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Effect of Testosterone on Reproductive and Immune Systems of Gilthead Seabream (*Sparus aurata* L.). Identificaction of a Constitutively Active Androgen Receptor Variant

Efecto de la Testosterona en el Sistema Reproductor e Inmunitario de la Dorada (*Sparus aurata* L.). Identificación de una Variante Constitutivamente Activa del Receptor de Andrógenos

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Memoria presentada por

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INDEX

ABBREVIATIONS

11KT	11-ketotestosterone
Ab	Antibody
Abs	Absorbance
AGs	Acidophilic granulocytes
AR	Androgen receptor
ARβ	Androgen receptor isoform beta
ARΔLBD	Androgen receptor with lack ligand-binding domain
βdiol	5α-androstae-3β-17βdiol
bp	Base pairs
ccl4	CC chemokine ligand 4
cDNA	Complementary DNA
CSFs	Colony stimulating factors
cyp	Cytochrome P450 enzymes
DHT	Dihydrotestosterone
Dmrt1	Double sex-and mab3-related transcription factor 1
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dpi	Days post-implant
E_2	17β-estradiol
EDCs	Endocrine disruption compounds
EDTA	Ethylenediaminetetraacetic acid
EE_2	17α- ethinylestradiol
ELISA	Enzyme-linked immune sorbent assay
ER	Estrogen receptor
F	Forward primer
FBS	Fetal bovine serum
FSH	Follicule stimulating hormone

FSH-R	Follicule stimulating hormone receptor
GSI	Gonadosomatic index
GTHs	Gonadotropins
Hf-FBS	Hormone free fetal bovine serum
HK	Head kidney
hsds	Hydroxysteroid dehydrogenases
IFN	Interferon
Ig	Immunoglobulin
il	Interleukin
ISM	*In situ* forming microparticle
LH	Luteinizing hormone
LH-R	Luteinizing hormone receptor
Ly	Lymphocytes
mAb	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MB	Body mass
MCSFR	Macrophage colony stimulating factor receptor
MG	Gonad mass
MH	Hepatic mass
mRNA	Messenger RNA
MØs	Macrophages
ND	Non-detected
NO	Nitric oxide
NRs	Nuclear receptors
ns	Non-significant
Nt	Nucleotides
OHT	11β-hydroxitestosterone

P	Progesterone
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PRRs	Pattern-recognition receptors
PS	Post-spawning
R	Reverse primer
RC	Reproductive cycle
RNA	Ribonucleic acid
RNase	Ribonuclease
RNIs	Reactive nitrogen intermediates
ROIs	Reactive oxygen intermediates
rps18	Ribosomal protein S18
R	Resting
RT	Reverse transcription
S	Spawning
sele	E-Selectin
SG	Spermatogenesis
sRPMI	RPMI-1640 culture medium supplemented with 0.35% NaCl
star	Steroidogenic acute regulatory protein
T	Testosterone
TI	Testicular Involution
tgfβ	Transforming growth factor β
tlr	Toll-like receptors
tnf	Tumor necrosis factor
VaDNA	*Vibrio anguillarum* genomic DNA

SUMMARY

During the development of this thesis, we want to analyze the effect of exogenous androgens, mainly testosterone (T), and the activity of androgen receptor (AR) in immune-reproductive interaction in the gilthead seabream (*Sparus aurata* L.). Furthermore, we described the presence of a mechanism, in which the unique AR gene can give diverse transcripts either in testis or immune organs.

The gilthead seabream is a marine, protandrous teleost fish with significant economic value in the Mediterranean aquaculture. The specimens of this specie are male during the two first years, and present a reproductive cycle (RC) divided in four stages. In first RC, the stages are spermatogenesis (SG), spawning (S), post-spawning (PS) and resting (R); in the second RC, the last stage is substituted by testicular involution (TI) stage. After spawning there are various changes in gonad, between which are a massive infiltration of leukocytes, being higher in TI and that can lead to sex change. For this motive this specie can being used as a model for studying the immune-reproductive interactions.

In first place, we wanted to observe the capacity of exogenous T to modify the balance of sex steroid hormones and on the physiology of the gonad (proliferation, apoptosis, leukocyte influx and immune status) of mature male specimens of the gilthead seabream. To this end, we used the in situ forming microparticle (ISM) system, which delivers sex steroids without promoting significant physiological alterations in gilthead seabream and that has been previously used in our laboratory to show that T modulates the inflammatory response of head kidney (HK) leukocytes in the gilthead seabream. The results suggest that in mature males, exogenous T can blocked the cell proliferation and increase the migratory influx of leukocyte, although decrease the ability to recognize and response to pathogens.

Secondly, we identified a truncated form of the AR generated by alternative splicing that lacks the ligand-binding domain (ARΔLBD) either in testis, HK and acidophilic granulocytes (AGs). We studied the modulation of this variant by androgens and immune stimulus in order to demonstrate its mediation in the role of androgens in the immune response. The results showed that T is able to regulate the testicular morphology through the modulation of the expression of ARΔLBD variant. Furthermore, T was also able to regulate the immunecompetence through ARΔLBD variant. Interestingly, ARΔLBD variant only is expressed in AGs but no in

macrophages (MØ). Furthermore, the stimulation with bacterial DNA, also regulated the alternative splicing of AR, but depending of the reproductive stage.

These data suggest a crosstalk between endocrine and immune stimuli in the regulation of AR alternative splicing and AGs function. Furthermore, T might be implicated, in the migratory influx into gonad and the physiological changes in gonad through by means of regulation of ARΔLBD.

I. INTRODUCTION

Fish are the largest group of vertebrates and it is composed of about 24,600 species, of which 23,700 are teleosts. This large diversity is manifested in aspects of the biology of this group as their way of life, physiology and reproduction. Furthermore, nowadays the demand of seafood has an exponential increase which implies that the fisheries exert high pressure on the marine system, compromising its capacity for renewal. Extractive fishing hardly covers 60% of the annual world fish production (FAO, 2012), a situation to which aquaculture is seen as the only way to satisfy the demand in the near future. So, FAO estimates that in 2030 more than 65% of the aquatic food will come from aquaculture. Moreover, Spain is the third member state of the European Union with a higher production of fish from aquaculture, 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 main objectives in the research of aquaculture.

Moreover, fish occupy a key phylogenetic position in the evolution of vertebrates representing the first animal group that has an innate and adaptive immune system well structured, so 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).

The effects that sexual steroid hormones exert on fish physiology are a prominent field of research, especially in view of the increasing levels of endocrine disrupting compounds (EDCs), including xenobiotic hormones, that are found in aquatic environments. Indeed, EDCs have been detected in these environments in concentrations capable of altering the reproductive physiology and behavior of fish (Nash *et al.*, 2004; Mortensen & Arukwe, 2007). Such studies highlight the complexity of the organism's responses to estrogenic EDCs; however, the effects of androgenic

EDCs in fish have been less well studied and most of them have focused on the alterations of the reproductive physiology that they provoke. Moreover, it is well known that androgenic modulation is acting through classical nuclear androgen receptor (AR). Nevertheless, the androgenic effects are poorly understood and contradictory results, which could be indicative of a more complexity in its regulation.

The gilthead seabream (*Sparus aurata* L.) is a seasonal breeding, marine, protandrous hermaphrodite teleost with a bisexual gonad. This species offers an interesting model for studying immune-reproductive interactions because testis undergoes abrupt morphological changes, especially after spawning, including a massive infiltration of acidophilic granulocytes (AGs), an immune cell type essential for the normal physiology of the gonad. The presence of this cell type in the gonad is activated by tissue-specific factors and hormones (Chaves-Pozo *et al.*, 2003, 2005a,b; Liarte *et al.*, 2007).

The research groups “Grupo de Investigación en Acuicultura Marina” (Instituto Español de Oceanografia, Centro Oceanográfico de Murcia) and "Innate Immune System of Teleost Fish" (University of Murcia) which have developed this PhD thesis, have a long history of collaboration in the study of aquaculture interest Mediterranean species such as sea bream (*Sparus aurata* L.), sea bass (*Dicentrarchus labrax* L.) or Shi drum (*Umbrina cirrosa* L.), species that are commercially important to the economy of the Region of Murcia and the whole country. In this context, the focus of this work is: i) to describe the androgenic effects, mainly testosterone (T), in the gonadal physiology, mainly in the local immune response, and ii) to identify a truncated form of the AR generated by alternative splicing that lacks the ligand binding domain (ARΔLBD) in head kidney (HK) and in AGs and macrophages (MØ), the main phagocytic cells in gilthead seabream, and its modulation by androgens and immune stimulus in order to demonstrate its mediation in the role of androgens in the immune response.

1. Reproductive system in teleosts.

The great diversity of fish also means that there is a great diversity in reproductive strategies and reproductive cycles (RCs).With respect to reproductive strategies we can find two principal categories: gonochorists and hermaphrodites (Devlin & Nagahama, 2002).

The gonochorists species develop a functional single sex throughout their life and they can be:

i) primary or differentiated, if it 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 testis;

iii) secondary, if it develops a hermaphrodite gonad that subsequently evolves toward a single sex.

The hermaphrodite species, however, develop two functional sexes throughout their life and they can be:

i) protandrous, if the gender developed before is male;

ii) protogynous by gender to develop is female;

iii) ambisexuales simultaneously if they develop both gonads.

On the other hand, the RCs also present a great diversity, depending on the environmental factors. The RCs can be classified based on the length of the laying period (Miura, 1999). So we can find species which lay:

i) for several weeks a year, mainly in species such as salmonids, typical of cold weather;

ii) for several months a year, in species typical of water temperature, such as gilthead seabream and common carp;

iii) throughout the year, in species typical of tropical climate.

This behavior is reflected in the RC, so in seasonal breading species, this cycle can be divided into four stages: gametogenic activity or spermatogenesis (SG),

spawning (S), post-spawning (PS) and resting (R) while species that laying throughout the year lacking PS and R stages (Scott *et al.*, 1989; Miura, 1999). However, despite this great diversity of strategies and RCs, morphological and functional characteristics of the gonad of teleosts are highly conserved even in hermaphroditic species (Le Gac & Loir, 1999). Thus, seminal tubules contain two types of cells: the germ cells and Sertoli cells. Both cell types form cysts within which develop and mature germ cells, being the development of all germ cells within a synchronous cyst (Grier, 1981, Billard *et al.*, 1982; Nagahama, 1983; Callard, 1991), and some species has also previtellogenic oocytes even during the active male. The interstitial tissue is composed by Leydig cells, MØs, fibroblasts, myeloid cells, blood vessels and collagen fibers (Grier, 1981; Nagahama, 1983; Loir *et al.*, 1995).

1.1. Endocrine regulation of reproductive processes.

The regulation of the processes implicated in the reproduction depends largely on gonadotropins (GTHs), synthesized and secreted by the pituitary. There is a duality of GTHs structurally homologous to the follicle stimulating hormone (FSH) and luteinizing hormone (LH) of mammals (Schulz *et al.*, 2001) either in teleosts such in other vertebrates. In gilthead seabream, it has been described the location of the cells producing FSH and LH in the pituitary during ontogeny (García-Ayala *et al.*, 2003). Moreover, receptor for these two hormones (FSH-R and LH-R) has been described in this species (Wong *et al.*, 2003). However, these hormones do not act directly on SG 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).

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 testis 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 up regulated 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. 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, 2002, 2003; Amer *et al.*, 2001; Hess, 2003. On the other hand, T and 11-ketotestosterone (11KT) are generally considered the major circulating male androgens, as well as the most potent ones (Borg, 1994), however the androgens derivated from T such 11β-hydroxitestosterone (OHT) or DHT have also been detected in breeding specimens of several fish species where they clearly have an *in vivo* androgenic effect (Borg, 1994; Margiotta-Casaluci & Sumpter, 2011). 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). Furthermore, T, an aromatizable androgen, is locally transformed into E_2, triggering estrogenic effects, while DHT, non-aromatizablee androgen, may also be locally transformed into 5α-androstae-3β-17βdiol (βdiol) (Mouriec *et al*., 2009), as a well-known estrogenic compound that strongly stimulates the transcriptional activity of mammalian estrogen receptor (ER) (Kuiper *et al.*, 1998).

