction, both testosterone and 11-ketotestosterone up-regulated different immune genes, such as immune receptors and pro-inflammatory cytokines, and down-regulated the anti-inflammatory cytokine, transforming growth factor (TGF) β (Águila et al., 2011). Taking into account the complexity of sex steroid hormone signalling through intracellular and membrane receptors and sex steroid hormone conversion through transformation in other derivatives (such as reduced derivatives or even 17β-estradiol) and bearing in mind that both testosterone and 11-ketotestosterone alter the macrophage gene expression and functions analyzed, it can not be discounted that macrophages convert testosterone into 11-ketotestosterone or another molecule capable of signalling through other receptors in this cell type. In this sense, mammalian macrophages lack AR but are able to respond to androgens through a membrane AR that triggers a Ca^{2+} influx (Benten et al., 2004). Moreover, mammalian testicular macrophages have a steroidogenic capability as they are able to produce and secrete 25-hydroxycholesterol, which affects Leydig cell steroidogenesis (Hales, 2002). In light of the above, further studies are needed to complete our understanding of the effect of androgens on fish innate immunity and macrophages.

2.2 Acidophilic granulocytes

The acidoplilic granulocytes of gilthead seabream display some functions similar to human neutrophils despite their opposite staining pattern. In brief, they are the most abundant circulating granulocytes and are recruited from the head-kidney to the site of inflammation (Chaves-Pozo et al., 2004, 2005c), where they attach themselves to, internalize and kill bacteria through the production of reactive oxygen intermediates (Chaves-Pozo et al., 2004, 2005c; Meseguer et al., 1994; Sepulcre et al., 2002). However, they also show a monocyte/macrophage-like behaviour as they are able to specifically target a tissue and respond to physiological stimuli by displaying modified functions, as do the monocytes/macrophages of mammals (Chaves-Pozo et al., 2005c; Stout & Suttles, 2004). In fact, gilthead seabream acidophilic granulocytes infiltrate the testis in a way that resembles an inflammatory process triggered by physiological stimuli, and their main activities are strongly inhibited by the testicular microenvironment in order to preserve reproductive functions (Chaves-Pozo et al., 2005b). Previous data showed that 17β-estradiol is related *in vivo* with the mobilization of acidophilic granulocytes from the head-kidney to the gonad and probably with the degree of this infiltration (Chaves-Pozo et al., 2007, 2008a). Interestingly, neither testicular nor head-kidney acidophilic granulocytes express any of the ER known in the gilthead seabream (Pinto et al., 2006; Liarte et al., 2011b). However, studies performed with conditioned medium from 17β-estradiol-treated macrophages suggest that some, but not all, the acidophilic granulocyte functions modified by the testicular microenvironment might be regulated by the factors produced by 17β-estradiol-treated

macrophages (Liarte et al., 2011b). In this sense, there is evidence that suggests a pro-inflammatory role for 17β-estradiol in the gilthead seabream, since it is able to stimulate *in vivo* specific leukocyte migration and promote acidophilic granulocytes infiltration into the gonad (Chaves-Pozo et al., 2007). However, *in vitro*, 17β-estradiol failed to promote chemiotaxis in purified acidophilic granulocytes, although it is produced a positive migration of leukocytes when head-kidney suspensions were exposed to 17β-estradiol (Liarte et al., 2011b).

In so far as androgens are concerned, acidophilic granulocytes constitutively express AR, the expression of which is modified by 11-ketotestosterone and testosterone, but only when the cells are co-stimulated with VaDNA (Águila et al., 2011). The effects of 11-ketotestosterone and testosterone on acidophilic granulocytes differ: while testosterone increased, 11-ketotestosterone decreased the expression of IL-1β and toll-like receptors (TLRs), although both up-regulated the VaDNA-induced expression of IL-1β (Águila et al., 2010).