The gonad is the main organ that synthesizes biologically active steroids *de novo* (Stocco, 2001), while the liver is not a typical steroid-producing organ, but rather a steroid-metabolizing organ (Arukwe, 2008). Both, *the novo* synthesis and metabolism of steroid hormones are controlled by rapid changes in the activities of the steroidogenic enzymes, by the availability of substrate or by changes in the amounts of the steroidogenic enzymes through the regulation of the specific mRNAs encoding them (Miller, 1989).

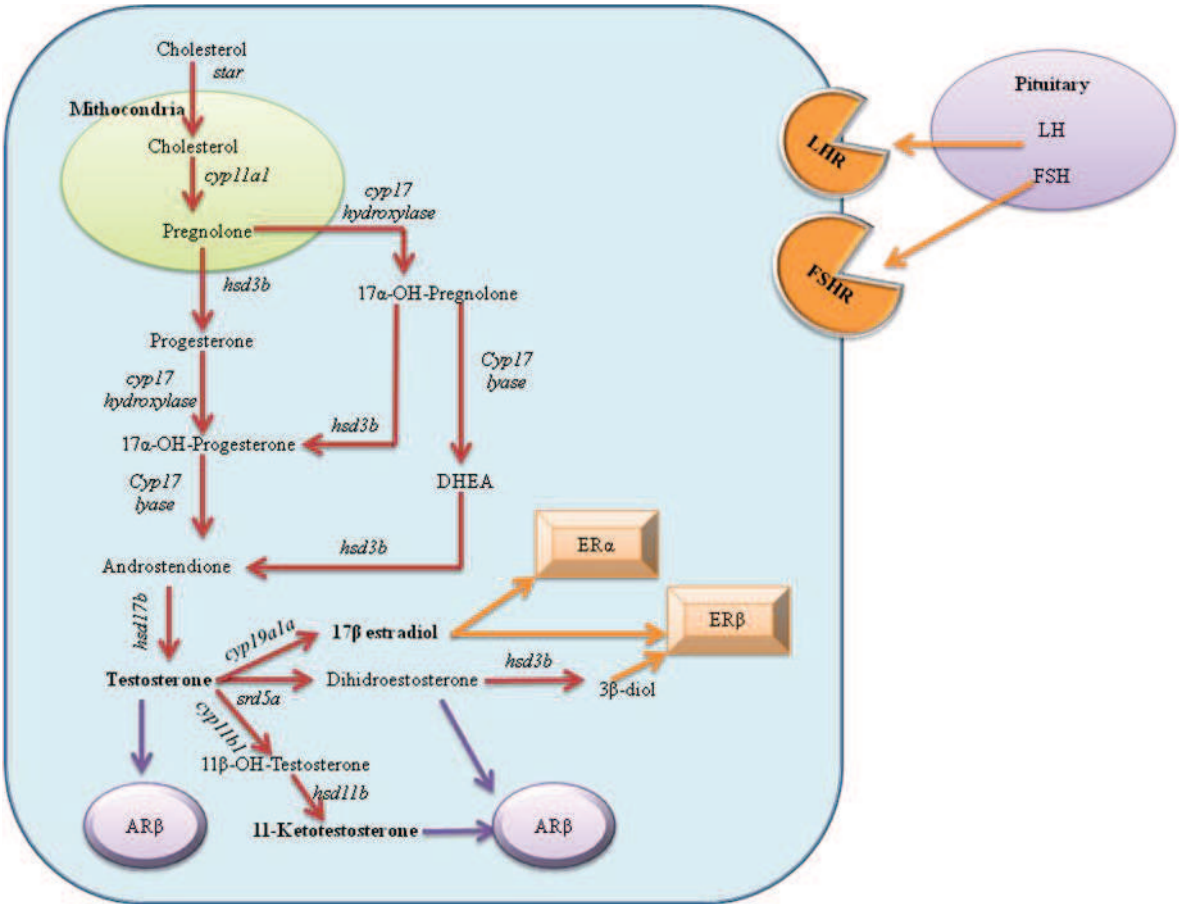

Figure 1. Schematic representation of the key steps involved in steroidogenesis in teleosts (modified from Villanueve *et al.*, 2007).

1.2. Reproductive system of gilthead seabream.

Among the hermaphrodites, the bream has a peculiar morphology characterized by a heterosexual gonad. In this group, the gonad is defined as a type of ovotestis consisting in an ovarian area in mediodorsal portion of the gonad and testicular area in lateroventral portion, both bounded by a wall of connective tissue (Sadovy & Shapiro, 1987; Besseau & Faliex 1994; Brusle-Sicard & Fourcault, 1997).

The gilthead seabream is a seasonally breeding, marine, protandrous hermaphrodite teleost. Specimens are males in the western of Mediterranean area, at least during the first two RCs. Males present a bisexual gonad, which has a functional testicular area and a non-functional ovarian area (Chaves-Pozo *et al.*, 2005a; Liarte *et al.*, 2007) The RCs of gilthead seabream males are divided into four stages: SG, S, PS, and R or testicular involution (TI) stage depending on whether the fish are in their first

or second RC (Chaves-Pozo *et al.*, 2005a; Liarte *et al.*, 2007) (Figure 2). Spermatogonia stem cell, primary spermatogonia and Sertoli cell are always present in testis, although with variable proliferation rates. In the last stage of SG, spermatocytes, spermatids and spermatozoa appear (Chaves-Pozo *et al.*, 2010). During PS the proliferative events resumes, although necrotic areas and apoptotic germ cells are also observed (Chaves-Pozo *et al.*, 2003, 2005a). In R, there is an increase of the number of proliferative cells and no apoptotic cells where observed, while in TI stage the number of apoptotic cells increase (Liarte *et al.*, 2007). Respect the ovarian area, this shows a proliferative activity during the last stages, R and TI, at a rate that does not differ from the proliferative activity (Micale *et al.*, 1987; Chaves-Pozo *et al.*, 2005a, Liarte *et al.*, 2007).

The abrupt morphological changes that occur in testis, especially after S of second RC, including a massive infiltration of AGs, an immune cell type essential for the normal physiology of the gonad, and whose presence in the gonad is actively regulated by tissue-specific factors and hormones (Chaves-Pozo *et al.*, 2003, 2005a,b; Liarte *et al.*, 2007). In fact, an endogenous increase of the E_2 plasma levels in male specimens is correlated with AGs migration into the testis after S (Chaves-Pozo *et al.*, 2008a, 2012), while exogenous E_2 or 17α-ethinylestradiol (EE_2), a synthetic estrogen,accelerates the final events of spermatogenesis and promotes a massive leukocyte infiltration in the testis by up-regulating the expression of several genes involved in regulating leukocyte trafficking (Chaves-Pozo *et al.*, 2003, 2008b; Liarte *et al.*, 2007; Cabas *et al.*, 2011). Moreover, short-term exposure to E_2 inhibits the proliferation of spermatogonia in their early stages, while long-term exposure to E_2 restarts Sertoli cell proliferation (Chaves-Pozo *et al.*, 2007). Interestingly, both estrogenic compounds deplete the plasmatic androgen levels of gilthead seabream concomitantly with the suppression of S, suggesting that the induction of S is an androgen-dependent issue (Chaves-Pozo *et al.*, 2008b; Cabas *et al.*, 2011). However, the role of androgens, 11KT and T, is less known, and has been suggested that they play different and specific roles in the testicular physiology of gilthead seabream as they peak at different stages (Chaves-Pozo *et al.*, 2008a). Moreover, their profiles suggest that T is not essential in the testicular regression process that occurs after S, when the infiltration of leukocytes is enhanced (Chaves-Pozo *et al.*, 2005a).

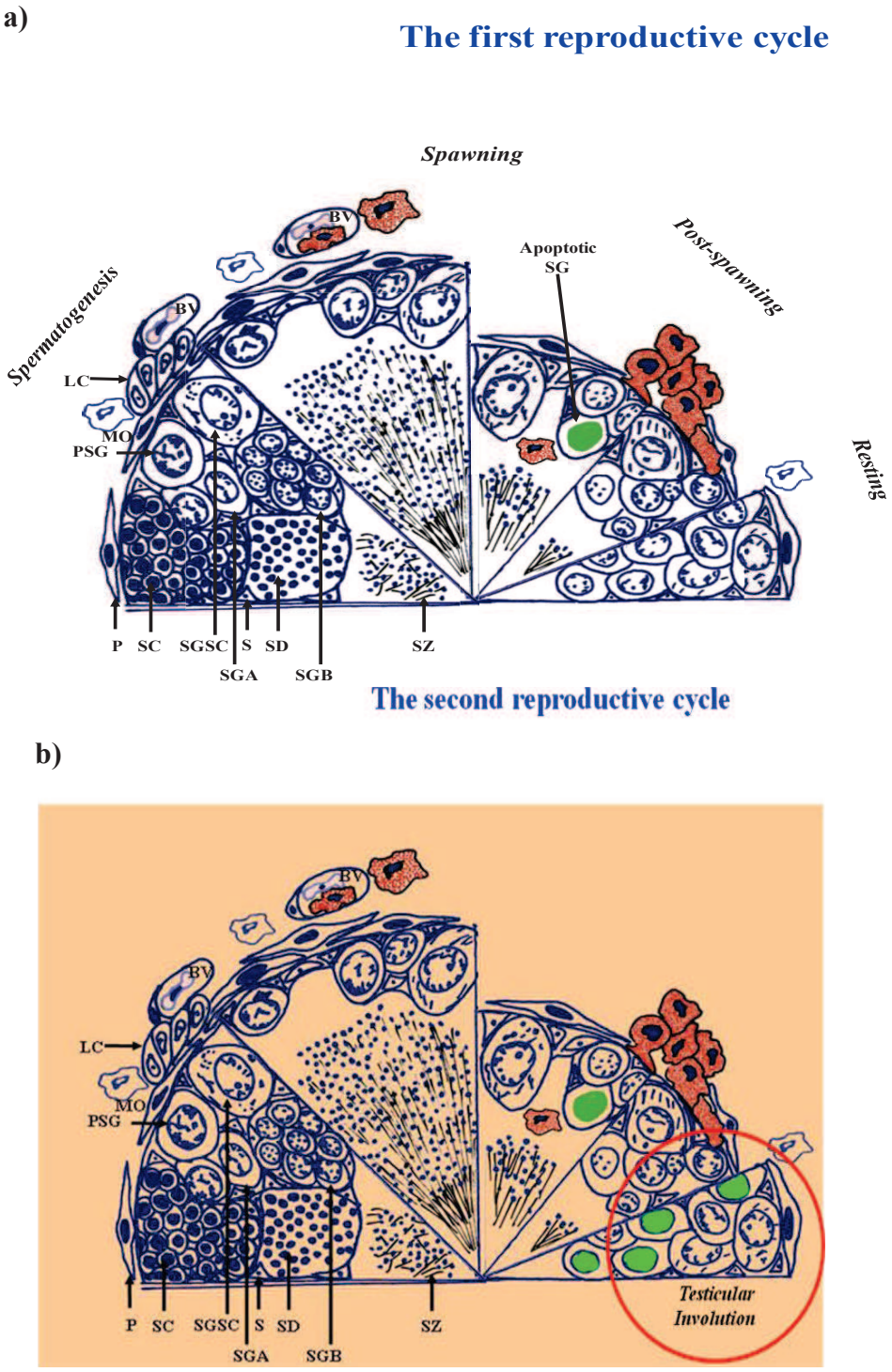

Figure 2. Schematic illustration of the spermatogenetic cycle in the gilthead seabream testis during the first (a) and second (b) reproductive cycle. Germinal compartment; SGSC, spermatogonium stem cell; PSG, primary spermatogonium; SGA, spermatogonia A; SGB, spermatogonia B; SC, spermatocyte; SD, spermatid; S, Sertoli cell; P, peritubular cell; MO, macrophage; AG, acidophilic granulocyte; BV, blood vessel, LC, Leydig cell (Modified García-Ayala *et al*., 2010).

2. Immune system in teleosts.

The immune system, in teleost fish, displaying cellular and humoral responses that have the characteristics of specificity and memory, those characteristics are in birds and mammals too (Van Muiswinkel, 1995). Teleosts are the first animal group that has an innate and adaptive immune system well structured and differenciated. The innate response comprises:

i) physical barriers (epithelium and mucosa).

ii) cellular effectors (phagocytic cells and nonspecific cytotoxic cells).

iii) humoral factors (complement and other acute phase proteins).

The adaptive response is composed by:

i) cellular components (lymphocytes).

ii) humoral components (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 & Clem, 1977; Avtalion, 1981; Abruzzini *et al.*, 1982; Clem *et al.*, 1984, 1985, 1991).

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 the kidney, a primary organ, the par excellence hematopoietic organ. Kidney consists of two parts: the anterior or cephalic (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 MØs, lymphocytes (Ly) and AGs. AGs are the major cell type participating in innate host responses, while the HK is the central immune organ that provides a source for AGs (Sepulcre *et al.*, 2002; Chaves-Pozo *et al.*, 2007). Regarding to secondary lymphoid organ, the spleen is the most important but presents few Ly although may increase in number by administration of an antigen.

2.1. Innate immune system.

In teleosts, the first line of defense against invasion of microorganisms consists of 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.

A variety of leukocytes are implicated in cellular response of the fish, they include phagocytes (monocytes/MØs and granulocytes) and nonspecific cytotoxic cells (Secombes, 1996). Phagocytes are more important in for its capacity to eliminate viruses, bacteria and parasites (Rowley *et al.*, 1988; Secombes & Fletcher., 1992; Sepulcre *et al.*, 2002) and, moreover, they can be the initiator of activation and regulation of the specific immune response (Clem *et al.*, 1985, 1991; Vallejo *et al.*, 1992). As in mammals, the process of phagocytosis in fish has the same steps and 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 respiratory burst and is independent of mitochondrial respiration. This production has bactericidal activity (Sharp & Secombes, 1993; Skarmeta *et al.*, 1995).

ii) The production of nitric oxide (NO) and other nitrogen reactive intermediates (RNIs).

AGs as the more active and abundant phagocytic cell describe in gilthead seabream (Sepulcre *et al.*, 2002; Chaves-Pozo *et al.*, 2004a). Furthermore, 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, 2007), produce cytokines 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), with the exception of tlr3 (Sepulcre *et al.*, 2007).

On the other hand, there are a wide variety of substances (humoral effectors) that act on the innate defense of fish (Alexander & 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,

vi) complement components that perform several functions, among which leukocyte chemotactic activity (Lamas & Ellis, 1994), opsonisation (Sakai, 1984a), inactivation of certain toxins (Von Eschen & Rudbach, 1974; Ellis, 1980; Sakai, 1984b), the bactericidal activity (Sakai, 1983), cytotoxicity and viral inactivation (Sakai, 1992), are the most remarkable.

2.2. Adaptive immune system.

Ly are key mediators of the adaptive immune response with the production of antibodies (Ab). *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 & Clem, 1982; DeLuca *et al.*, 1983; Secombes *et al.*, 1983; Navarro *et al.*, 1993), suggesting that the surface of Igs may be a marker for B cell-like cells and allowing the isolation of the two cell populations of Ly of teleost: Ig+ e Ig-. Further, performing some functional studies, it has been shown that these two populations for 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). Interestingly, IgD (Edholm *et al.*, 2011) and IgT (Hansen *et al.*, 2005; Danilova *et al.*, 2005) have been also identified in fish. Although the functional relevance of IgD remains to be

determined, as in mammals, the teleost-specific IgT seems to be specialized in mucosal immunity and, therefore, it would be the functional equivalent to mammalian IgA (Zhang *et al.*, 2010). The existence of T cells in the teleost by the identification of T cell receptor (TCR) has been recently confirmed (Haire etl al., 2000; Wang *et al.*, 2001; Wermenstam & Pilstrom, 2001; Nam *et al.*, 2003).

2.3. Regulatory molecules of the immune response

The innate immune response operates on the basis of special receptors, the pattern-recognition receptors (PRRs) which recognize microbial mots known as pathogen-associated molecular patterns (PAMPs) (Akira & Takeda, 2004). Among PRRs, tlrs are the major in the recognition of microbian components and the induction of immune responses (Akira & Takeda, 2004). Tlr are presented in plants and have been conservated in invertebrates and vertebrates (Aderem & Ulevithc, 2000; Medzhitov & Janeway, 2000). In fish, tlrs have been described in pufferfish (*Fugu rubripes*), zebrafish (*Danio rerio*) and gilthead seabream (Oshiume *et al.*, 2003; Jault *et al.*, 2004: Sepulcre *et al.*, 2007).

On the other hand, the recognition of PAMPs by tlrs induces signals that result in the production of pro-inflamatory cytokines and ROIs (Sepulcre *et al.*, 2007). Cytokines are proteins (usually glycoproteins) with a low molecular weight 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). Cytokines mediate effector phases in both innate and adaptive immunity (Abbas *et al.*, 2001). In the innate immunity, cytokines are produced mainly by mononuclear phagocytes and so are usually called monokines, which are produced by mononuclear phagocytes in response to microorganisms and upon T-cell antigen stimulation as part of adaptive immunity. However, in the adaptative response, the most of the cytokines involved are lymphokines, which are produced by activated T-Ly. These molecules present a double function, either regulating the proliferation and differentiation of different Ly populations or participating in the activation and regulation of inflammatory cells (mononuclear phagocytes, neutrophils and eosinophils). Other cytokines are produced by both cell type (Ly and mononuclear phagocytes) which are known as colony stimulating factors (CSFs) and 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 adaptive immunity, and regulate the inflammatory and immune response.
- Their secretion is brief and auto-limited. In general, cytokines are not stored as preformed 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 & Harmsen, 1984; Lawrence, 1996; Yin *et al.*, 1997), pro-inflammatory cytokines, as interleukine 1β (il1β) (Jang *et al.*, 1995a, b; Zou *et al.*, 1999a, b; Fujiki *et al.*, 2000), chemokines, as ccl4 (Daniels *et al.*, 1999; Fujiki *et al.*, 1999; Laing *et al.*, 2002), immunosuppressive or anti-inflammatory cytokines, as transforming growth factor β (tgf-β) (Sumathy *et al.*, 1997;

Laing *et al.*, 1999; Harms *et al.*, 2000) and interferons (IFNs) (Congleton & Sun, 1996; Collet & Secombes, 2002; Hansen & La Patra, 2002).

3. Immune-reproductive interactions.

The interaction between immune and reproductive system is widely accepted. Hormones, in general, and sex steroids specifically, may affect the genes, immune responses, and behaviors that influence susceptibility and resistance to infection (Klein, 2000). The differences between the immune function of males and females have frequently been reported in mammals (Cohns, 1979; Bouman *et al.*, 2005). 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 & Kovacs, 1996). These differences have also been reported in avian (Romano *et al.*, 2011), in reptile (Svensson *et al.*, 2009) and fish (Hoeger *et al.*, 2005). In mammals, female generate higher levels of Ab in response to exogenous antigens (Adori *et al.*, 2010; Friedman, 2010; Yeh & Chen, 2010) and show a lower incidence of tumors and better resistance against viral and parasitic infections (Filipin Mdel *et al.*, 2010; Gillgrass *et al.*, 2010), although this is accompanied by sharp susceptibility to developing auto immune syndromes (Fairweather *et al.*, 2008; Lee & Chiang, 2012).

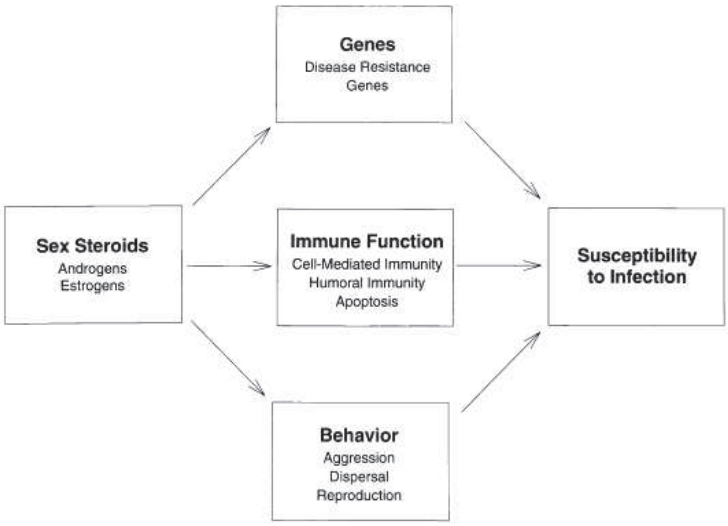

Figure 3. Schematic representation of alternative hypotheses for how sex steroid hormones affect susceptibility to infection. Sex steroid hormones, more specifically androgens in males and estrogens in females, may cause sex differences in infection by altering host immunity, genes, or behaviors that affect susceptibility and resistance to infection (Klein, 2000).

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 the physiology of immune cells. It is known that, in mammalian, there are populations of testicular MØs, Ly and mast cells. The resident leukocyte populations in rat testis interact and regulate testicular function. Thus, MØs regulate Leydig cell development, SG and steroidogenesis through the production of protein factors or soluble lipid (Hedger, 1997; Nes *et al.*, 2000). Furthermore, factors produced by T cells and MØ, along with the production of tgf-β, activin or Fas ligand by Sertoli cells, guarantee testicular immunesuppression 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.

In human neutrophils, the E_2 regulates its own receptors (Molero *et al.*, 2002), these evidences, along with many others, show that estrogen has an immune modulatory role (Straub, 2007). The estrogens have anti-inflammatory but they also can have pro-inflammatory roles depending on the above-mentioned influencing factors (Straub, 2007). On the other hand, the role of the androgens seem to have a suppressive effect on immune responses of vertebrates (Schuurs *et al.*, 1990; McCruden *et al.*, 1991; Paavonen, 1994; Olsen & Kovacs, 1996) and increases susceptibility toward numerous infectious diseases (Alexander & Stimson, 1988; Roberts *et al.*, 1996). It has been proposed that T modifies the production of cytokines by leukocytes (Posma *et al.*, 2004), decreases *tlr4* expression in MØs and alters the sensitivity of these to *tlr4* ligands (Rettew *et al.*, 2008). Moreover, immune challenges and inflammatory conditions reduce T plasma levels (Greiner *et al.*, 2010). It is known that T also can induce a change in T cells from a resistance-promoting phenotype to a susceptibility-mediating phenotype, thus enabling the T cells to mediate the suppressive effect of testosterone (Benten *et al.*, 1991). Male mice infected with the fungus, *Candida albicans*, have lower antibody responses than females; in contrast, castrated males have similar antibody responses to females suggesting that T may suppress immune responses to *C. albicans* (Rifkind & Frey, 1971). However, some studies present results when T doesn't have an immune suppressor effect, for example, in red-winged blackbirds (*Agelaius phoeniceus*), artificially high to very high levels of T did not suppress secondary

humoral immune responses (Hasselquist *et al.,* 1999), and the same for primary humoral immune responses in black-headed gulls (*Larus ridibundus*; Ros *et al.,* 1997). Furthermore, male rats infected with Seoul virus produce higher antibody responses and shed virus longer than females, however, castration of adult males has no effect on antibody production or virus shedding (Klein *et al.*, 2000). All these data suggest that *T* can have both immunosuppressive and immunostimulatory effects.

3.1. Immune-reproductive interactions in teleosts.

The fish is the most primitive vertebrate and physiological activities including the immune function are greatly influenced by the environment. In most cases the outbreak of fish disease is dependent on environmental and endogenous factors, and unfavorable conditions result in opportunistic infection. Therefore, management of fish health based on the information provided by endocrine-immune interactions is essential to prevent fish diseases (Yada & Nakanishi, 2002).

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 & Sumpter, 1989; Besseau & Faliex, 1994; Bruslé-Sicard & Fourcault, 1997; Lo Nostro *et al*., 2004). Thus, in the SG and S stages some MØ have been described in the interstitial tissue of the rainbow trout testis (Loir *et al.*, 1995), whereas in the PS stage a high population of phagocyte cells has been described in several teleost fish (Henderson, 1962; Shrestha & Khanna, 1976; Scott & Sumpter, 1989; Loir *et al.*, 1995). In general, these leukocytes are not considered as a testicular leukocyte population, but infiltrate the organ, to a greater or lesser extent, depending on the stage of the RC.