2.3 Lymphocytes

T and B lymphocytes are the acknowledged cellular pillars of adaptative immunity. T cells are primarily responsible for cell-mediated immunity, while B lymphocytes are responsible for humoral immunity, but, in conjunction with other cell types, both mediate effective adaptive immunity (Pancer & Cooper, 2006). Recently, in long-term leukocyte cell lines of T-cells and B-cells from channel catfish, the differential expression of ERα and ERβ was described. Thus, ERα is expressed in both cell types, while only T-cells express ERβ2 (Iwanowicz & Ottinger, 2009). In the gilthead seabream, lymphocytes only express the ERα gene (Liarte et al., 2011b). In mammals, however, B lymphocytes express both ERα and ERβ genes, while there is debate as to whether or not T cells contain classical nuclear ER (Benten et al., 1998; Harkonen & Vaananen, 2006). *In vitro* functional assays demonstrated that 17β-estradiol stimulates lymphocyte proliferation (Cook et al., 1994).

To determine whether fish lymphocytes respond to androgens, the classical chemical characterization of AR was performed in salmonid lymphocytes (Slater et al., 1995). In these species, 11-ketotestosterone inhibits lymphocyte proliferation, while testosterone reduces the number of antibody-producing cells and acts with cortisol to produce a greater inhibitory effect (Cook et al., 1994; Slater & Schreck, 1993).

2.4 Endothelial cells

Leukocyte recruitment is an early and pivotal event in any inflammatory response. Since gilthead seabream acidophilic granulocytes are recruited from the blood stream into the testis in a process that might be orchestrated by 17β-estradiol (Chaves-Pozo et al., 2005b, 2007, 2008a), we investigated the role of the endothelium in this process. Leukocyte-endothelial interactions are a special case of cell sorting, in which the endothelium discriminates between circulating leukocytes in order to select cells for transmigration into surrounding tissue (Ebnet et al., 1996a). Endothelial cells play a singular role in this process, receiving information from the underlying tissue and transforming it into information that can be read rapidly by the passing leukocytes (Ebnet et al., 1996b). Accumulated evidence on mammalian models of cardiovascular disease points to the prominent role of estrogens in the ability of endothelial cells to trigger inflammation and participate in the leukocyte infiltration process (Nilsson, 2007; Straub, 2007). In mammals, endothelial cells constitutively express both ERα and ERβ, although ERα plays a prominent role in the

vascular physiology (Ihionkhan et al., 2002; Straub, 2007). Gilthead seabream endothelial cells constitutively express ERα and ERβ1 but not ERβ2 (Liarte et al., 2011c). However, few studies have been carried out into the effect of 17β-estradiol on endothelial cell physiology in fish. In the Japanese eel, 17β-estradiol stimulated the production of vascular endothelial cell growth factor in endothelial cells (Huang et al., 2006). In the gilthead seabream endothelial cell cultures, 17β-estradiol induced the expression of genes coding for chemokines, adhesion molecules and MMPs, which agrees with previous studies that demonstrated that 17β-estradiol promotes acidophilic granulocyte infiltration into the testis (Chaves-Pozo et al., 2007). These effects contrast with that which occurs in mammals, where 17β-estradiol inhibits *in vivo* the migration of leukocytes into inflamed areas and exerts tissue-protective activities through the down-regulation of adhesion molecules and the proforms of MMPs (Straub, 2007). On the other hand, 17β-estradiol did not affect the expression in endothelial cells of the genes encoding major pro-inflammatory cytokines, such as IL-1β, IL-6 and TNFα, which may prevent the detrimental effects of 17β-estradiol-induced inflammation through leukocyte recruitment (Liarte et al., 2011c).

Little is known about androgens and their receptors in fish endothelial cells. There are very recent studies that suggest that androgens influence fish endothelial cell physiology, although further effort is needed to really understand how androgens affect endothelial cells and their molecular pathways. Trout testicular endothelial cells possess AR, as located by immunocytochemistry (Galas et al., 2009). In the gilthead seabream, recent studies determined that testosterone up-regulated TNFα, cyclooxigenase 2 (Cox2) and IL-1β, and down-regulated TGFβ and aromatase (the enzyme that transforms testosterone into 17β-estradiol) gene expression (Águila et al., 2011).