As in mammals, in teleosts, pituitary and steroids hormones are able to regulate the functions and reactions of leukocytes. In tench (*Tinca tinca* L.), T injection did not suppress the lytic activity of plasma or the response of HK phagocytes, however T-induce lymphopenia of the spleen (Vainikka *et al.*, 2005). 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 MØs (Watanuki *et al.*, 2002). These hormones inhibit the phagocytic activity of carp MØ 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 other studies in fish, T did not suppress the leukocyte phagocytosis or cause apoptosis of leukocytes (Law *et al.*,

2001; Saha *et al.*, 2003). The potential effects of androgens on immunity in fish are poorly understood and present contradictory results, while T increase innate immune parameters (Cuesta *et al.*, 2007), 11KT seems to induce a decrease of immune response (Yamaguchi *et al.*, 2001; Watanaki *et al.*, 2002; Kurtz *et al.*, 2007)

In gilthead seabram, previous studies in our laboratory have suggested that sex hormones might be key regulators of leukocyte functions in the gonad. Thus, an AGs infiltration in the testis, orchestrated by gonadal factors including sex steroid hormones, has been observed to be related to:

i) PS and TI 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.

iv) Experimental induction of E_2 increasing serum levels in spermatogenically active males correlated with an acceleration of the final events of SG and an inhibition of the proliferation of spermatogonia in early stages.

However, when AGs infiltrate the testis, they show heavily impaired ROIs production and phagocytic activity (hardly 1% of the testicular AGs are able to phagocytose) 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, b, 2007, 2008a). Furthermore, 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, MØ and Ly have also been observed in the interstitial tissue (Liarte *et al.*, 2007; Chaves-Pozo *et al.*, 2008a). However, the number of testicular MØ remains steady throughout the RC when the specimens are males, while no data related to Ly are available (Chaves-Pozo *et al.*, 2008a). These observations, which were 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, T and 11KT seem to play different and specific roles in the testicular physiology of the gonad of gilthead seabream (Chaves-Pozo *et al.*, 2008a). Moreover, T and 11KT plasmatic levels are correlated with the expression of extracellular matrix that takes part in inflammatory processes (Castillo-Briceño *et al.*, 2009, 2011). Recent studies show that, in gilthead seabream, exogenous T, *in vivo,* regulated the expression of cytokines, chemokines and immune receptors (Castillo-Briceño *et al.*, 2013), and increased the phagocytic activity and the production of ROIs. Moreover, *in vitro* treatment of HK leukocytes with T and 11KT was not able to modulate the production of ROIs, although T induced the expression of the pro-inflammatory cytokine *il1β* (Águila *et al.*, 2013), despite the fact that E_2 showed a suppressive effect on the production of ROIs and the VaDNA-stimulated production of IL-1β (Chaves-Pozo *et al.*, 2003). However, 11KT was able to strongly inhibit the expression of all genes in both non-activated and VaDNA-activated AGs and exerted the opposite effect in MØs (Águila *et al.*, 2013). The different responses of AGs and MØs to androgen exposure is interesting and might help to explain the opposite reports on the effect of androgens on vertebrate immune responses, particularly in fish where several studies report their immunosuppressive roles (Yamaguchi *et al.*, 2001; Watanuki *et al.*, 2002; Kurtz *et al.*, 2007). Therefore, all these results point to the extremely complex crosstalk between immune and reproductive systems.

4. Androgen receptors.

Traditionally, androgens act via classical nuclear androgen receptor (AR), nevertheless non-genomic pathways have also been described (Rahman & Christian, 2007). Furthermore, AR duplication is common in fish, a particularity that leads to several androgenic forms with different binding affinities for T, 11KT and DHO (Sperry and Thomas 1999a,b; Blázquez & Piferrer, 2005; de Waal et al, 2008; Douard *et al.*, 2008).

The AR is a member of the steroid receptor transcription factor family that mediates in the action of the androgens. The structural organization of the AR gene, mature spliced mRNA is organized in eight exons, and the protein is divided into three domains, according its structure and function (Figure 4): i) amino-terminal domain (NTD), encoded mostly by exon 1; ii) DNA-binding domain (DBD), encoded by exon 2 and 3, and two zinc-fingers are included in part of exon 4; iii) and ligand-binding

domain (LBD), which is encoded by part of exon 4 and exons 5,6,7 and part of exon 8. The two last domains, DBD and LBD, are highly conservated from teleosts to mammals. Most of vertebrates have only one AR, however in teleosts, two main isoforms, ARα and ARβ, are present, which display different tissue distributions with ARα present only in brain and ARβ found in brain and in gonad (Sperry & Thomas, 1999a). These two isoforms present different binding affinities for androgens, mediate the different actions of androgens in different tissues (Sperry & Thomas, 2000). The AR isoform most similar to ARβ in fishes appears closest to that of other vertebrates in sequence homology and androgen binding and is the probable ancestral vertebrate AR (Sperry & Thomas, 1999a; Harbott *et al.*, 2007; Gorelick *et al.*, 2008).

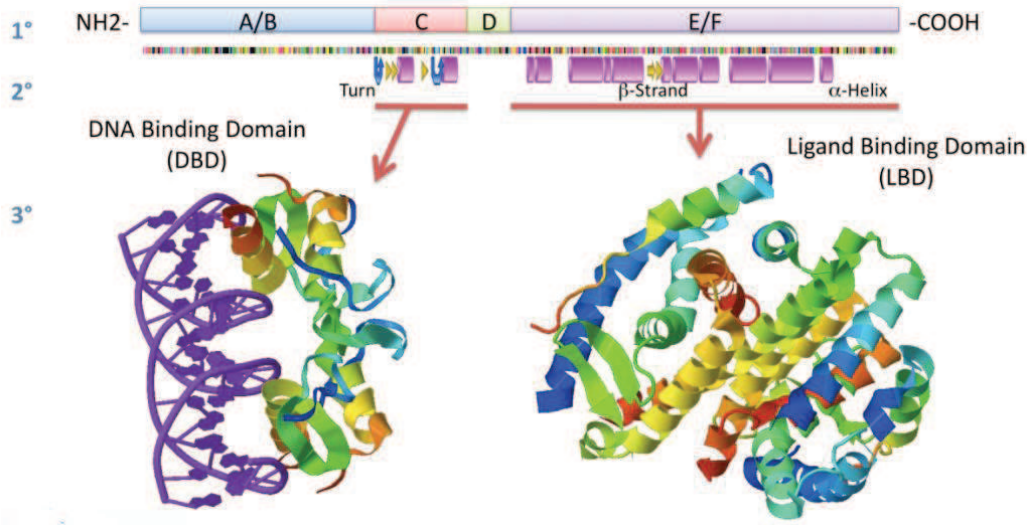

Figure 4. Schematic illustration of structure of nuclear receptors (NRs). Nuclear Receptor Resource (Wahli *et al.*, 1991; Chawla *et al.*, 2001).

AR is localized in the cytoplasm where it engaged with other molecular, chaperones (Dehm & Tindall, 2011). Through the binding with androgens, the receptor suffer a conformational change and there is to nuclear translocation (Cutress *et al.*, 2008) where the receptor acts as transcription factor and bind to specific DNA response elements in target gene promoter, causing activation or repression of transcription and subsequently protein synthesis (Beato, 1989; Roy *et al.*, 1999; Zhou *et al.*, 1994; Quigley *et al.*, 1995; Heinlein & Chang, 2002). The primary mechanism of action for androgen receptors is a direct regulation of gene transcription. However, several studies

suggest that some androgen responses are involved non-classical and non-genomic mechanism (Foradori *et al.*, 2007).

The AR is important for reproductive functions in males through the binding with androgens, it is involved in differentiation of the efferent duct system for germ cells (Hannema & Hughes, 2007), SG (De Gendt *et al.*, 2004), reproductive behavior and secondary sexual characteristics (Sato *et al.*, 2004, Soma 2006). In prepubertal and immature teleost fish the androgens can induce SG (Miura *et al.*, 1991; Cavaco *et al.*, 1998; Campbell *et al.*, 2003), however many aspects of the role of androgens in adults teleost SG remain elusive. On the other hand, the AR is also important in females (Shiina *et al.*, 2006; de Waal *et al.* 2008). On the other hand, the AR shows a widespread expression pattern over different tissues, suggesting a broad spectrum of androgen-induced biological activities (de Waal *et al.*, 2008).

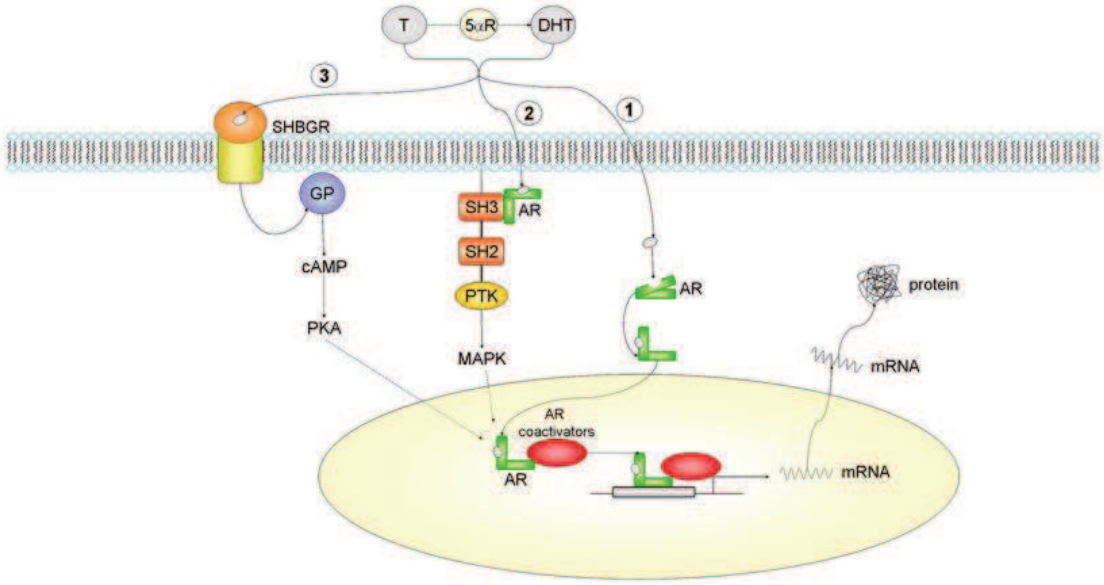

Figure 5. Androgen actions via intracellular androgen receptor mediated pathways. T can be converted to DHT by the 5αR enzyme. 1) In the classical pathway, androgen freely passes through the membrane bi-layer and binds cytoplasmic androgen receptor (AR). Bound AR translocates to the nucleus, binds to a DNA response element on a promoter of an androgen responsive gene and stimulates transcription. 2) Bound AR interacts with the SH3 domain of the tyrosine kinase c-Src to activate the MAPK pathway and influence AR-mediated transcription via phosphorylation of coactivator/ receptor complexes. 3) Androgen bound to steroid hormone binding globulin (SHBG) can activate SHBG receptor (SHBGR) and lead to an increase in PKA activity. PKA may influence AR-mediated transcription via alteration of phosphorylation status of AR and AR coregulators. Abbreviations: T = testosterone, DHT = dihydrotestosterone, 5αR = 5 alpha reductase enzyme, AR = androgen receptor, PKA = protein kinase A, GP = g-protein, SH2 = Src homology domain 2, SH3 = Src homology domain 3, PTK = protein tyrosine kinase, MAPK = mitogen-activated protein kinase, SHBGR = steroid hormone binding globulin receptor, cAMP = cyclic adenosine monophosphate. (Foradori *et al.*, 2007).

As we saw in the previous section, in teleost fish, sex steroid hormones can modify the immune response, but the results are contradictory. The effects of estrogens and androgens are mediated by nuclear steroid receptors, ER and AR) respectively, although other alternative pathways have also been described for the effects of sex steroids hormones. In this respect, AR has been found in leukocytes, firstly describe in salmonids (Slater *et al.*, 1995) and in immune competent tissues of sea bass (Blázquez & Piferrer, 2005), zebrafish (de Waal *et al.*, 2008) and gilthead seabream (Castillo-Briceño *et al.*, 2013; Águila *et al.*, 2013). Interestingly, in gilthead seabream, although AGs influx to the gonad is related with the increase of E_2 serum levels, these cells do not present any of the three receptor estrogens (ERs) (Pinto *et al.*, 2006; Liarte *et al.*, 2011), moreover, the effect of the androgens are contradictory. So this data, might suggest that non-genomic and alternatives pathways are implicated in the regulation of immune cells activities and the interaction immune-reproductives.

5. Alternative splicing.

Alternative splicing of precursor messenger RNA (pre-mRNA) was described 30 years ago (Nilsen and Graveley, 2012). Alternative splicing is a process for increasing functional diversity from a limited set of genes, by which several mRNA are generated from a single gene (Dehm & Tindal, 2011; Laurentino *et al.*, 2013). This mechanism is correlated with the complexity of organisms, and could play an important role in different processes, mainly developmental processes (Foulkes *et al.*, 1992; Nilsen & Graveley, 2010; Laurentino *et al.* 2013). The estimated number of genes that encode more than one protein as a result of alternative splicing of a pre-mRNA has steadily risen over time (Nilsen and Graveley, 2012). Alternative splicing involves the differential use of splice sites to create protein diversity. Nearly all instances of alternative splicing result from the use of one or more of four basic modules (Figure 6): a) alternative 5′ splice-site choice, b) alternative 3′ splice-site choice, c) cassette-exon inclusion or skipping, and d) intron retention.

The number of mechanisms that are known to be involved in splicing regulation approximates the number of specific splicing decisions that have been analyzed in any detail, and the most extensively studied cases of alternative splicing are regulated alternative splicing events. The regulation in this context means that distinct splicing patterns are observed in different cellular environments, can be tissue specific or

dictated by developmental (Sánchez, 2008) or differentiation-specific (Boutz *et al.*, 2007; Makeyev *et al.*, 2007;). In addition, some alternative splicing patterns have been shown to be modulated in response to external stimuli, such as depolarization of neurons (Xie *et al.*, 2001) or activation of signal transduction cascades (Shin *et al.*, 2004; Lynch, 2007).

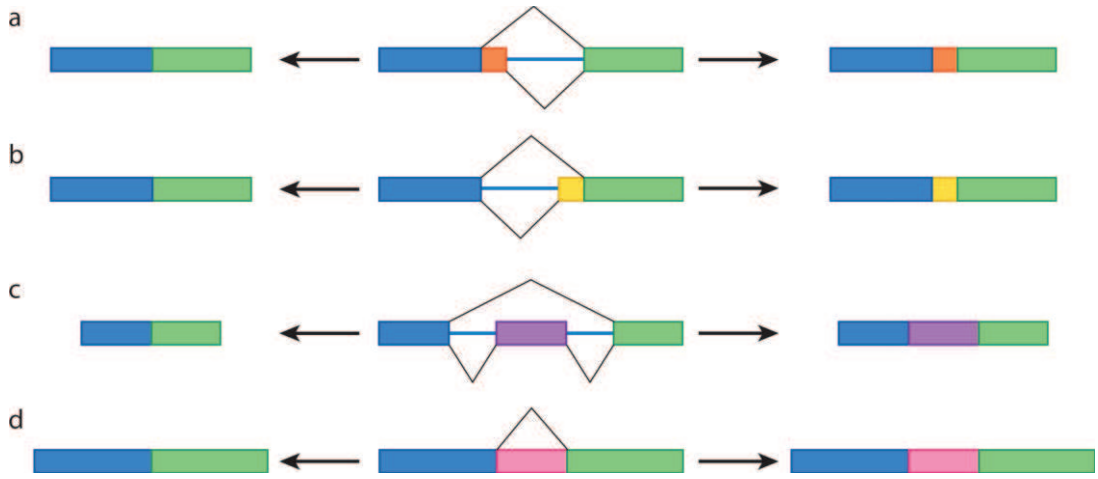

Figure 6. Types of alternative splicing. There are four basic types of alternative splicing: alternative 5′ splice-site selection (**a**), alternative 3′ splice-site selection (**b**), cassette-exon inclusion or skipping (**c**) and intron retention (**d**). The rectangles in the centre represent pre-mRNAs. For each pre-mRNA, the black lines span the regions that can be spliced out, with the lines above corresponding to the mature mRNA shown on the left and the lines below to the mRNA on the right. That is, the mRNA that is synthesized when the central exon (or intron in **d**) is skipped is shown on the left, and the mRNA that is synthesized when this sequence is included is shown on the right. In **d**, the pink portion is considered an exon when included (right) and an intron when skipped (left) (Nilsen and Graveley, 2012).

Most processes of alternative splicing occur in reproductive and immune tissues. In testis, the alternative splicing is so abundant due to be the site of an extensive developmental process (Elliot & Grellscheid, 2006). On the other hand, the immune system also present great varieties of alternative splicing because it requires high degree of diversity and the ability of rapidly adapt and response to changes in environmental conditions (Lynch, 2004).

5.1. Alternative splicing of AR.

The alternatively splicing variants are commonly generated in steroid hormone receptors, and testis is the tissue where is most frequent (Hirata *et al.*, 2003; Yeo *et al.*,

2004; Elliott & Grellscheid, 2006). In humans, many AR variants exist, some occurs naturally in normal tissues and other are produced in unhealthy tissues by different mechanisms (Dehm & Tindall, 2011). A natural alternative splice of AR in human is AR45, is expressed in diverse tissues including heart, muscle, uterus, prostate, lung, and breast, with no apparent expression in brain. This alternative variant of AR yield a 45 KDa receptor isoform with all domains, only NTD is different to wild type NTD. This variant interacts with the full-length AR (ARfl), and it is a negative regulator of AR signaling (Ahrens-Fath *et al.* 2005). However, the most alternative splices are related with diseases. The alternative splices of AR can be divided into 2 groups:

a) Variants which alternative splice produces a loss of function of AR truncated protein (Figure 7).

b) Variants which have a gain of function of AR (Figure 8).

In the first case, the alternative splices of AR are related with androgen insensitivity syndrome (AIS). The truncated AR proteins have impaired function that prevents normal androgen signaling and proper development of internal and external male phenotypes (Brinkmann, 2001). The most severe AIS phenotype is complete AIS (CAIS), where individuals have a complete female appearance. Partial AIS (PAIS) includes a wide range of phenotypes including individuals with a primarily female appearance as well as individuals with a primarily male appearance (Dehm & Tindall, 2011). These pathologic transcripts present different mechanisms, the splicing and different truncated AR protein (Figure 7). Some variants produce complete protein but are not able to bind androgens, others give a truncated protein only with NTD, and others do not present DBD. However, most of these pathologic transcripts are produced from an altered gene sequence.

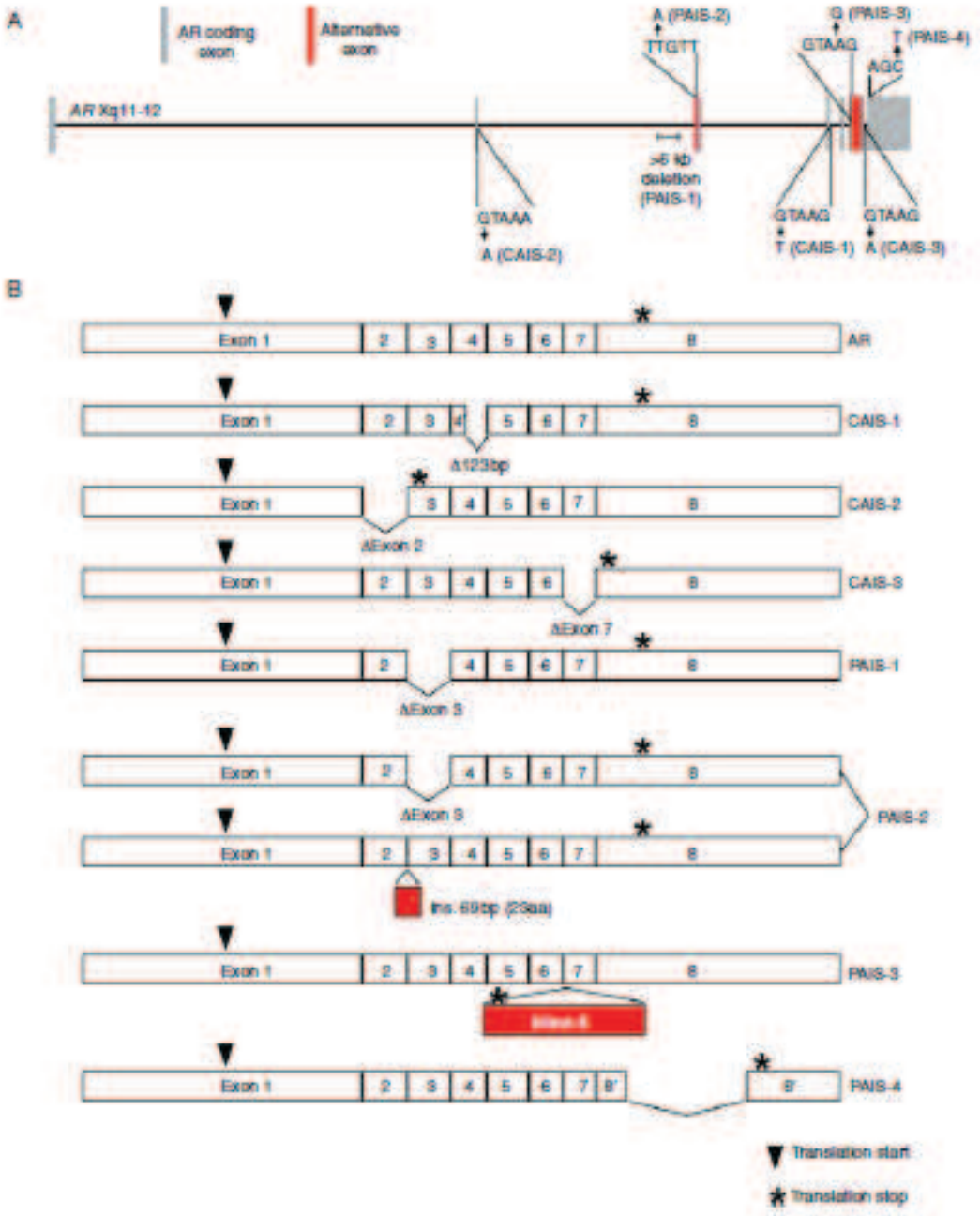

Figure 7. Mutations and structural alterations in the AR gene disrupt mRNA splicing patterns in androgen insensitivity syndrome (AIS). (A) Schematic of the AR gene locus with intronic point mutations and structural alterations that have been identified in individuals with complete AIS (CAIS) or partial AIS (PAIS). Predominant AR mRNA isoforms expressed in individuals with these specific gene alterations are illustrated in (B) (Dehm & Tindall, 2011).

On the other hand, AR varianst with gain of function are related with cancer, mainly, prostate cancer (PC). Most of the variants, in these cases, can give rise to truncated AR protein isoforms with lack the LBD (ARΔLBD), can bind DNA independent of androgens and present a constitutively activity (Dehm & Tindall, 2011). These variants are described, mainly, in cases of PC resistant to androgen depletion therapy (ADT), and some are the most variant in cases of castration resistant-PC (CRPC), in which the presence of these variants produce bone metastases and short survival in the individuals (Hörnberg *et al.*, 2011). However, the function of these variants might be different, some ARΔLBD forms do not interact with ARfl and act as an independent factor (Dehm & Tindall, 2011), while other variants are able to interact with ARfl and enhance the ligand-dependent and ligand-independence activity ((Dehm *et al.*, 2008; Dehm & Tindall, 2011; Hörnberng *et al.*, 2011), maybe through a mechanism of stabilization of protein or even with the induction of ARfl translocation to the nucleus in absence of the androgens (Dehm & Tindal, 2011).

However with the great variety of alternative splice of AR and the great diversity of functions in which they are implicated, the mechanisms of regulation of AR splicing are unclear. One possible mechanism for this increase in truncated AR isoform expression could be changes in expression or activity of factors that regulate AR, however, the alterations in AR splicing patterns can also be stable due to sequence or structural changes in the genomic DNA template (Dehm & Tindall, 2011).