3. Effects of endocrine disruptors on the immune response

In several species, the affinity for ER of several agonists, including natural estrogens like 17β-estradiol, estrone or estriol, and estrogenic disruptor compounds, like 17α-ethynilestradiol or diethylstilbestrol, has been tested. The different types of ER show differential binding preferences for ligands and their expression patterns are tissue-dependant (Iwanowicz & Ottinger, 2009). Taking into account that ER and AR are widely distributed in immune tissues, including the spleen, liver and anterior kidney (Lynn et al., 2008; Shved et al., 2009; Slater et al., 1995; Todo et al., 1999), the study of endocrine disruptor compounds as potential aquatic pollutants has taken on some importance for fish immunologists. Several anatomical and morphological changes were observed in lymphoid tissues following exposure to xenoestrogens and xenoandrogens. Spleno-somatic and hepato-somatic indices and thymus volume are affected by exposure to sex-steroids (androgens and estrogens) or to their related endocrine disruptor compounds (Grinwis et al., 2009; Kurtz et al., 2007; Tellez-Banuelos et al., 2009; van Ginneken et al., 2009). In the gilthead seabream the dietary intake of 17α-ethynilestradiol promotes the up-regulation of several genes related with leukocyte recruitment (e.g. E-selectin (sele), the CC chemokine-like 4 (CCL4), TNFα and IL-8). Moreover, the heavy chain of IgM and IgT genes has also been seen to be up-regulated (Cabas et al., 2011). An increase in the spleno-somatic index was also recorded.

Surprisingly, *in vitro* 17α-ethynilestradiol treatment of gilthead seabream endothelial cells dramatically reduces the expression of chemokines, adhesion molecules and MMPs in

VaDNA-activated endothelial cells unlike in 17β-estradiol-treated endothelial cells (see point 2.4). Although, the differential expression profile in stimulated 17α-ethynilestradiol-treated endothelial cells, compared with 17β-estradiol-treated endothelial cells, indicates that this compound would be able to impair the recruitment and activation of fish leukocytes, other molecular pathways might promote an inflammatory process in the gonad *in vivo,* as described by Cabas et al. (2011). These data show the complex effect of endocrine disruptor compounds on immune functions and the need to deepen our knowledge of their molecular action mechanism. As also occurs in mammals, 17β-estradiol, but not 17α-ethynilestradiol, significantly enhances nitric oxide production in gilthead seabream endothelial cells, indicating that some estrogens regulate nitric oxide production by endothelial cells from fish to mammals (Arnal et al., 1996; Liarte et al., 2011c; Nilsson, 2007). As far as we know, most studies on this topic have dealt with the effects of estrogenic and anti-androgenic disruptor compounds on reproductive functions. It is known that these disruptor compounds mainly affect several enzymes in the steroidogenic pathway, such as 20β-hydroxysteroid deshydrogenase, 17β- hydroxysteroid deshydrogenase and 11β-hydroxysteroid deshydrogenase, aromatase and 5α-reductase (Rempel & Schlenk, 2008). Further studies are needed into androgenic disruptor compounds as well as into estrogenic, anti-androgenic and androgenic disruptor compound mixtures to better understand how chemically and pharmaceutically polluted water might affect the reproductive and immune function of fish. Future studies and analyses along these times are being undertaken in our laboratory.

4. Conclusion

It is known that both estrogens and androgens modulate the fish immune response, although the molecular mechanisms by which they act are not completely understood. *In vivo* and *in vitro* analyses have demonstrated that gilthead seabream leukocyte (macrophages, acidophilic granulocytes and lymphocytes) express intracellular AR and/or ER, whose expression pattern upon stimulation depend on the cell type and the stimuli in question. Estrogens and androgens compromise the immune response, affecting cell types other than leukocytes. Thus, endothelial cells are involved in the leukocyte trafficking that occurs during the inflammatory process and their activities are also modulated by sex steroids. A wide variety of chemicals discharged from industrial and municipal sources has been reported to disrupt the endocrine system of animals via the food chain and contaminated water. Some of these contaminants have a widespread presence in the aquatic environment. Although, current knowledge concerning the sensitivity of marine fish to estrogenic and androgenic chemical in the environment is limited, we have seen that the most widespread (estrogenic) disruptor compound drastically affects leukocyte trafficking and recruitment into tissues. The short time of exposure (3 hours) used in our *in vitro* experiments suggests that, together with ER and AR activation, some transcription-independent non-genomic actions might be acting on sex steroid hormones-stimulated leukocytes. Taking all this into account, further effort will focus on the cloning and characterization of membrane AR and ER, their expression pattern in immune cells and the molecular characterization of the way of which estrogenic and androgenic compounds disrupt the molecular signalling pathways of intracellular and membrane androgen and estrogen receptors.

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