To date, the knowledge of the presence of splice AR variants is limited to humans, mainly in pathological conditions. Recently, some AR variants have been identified in healthy human tissues, and the presence of these variants in other vertebrates like fish have been demonstrated (Laurentino *et al.*, 2013). The conservation of AR splicing pattern in different tissues and in evolutionarily distant vertebrate species could indicate the functional importance of these AR forms (Laurentino *et al.*, 2013). Interestingly, in gilthead seabream, an alternative splice of AR, which produce AIS in humans (Hellwinkel *et al.*, 1999), has been described in testis (Laurentino *et al.*, 2013). This variant is characterized by the deletion of exon 2 and introduction of a premature stop, giving a protein only with NTD, this variant is known as $AR\Delta 2^{stop}$. However, gilthead seabream could present more alternative transcripts due to the complexity of reproductive cycle, in which diverse processes are implicated and that can perform to the sex change in males after the second reproductive cycle.

Figure 8. Alternatively spliced AR isoforms identified in prostate cancer (PCa). (A) Schematic of the AR gene locus with locations of alternatively spliced, cryptic exons illustrated in red. The location of an intragenic tandem duplication identified in 22Rv1 cells is indicated. (B) Alternative splicing of cryptic exons in the AR locus or exon skipping gives rise to COOH-terminally truncated AR mRNA isoforms that encode constitutively active, ligand-independent transcription factors. CE20 denotes the use of an alternative splice acceptor site for splicing of exon CE2 downstream from exon 3 (Dehm & Tindall, 2011).

II. OBJECTIVES

The specific objectives of this work are:

1. To evaluate the effect of exogenous testosterone on the sex steroid hormone balance and the gonadal physiology of gilthead seabream males.
2. To characterize at molecular and functional levels a constitutively active androgen receptor variant.

CHAPTER I. *Testosterone implants modify the steroid hormone balance and the gonadal physiology of gilthead seabream (Sparus aurata L.) males.*

Abstract.

Androgens can induce complete spermatogenesis in immature or prepubertal teleost fish; however, many aspects of the role of androgens in adult teleost spermatogenesis remain elusive. We used the *in situ* forming microparticle (ISM) system containing 1 mg of testosterone (T)/kg body weight (T-ISM) in a homogenous population of gilthead seabream at testicular involution stage to study *in vivo* the effects of T on the sex steroid hormone balance and on the physiology of the gilthead seabream gonad. The levels of T, 11-ketotestosterone (11KT) and 17β-estradiol (E_2) in plasma, gonad and liver were determined in T-ISM implanted specimens after 7, 14, 21 and 28 days. The effect of T-ISM was evaluated on (i) *de novo* synthesis and metabolism of T in the gonad and liver by measuring the gene expression levels of the main steroidogenic proteins involved, (ii) the progress of spermatogenesis, (iii) the presence of different leukocyte cell types in the gonad, and (iv) the mRNA expression of some genes involved in the leukocyte migratory influx into the gonad and of some immune-relevant molecules. T-ISM implants promote an increase of T up to supra-physiological levels which induce a depletion of E_2 levels and maintain the 11KT levels at physiological concentrations. The gene expression profile of some steroidogenic enzymes in gonad and liver ruled out the transformation of T into estrogenic compounds following T-ISM implantation whereby androgens may also be involved in the leukocyte migratory influx, which occurred even when cytokine, chemokine and cell adhesion molecule gene expressions were down-regulated. Moreover, T-ISM implants block germ cell proliferation, although increased *dmrt1* gene expression may prevent the complete depletion of germ cells in the gonad. Furthermore, T down-regulated the expression of several *tlr* genes, which may result in the inhibition of the immune response in the gonad through the impaired ability to recognize and respond to pathogens.

Keywords: Testosterone, steroidogenic enzymes, leukocytes, testis, gilthead seabream

1. Introduction.

Androgens can induce complete spermatogenesis in immature or prepubertal teleost fish (Campbell *et al.*, 2009; Cavaco *et al.*, 1998; Miura *et al.*, 1991); however, many aspects of the role of androgens in adult teleost spermatogenesis remain elusive. It is clear that androgens (testosterone, T, and 11-ketotestosterone, 11KT) are essential for controlling sexual differentiation and spermatogenesis in males by binding to androgen receptor (AR), thereby activating or repressing the expression of specific genes (Borg, 1994; Devlin & Nagahama, 2002; Leet *et al.*, 2011). T levels increase in both males and females during gonadal development, while 11KT is considered to be the dominant androgen in males (Borg, 1994). However, other androgens derived from T, such as 11β-hydroxytestosterone (OHT) or dihydrotestosterone (DHT), have also been detected in breeding specimens of several fish species where they clearly have an *in vivo* androgenic effect (Borg, 1994; Margiotta-Casaluci & Sumpter, 2011). Furthermore, T, an aromatizable androgen, is locally transformed into 17β-estradiol (E_2), triggering estrogenic effects, while DHT, a non-aromatizable androgen, may also be locally transformed into 5α-androstane-3β, 17β-diol (βdiol) (Mouriec *et al.*, 2009), a well-known estrogenic compound that strongly stimulates the transcriptional activity of mammalian estrogen receptor (Kuiper *et al.*, 1998). The gonad is the main organ that synthesizes biologically active steroids *de novo* (Stocco, 2001), while the liver is not a typical steroid-producing organ, but rather a steroid-metabolizing organ (Arukwe, 2008). Both, *de novo* synthesis and metabolism of steroid hormones are controlled by rapid changes in the activities of the steroidogenic enzymes, by the availability of substrate or by changes in the amounts of the steroidogenic enzymes through the regulation of the amounts of the specific mRNAs encoding them (Miller, 1989).

The gilthead seabream (*Sparus aurata* L.) is a seasonally breeding, marine, protandrous hermaphrodite teleost with a bisexual gonad, which has a functional testicular area and a non-functional ovarian area, at least during the two first reproductive cycles (RCs). The reproductive cycle is divided into four stages: spermatogenesis, spawning, post-spawning, and resting or testicular involution stage depending on whether the fish are in their first or second RC (Chaves-Pozo *et al.*, 2005a; Liarte *et al.*, 2007). The testis of this specie undergoes abrupt morphological changes, especially after spawning, including a massive infiltration of acidophilic granulocytes (AGs), an immune cell type essential for the normal physiology of the

gonad, and whose presence in the gonad is actively regulated by tissue-specific factors and hormones (Chaves-Pozo, 2003, 2005a, b; Liarte *et al.*, 2007). In fact, an endogenous increase of the E_2 plasma levels in male specimens is correlated with AGs migration into the testis after spawning (Chaves-Pozo *et al.*, 2008a; 2012), while exogenous E_2 or 17α-ethinyl estradiol (EE_2) accelerates the final events of spermatogenesis, and promotes a massive leukocyte infiltration in the testis by up-regulating the expression of several genes involved in regulating leukocyte trafficking (Liarte *et al.*, 2007; Chaves-Pozo *et al.*, 2003, 2008b; Cabas *et al.*, 2011a). Moreover, short-term exposure to E_2 inhibits the proliferation of spermatogonia in their early stages, while long-term exposure to E_2 restarts Sertoli cell proliferation (Chaves-Pozo *et al.*, 2007). Interestingly, both estrogenic compounds deplete the plasmatic androgen levels of gilthead seabream concomitantly with the suppression of spawning, suggesting that the induction of spawning is an androgen-dependent issue (Chaves-Pozo *et al.*, 2008b; Cabas *et al.*, 2011a). Furthermore, 11KT and T, the main androgens in fish, play different and specific roles in the testicular physiology of gilthead seabream as they peak at different stages (Chaves-Pozo *et al.*, 2008a). Moreover, their profiles suggest that T is not essential in the testicular regression process that occurs after spawning, when the infiltration of leukocytes is enhanced (Chaves-Pozo *et al.*, 2005a). Although, most studies on reproductive dysfunctions in aquatic species have focused on endocrine disrupting chemicals (EDCs) with estrogenic action (Leet *et al.*, 2011), recent studies have highlighted the occurrence of androgenic environmental contaminants with adverse effects on reproduction and development (Ekman *et al.*, 2011).

The above observations prompted us to examine the effect of T on the balance of sex steroid hormones and on the physiology of the gonad (proliferation, apoptosis, leukocyte influx and immune status) of mature male specimens of the gilthead seabream, taking into account the local transformation of T in gonad and liver by measuring the expression levels of the genes coding for the main steroidogenic proteins involved. To this end, we used the *in situ* forming microparticle (ISM) system, which delivers sex steroids without promoting significant physiological alterations in gilthead seabream and has been used to show that T modulate the inflammatory response of head-kidney leukocytes in the gilthead seabream (Castillo-Briceño *et al.*, 2013).

2. Materials and Methods.

2.1. Animals and experimental design.

Healthy mature gilthead seabream (*Sparus aurata* L., Actinopterygii, Perciformes, Sparidae) males of 482 ± 7.45 g mean body weight (bw), at the spermatogenesis stage of their second RC, were bred and kept at the Centro Oceanográfico de Murcia (IEO, Mazarrón, Murcia). The fish were kept in 2 m^3 tanks with the water temperature ranging from 14.6 to 17.8 ºC, a flow-through circuit, a suitable aeration and filtration system and a natural photoperiod. The environmental parameters, mortality and food intake, were recorded daily.

Spermiogenic active males, determined by gentle abdominal massage, were separated (December) from non-spermiogenic ones and reared for 30 days (January). The specimens were anesthetized with 40 µl/l of clove oil before handing and injected them; after that three experimental groups were established: (i) fish (n=26) injected with ISM implants without T (control); (ii) fish (n=26) injected with T-ISM containing 1 mg T/kg bw, and (iii) fish (n=26) not injected. The specimens were fed three times a day *ad libitum* for 28 days with a commercial pellet diet (44% protein, 22% lipids, Skretting, Spain) and fasted for 24 h before sampling (7, 14, 21 and 28 days post-implantation, dpi). Six fish (group and sampling time) were anesthetized with 40 µl/l of clove oil and blood was obtained from the caudal peduncle using ammonium-heparinized syringes and the plasma samples, obtained by centrifugation (10,000g, 1 min, 4 ºC), were immediately frozen in liquid nitrogen and stored at -80 ºC until use. Then, specimens were decapitated and weighed, and the gonads and liver were removed, weighed and embedded in TRizol or immediately frozen in liquid nitrogen and stored at -80ºC until processed for gene expression or sex steroid hormone levels analysis, respectively. The gonads were also processed for light microscopy. 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. In situ forming microparticle (ISM) system.

ISM implants have previously been used in gilthead seabream (Castillo-Briceño *et al.*, 2013). Briefly, polymer solutions were prepared by mixing poly-D,L-lactate-co-

glycolate PLGA (P2066-1G, Sigma) with the solvent 2-pyrrolidone (Fluka Analytical) 20 % (w/w), in glass vials until a clear solution was formed. T (Sigma) was dissolved in polymer solution 10% (w/w) and the ISM implants were prepared by emulsifying the steroid-containing polymer solution into peanut oil (external oil phase) at a polymer to oil phase ratio of 0.1:1 in a sonication bath (Ultrasons 3000512, P Selecta) for 10 minutes at room temperature. Pluronic F127 (Sigma) 1 % (w/w) based on the amount of the total formulation, and aluminum monostearate (Fluka Analytical) 2 % (w/w) based on the oil phase, were added to increase the stability of the emulsions. T-ISM implants were prepared with 1 mg of T/kg bw and the implants, both ISM (without T) and T-ISM, were placed by an intramuscular injection, approximately 2 cm below the dorsal fin, in order to achieve plasma concentrations above physiological levels (up to 2 ng/ml) (Chaves-Pozo *et al.*, 2008a).

2.3. Gonadosomatic and hepatosomatic indexes.

The gonadosomatic index (GSI) was calculated as an index of the reproductive stage [100*(MG/MB) (%)] and the hepatosomatic index (HSI) was calculated as an index of the toxic effect of the treatment [100*(MH/MB) (%)], where MG is gonad mass (in grams), MH is hepatic mass (in grams) and MB is body mass (in grams).

2.4. Analytical techniques.

Plasma and tissue levels of T, 11KT and E_2 were quantified by ELISA following the method previously used in gilthead seabream (Chaves-Pozo *et al.*, 2008a). Steroids were extracted from 40 µl of plasma or 10 µl of tissue (1g/ml) homogenized in lysis buffer [50 mM phosphate buffer with 10 mM DTT (Sigma) and 250 mM sucrose (Sigma)] in 1.3 ml of methanol (Panreac). Then, the methanol were evaporated at 37º C and the steroids were resuspended in 400 µl of reaction buffer [0,1 M phosphate buffer with 1mM EDTA (Sigma), 0.4 M NaCl (Sigma), 1,5 mM NaN_3 (Sigma) and 0.1% albumin from bovine serum (Sigma)] and 50 µl were used in each well so that 5 µl of plasma or 1.25 µl of homogenized tissue were used in each well for all the assays. T, 11KT and E_2 standards, 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 x 10^{-4} to 5 ng/ml (0.03-250 pg/well), a blank and a non-specific binding control (negative control) were established in all the

assays. Standards and extracted plasma and tissue homogenated samples were run in duplicate and all the measures were corrected with the blank and negative control. The lower limit of detection for all the assays were 6.1 pg/ml for plasma and 30.5 pg/mg for tissue samples. The intra-assay coefficients of variation (calculated from sample duplicates) were 13.2 ± 2.3% for T, 12.6 ± 2.4% for 11KT and 6.2 ± 1.2% for E_2 assays in plasma and 21.7 ± 2.6% for T, 5.4 ± 1.1% for 11KT and 22.5 ± 3.1% for E_2 assays in tissue homogenates analysis. 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).

2.5. 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 degree of development of each specimen. The sections were subjected to an indirect immunocytochemical method using three monoclonal antibodies (mAbs) specific to: i) gilthead seabream AGs (G7) [24], ii) gilthead seabream immunoglobulin M (IgM, Aquatic Diagnostic) to detect B lymphocytes, or iii) proliferating cell nuclear antigen (PCNA, Sigma) to analyze cell proliferation at the optimal dilution of 1:100, 1:250 or 1:1000, respectively, as described previously [25]. The specificity of the reactions was determined by omitting the first antiserum. Some other sections, obtained from gonads fixed in 4% paraformaldehyde solution, were subjected to *in situ* detection of DNA fragmentation (TUNEL) assay to identify apoptotic cells (*in situ* cell death detection kit; Roche), as described previously (Chaves-Pozo *et al*., 2007). Slides were examined with an Axiolab (Zeiss) light microscope.

2.6. Analysis of gene expression.

Total RNA was extracted from gonad fragments with TRIzol reagent (Invitrogen) following the manufacturer's instructions, pooled using the same amount of RNA from 3 fish per group and sampling day (2 pools per group and sampling time) and treated with DNase I, amplification grade (1 unit/µg RNA, Invitrogen). SuperScript III RNase H− Reverse Transcriptase (Invitrogen) was used to synthesize first strand cDNA with oligo-dT18 primer from 1 µg of total RNA, at 50 ºC for 60 min.

The expression of genes coding for (i) steroidogenesis-related molecules: steroidogenic acute regulatory protein (*star*), cholesterol side chain cleavage cytochrome P450 (*cyp11a1*), steroid 11-beta-hydroxylase (*cyp11b1*), aromatase (*cyp19a1a*), 11β- hydroxysteroid dehydrogenase (*hsd11b*), 5α reductase (*srd5a*) and 3β-hydroxysteroid dehydrogenase (*hsd3b*), (ii) testicular specific, double sex-and mab3-related transcription factor 1 (*dmrt1*); (iii) CC chemokine *ccl4* and leukocyte adhesion molecule E-selectin (*sele*), and (iv) relevant-immune molecule genes: interleukin 1β (*il1b*), tumour necrosis factor α (*tnfa*), transforming growth factor β1 *(tgfb1*), and toll-like receptor (*tlr*)*5s*, *tlr5m* and *tlr9* 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.*, 2008b). 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 wells and repeated at least twice. Less than 2 % variation in *rps18* gene expression was observed between biological replicates.

Table 1. Gene accession numbers and primer sequences used for gene expression analysis.

Gene	Accession number	Name	Sequence (5'-3')	Use
Star	AM905934	F1	ACATCGGGAAGGTGTTCAAG	Real time PCR
		R1	TCTCTGCAGACACCTCATGG	
cyp11a1	FM159974.1	F	CGCTGCTGTGGACATTGTAT	Real time PCR
		R	CATCATGTCTCCCTGGCTTT	
cyp11b1	FP332145	F	GCTATCTTTGGACCCCATCA	Real time PCR
		R	CTTGACTGTGCCTTTCAGCA	
cyp19a1a	AF399824	F2	CAATGGAGAGGAAACCCTCA	Real time PCR
		R2	ATGCAGCTGAGTCCCTGTCT	
hsd11b	AM973598	F	AGACATGGGCAACGAGTCAG	Real time PCR
		R	TCCACATCTCCCTCCCACAT	
srd5a	AM958800	F	TGCACTTTCGTGACTCTGCT	Real time PCR
		R	TTTCGCACAAGACGTCCAGA	
hsd3b	HS985587	F	GGAGGACAAACTGGTGGAGG	Real time PCR
		R	ACATTCTCCGTTCCGGTGAC	
dmrt1	AM493678	F	GATGGACAATCCCTGACACC	Real time PCR
		R	GGGTAGCGTGAAGGTTGGTA	
ccl4	AM765840	F1	GCTGTGTTTGTGCTGATGCT	Real time PCR
		R1	GCTGGCTGGTCTTTTGGTAG	
Sele	AM749963	F1	GACAGTGAGCAGGCGTACAA	Real time PCR
		R1	ATCGCTTCATGATCCACACA	
il1b	AJ277166	F2	GGGCTGAACAACAGCACTCTC	Real time PCR
		R3	TTAACACTCTCCACCCTCCA	
tnfa	AJ413189	FE1	TCGTTCAGAGTCTCCTGCAG	Real time PCR
		RE3	CATGGACTCTGAGTAGCGCGA	
tgfb1	AF424703	F	AGAGACGGGCAGTAAAGAA	Real time PCR
		R	GCCTGAGGAGACTCTGTTGG	
tlr5s	AM953332	F	CTTCCTGAGCCAAGTTGAGG	Real time PCR
		R	TCAGCTTGTGGAGGTCCTTT	
tlr5m	AM296028	F	CCTGTCTGCAACTGTCAGGA	Real time PCR
		R	TGTGGATCTGGTTCAAGCTG	
tlr9	AY751798	F2	GGAGGAGAGGGACTGGAT	Real time PCR
		R2	GATCACACCGTCACTGTCTC	
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC	Real time PCR
		R	CTTCTGCCTGTTGAGGAACC	

2.7. Calculation and statistics.

The quantification of IgM+ and G7+ immunostained areas was calculated as the mean value ± SEM of the stained area/total area of 24 randomly distributed optical areas at 200 x 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).

All data were analyzed by one-way ANOVA and a *post hoc* test (Tukey Honestly Significant Difference) to determine differences between groups ($P \leq 0.1$). Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. Non-normally distributed data were log-transformed prior to analysis and a non-parametric Kruskal–Wallis test, followed by a multiple comparison test, was used when data did not meet parametric assumptions. In addition, non-parametric Pearson correlation tests were applied to test correlations among hormonal levels and gene expression levels for each condition. Statistical analyses were conducted using Statgraphics 15.0 (StatPoint, Inc). All data are presented as mean of standard error to the mean (SEM). Significance level (P) was fixed at 0.1 ($P < 0.1$*; $P < 0.05$**; $P < 0.01$***).

3. Results.

When spermiogenic active gilthead seabream males were separated from their non-spermiogenic counterparts and reared for 30 days, the plasmatic levels of sex steroids and the morphology of the testis of these specimens were similar to those observed at the testicular involution stage of the second RC (Liarte *et al*., 2007; Chaves-Pozo *et al*., 2008).

The implants themselves (without T) had no significant effect on the testicular morphology, GSI, HSI (Table 2) and plasmatic sex hormone levels (Table 3), when compared with non-injected fish with the exception of the plasma levels of E_2 which increased after 21days of the implantation.

3.1. T-ISM implants increase the gonadosomatic and hepatosomatic indexes.

GSI increased at 14 dpi, while HSI increased at 7, 14 and 21 dpi in T-ISM treated fish (Table 2).

Table 2: Gonadosomatic (GSI) and hepatosomatic (HSI) indexes of non injected, control (ISM implants without T) and T-ISM implanted gilthead seabream specimens.

		Days post-implant			
		7	**14**	**21**	**28**
GSI (%)	Non injected	0.55 ± 0.08^{a}	0.48 ± 0.1^{a}	0.34 ± 0.03^{a}	0.47 ± 0.07^{a}
	Control	0.43 ± 0.09^{a}	0.51 ± 0.05^{a}	0.60 ± 0.1^{a}	0.58 ± 0.1^{a}
	T-ISM	0.53 ± 0.1^{a}	1.83 ± 0.9^{b}	0.51 ± 0.1^{a}	0.4 ± 0.03^{a}
HSI (%)	Non injected	1.25 ± 0.1^{a}	1.18 ± 0.1^{a}	1.25 ± 0.1^{a}	1.09 ± 0.1^{a}
	Control	1.27 ± 0.1^{a}	1.20 ± 0.1^{a}	1.24 ± 0.1^{a}	1.42 ± 0.1^{a}
	T-ISM	1.48 ± 0.1^{b}	1.38 ± 0.1^{b}	1.60 ± 0.2^{b}	1.30 ± 0.1^{a}

Different letters denote statistically significant differences between the groups according to a Tukey post hoc test.

3.2. T-ISM implants modify the balance of sex steroid hormones.

In the plasma of T-ISM implanted fish, T levels were much higher than T levels in control fish at 7 dpi and then gradually fell until the end of the experiment, although they were still statistically higher than in control fish (Table 3). However, 11KT levels were lower than in the control fish reaching not detectable levels at the end of the experiment (Table 3) while E_2 levels were lower at all times points (Table 3). In the gonads of T-ISM implanted fish, T levels were lower than T levels in control fish at 14 dpi but were significantly higher at 21 and 28 dpi (Table 3), while 11KT levels were lower than in control fish on days 7, 14 and 28 (Table 3) and E_2 levels were similar than in control fish at 7 dpi and progressively decreased from day 14 onwards (Table 3). In the liver of T-ISM implanted fish, T levels progressively increased from day 7 onwards (Table 3). However, 11KT was lower than 11KT levels in control fish at days 7 and 28 but higher at day 14 (Table 3), while E_2 levels was only lower than in control fish at 14 dpi (Table 3). Interestingly, only in T-ISM implanted fish did the plasma levels of T positively correlate with E_2 and 11KT (Table 4) while the gonadal levels of E_2 were positively correlated with 11KT (Table 5).

Table 3: Sex steroid hormone levels in non injected, control (ISM implants without T) and T-ISM injected gilthead seabream.

			Days post-implant			
			7	**14**	**21**	**28**
plasma	**T (ng/ml)**	Non injected	0.72 ± 0.2^{a}	0.62 ± 0.2^{a}	0.50 ± 0.1^{a}	0.44 ± 0.3^{a}
		Control	0.79 ± 0.2^{a}	0.54 ± 0.1^{a}	1.68 ± 1.2^{a}	0.23 ± 0.03^{a}
		T-ISM	11.49 ± 6.08*	3.99 ± 3.47	1.82 ± 0.86	0.84 ± 0.39*
	11KT (ng/ml)	Non injected	0.11 ± 0.07^{a}	0.04 ± 0.02^{a}	0.09 ± 0.05^{a}	0.02 ± 0.01^{a}
		Control	0.1 ± 0.02^{a}	0.05 ± 0.02^{a}	0.05 ± 0.01^{a}	0.06 ± 0.02^{a}
		T-ISM	0.09 ± 0.02	0.05 ± 0.05	0.04 ± 0.01	ND
	E_2 (ng/ml)	Non injected	2.29 ± 1.0^{a}	0.51 ± 0.1^{a}	0.87 ± 0.2^{a}	0.42 ± 0.1^{a}
		Control	1.14 ± 0.1^{a}	1.02 ± 0.2^{a}	2.04 ± 0.1^{b}	0.95 ± 0.2^{a}
		T-ISM	0.51 ± 0.24*	0.01 ± 0.01***	0.20 ± 0.09***	0.04 ± 0.01***
gonad	**T (ng/ml)**	Control	28.67 ± 0.33	68.60 ± 0.21	ND	ND
		T-ISM	10.77 ± 0.04	9.30 ± 0.16**	25.96 ± 0.08***	21.81 ± 0.33***
	11KT (ng/ml)	Control	101.08 ± 0.36	2.63 ± 0.02	0.76 ± 0.01	369.93 ± 4.41
		T-ISM	4.32 ± 0.04**	ND	0.56 ± 0.01	0.23 ± 0.00*
	E_2 (ng/ml)	Control	7.36 ± 0.06	2.54 ± 0.02	4.52 ± 0.06	0.16 ± 0.00
		T-ISM	13.29 ± 0.12	0.39 ± 0.00*	0.04 ± 0.00*	ND
liver	**T (ng/ml)**	Control	ND	ND	ND	ND
		T-ISM	218.35 ± 2.10**	1809.79 ± 7.48***	2029.66 ± 15.25***	4181.12 ± 57.68***
	11KT (ng/ml)	Control	168.49 ± 2.31	0.10 ± 0.00	205.28 ± 4.10	1983.45 ± 22.23
		T-ISM	ND	15280.97 ± 303.68**	115.33 ± 2.24	0.48 ± 0.01***
	E_2 (ng/ml)	Control	0.03 ± 0.00	2.39 ± 0.01	0.95 ± 0.01	0.09 ± 0.00
		T-ISM	ND	0.65 ± 0.01*	0.76 ± 0.01	0.03 ± 0.00

Different letters denote statistically significant differences between non-injected and control groups according to a Tukey post hoc test (P≤0.05). Asterisks denote statistically significant differences between control and T-ISM groups according to a Tukey post-hoc test (*P ≤ 0.1, **P ≤ 0.05, ***P ≤ 0.01).

Table 4: Correlations observed in plasma between the sex hormone levels in ISM (implants without T) (white squares) and T-ISM (gray squares) implanted fish groups. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference P. Written in bolds are the parameters that correlated.

plasma	T	11KT	E_2
T		**0.76** **0.00**	**0.74** **0.00**
11KT	0.12 0.58		0.40 0.06
E_2	0.27 0.22	-0.11 0.61	

3.3. T-ISM implants modify the steroidogenic enzyme gene expression in gonad and liver.

T-ISM implants slightly affect the expression of genes that code for steroidogenic enzymes in gonad (Fig. 1) and in liver (Fig. 2).

In the gonad (Fig. 1a-g), the main differences were observed 28 dpi when a complete block of the expression of *star* was observed (Fig. 1a), coinciding with an up-regulation of the transcript levels of *cyp11b1* (Fig. 1c), *cyp19a1a* (Fig. 1d), *hsd11b* (Fig. 1e), and *srd5a* (Fig. 1f). The gonad transcript levels of *cyp19a1a* (Fig. 1d) and *hsd11b* (Fig. 1e) were also up-regulated at 7 dpi, while the transcripts of *srd5a* (Fig. 1f) and *hsd3b* (Fig. 1g) were down-regulated at 14 dpi and also the transcript of *cyp19a1a* at 21 dpi (Fig. 1d). The levels of T and the expression of *cyp11b1* and the transcript levels of *cyp19a1a* and *cyp11a1* were positively correlated in control fish but not in T-ISM implanted fish (Table 5). Moreover, the gonad levels of 11KT and the expression of *cyp11a1*, together with the transcript levels of *star* and *cyp19a1a*, *hsd11b*, *srd5a* or *hsd3b* negatively correlated in T-ISM implanted fish but positively or not correlated in control fish (Table 5*)*. Positive correlations in T-ISM implanted fish but not in control fish were observed between the expression levels of *cyp11b1* and *cyp19a1a* or *hsd3b*, of *cyp19a1a* and *hsd11b*, *srd5a* or *hsd3b*, and of *hsd11b* and *srd5a* or *hsd3b* (Table 5).

Table 5: Correlation observed in gonad between the sex hormone levels and the steroidogenic enzyme gene expressions analyzed in ISM (without T; white squares) and T-ISM (gray squares) implanted fish groups. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference P. Written in bolds are the parameters that correlated.

Gonad	T	11KT	E_2	*star*	*cyp11a1*	*cyp11b1*	*cyp19a1a*	*hsd11b*	*srd5a*	*hsd3b*	*dmrt1*
T		-0.19	-0.31	-0.3	-0.08	0.05	0.19	0.23	0.24	0.26	-0.05
		0.51	0.26	0.25	0.78	0.85	0.48	0.39	0.36	0.33	0.85
11KT	-0.24		**0.81**	0.29	**-0.61**	-0.36	-0.07	-0.31	-0.20	-0.12	-0.31
	0.42		**0.00**	0.31	**0.02**	0.20	0.82	0.28	0.50	0.69	0.29
E_2	0.10	-0.22		0.18	-0.47	-0.29	-0.12	-0.29	-0.25	-0.20	-0.21
	0.72	0.44		0.51	0.08	0.29	0.66	0.30	0.37	0.48	0.45
star	-0.07	-0.31	-0.02		-0.03	-0.34	**-0.50**	**-0.56**	**-0.67**	**-0.56**	0.01
	0.81	0.28	0.94		0.88	0.10	**0.01**	**0.02**	**0.00**	**0.02**	0.97
cyp11a1	-0.14	**0.71**	-0.17	**-0.76**		0.16	0.03	-0.02	-0.02	-0.01	-0.02
	0.62	**0.00**	0.53	**0.00**		0.45	0.90	0.92	0.92	0.95	0.93
cyp11b1	**0.64**	-0.29	0.32	-0.05	0.11		**0.78**	0.31	**0.68**	**0.63**	**0.52**
	0.01	0.31	0.22	0.82	0.62		**0.00**	0.16	**0.00**	**0.00**	**0.01**
cyp19a1a	-0.38	0.38	-0.04	**-0.64**	**0.84**	0.10		**0.66**	**0.92**	**0.92**	-0.08
	0.16	0.18	0.9	**0.001**	**0.00**	0.66		**0.00**	**0.00**	**0.00**	0.71
hsd11b	-0.22	-0.19	-0.33	-0.10	0.28	-0.39	0.34		**0.88**	**0.87**	-0.29
	0.43	0.51	0.21	0.69	0.19	0.07	0.11		**0.00**	**0.00**	0.17
srd5a	0.5	0.18	-0.16	0.28	0.18	**0.63**	-0.06	-0.10		**0.99**	-0.07
	0.06	0.53	0.55	0.26	0.42	**0.00**	0.77	0.66		**0.00**	0.73
hsd3b	0.18	-0.23	-0.19	**0.76**	-0.39	0.23	-0.41	-0.1	**0.76**		-0.14
	0.55	0.47	0.51	**0.00**	0.08	0.34	0.06	0.67	**0.00**		0.52
dmrt1	**0.71**	-0.14	0.41	-0.2	0.15	**0.93**	0.06	-0.41	**0.50**	0.14	
	0.00	0.65	0.11	0.36	0.49	**0.00**	0.77	0.05	**0.01**	0.55	

In the liver, the *cyp11b1* transcripts were undetected in all the fish groups studied (data not shown). The transcript levels of *cyp19a1a* (Fig. 2a) was down-regulated at 14 dpi and undetected at 21 and 28 dpi, while the transcript of *hsd11b* (Fig. 2b), *srd5a* (Fig. 2c) and *hsd3b* (Fig. 2d) were up-regulated at 14 dpi and down-regulated at 21 dpi and also at 28 dpi in the case of *hsd3b*. In the liver, the transcript levels of *hsd11b* correlated with the 11KT levels in T-ISM implanted fish but not in control fish (Table 6).

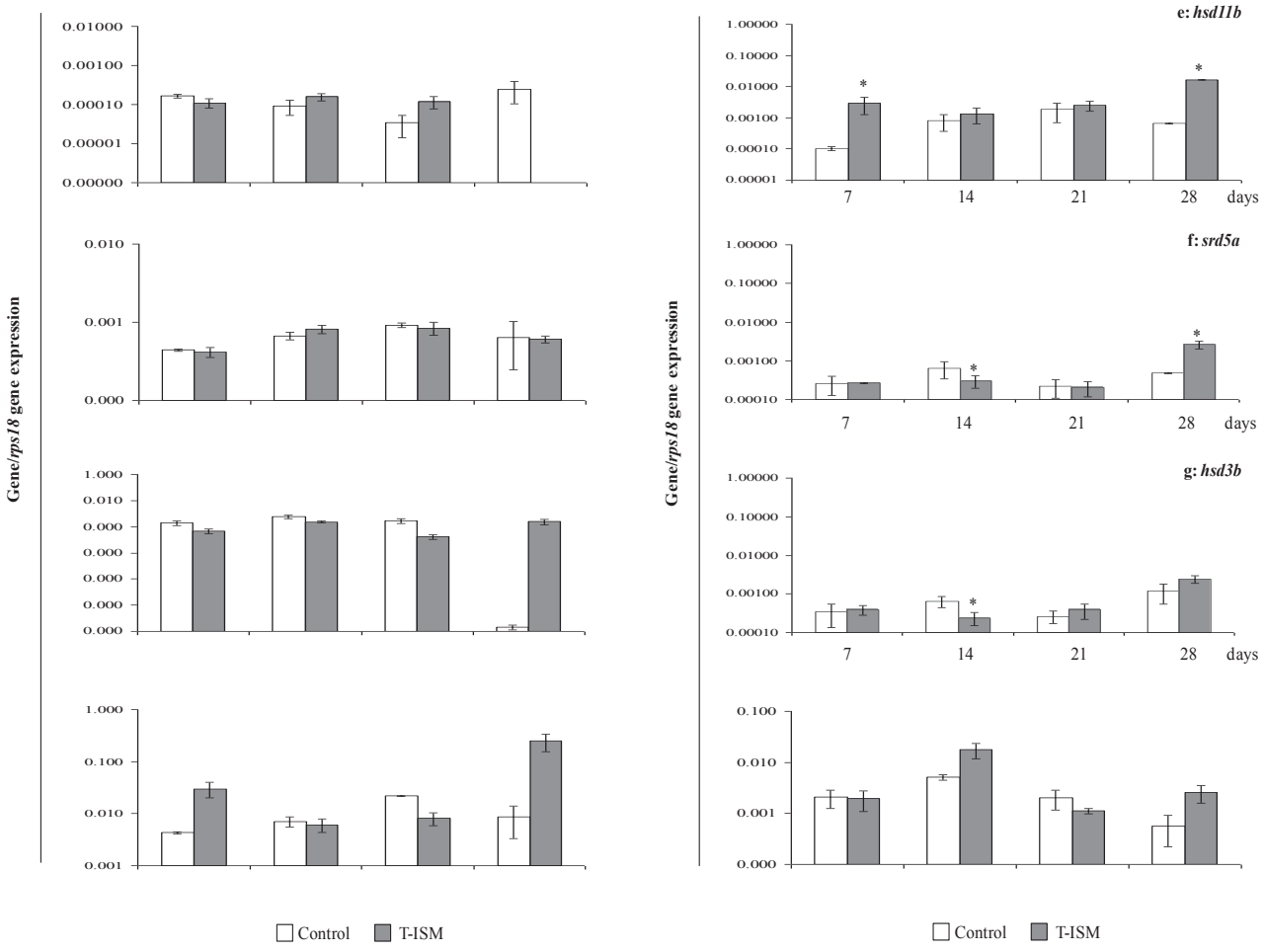

Figure 1. "Expression of genes that code for steroidogenic-relevant molecules and *dmrt1* in the gonad of gilthead seabream after T-ISM implants". The expression of *star* (a), *cyp11a1* (b), *cyp11b1* (c), *cyp19a1a* (d), *hsd11b* (e), *srd5a* (f), *hsd3b* (g) and *dmrt1* (c) genes of control (ISM implants without T) or T-ISM implanted specimens at 7, 14, 21 and 28 days post-implantation. The mRNA levels of all genes were studied by real-time qRT-PCR. Data represent means ± S.E.M. of duplicate samples corresponding to two independent pools obtained after mixing the same amount of mRNA from 3 fish (n=6 fish/group). Asterisks indicate significant differences compared with control group (*P ≤ 0.1, **P ≤ 0.05, ***P ≤ 0.01). Letters denote significant differences between different time points in T-ISM implanted fish group (P≤ 0.1). ND, non detected.

3.4. T-ISM implants modify the gonadal physiology.

The gonadal *dmrt1* transcripts were up-regulated in T-ISM implanted fish at 14 and 28 dpi (Fig. 1h) and correlated with T levels or with the *srd5a* transcript levels in control but not in T-ISM implanted fish (Table 5).

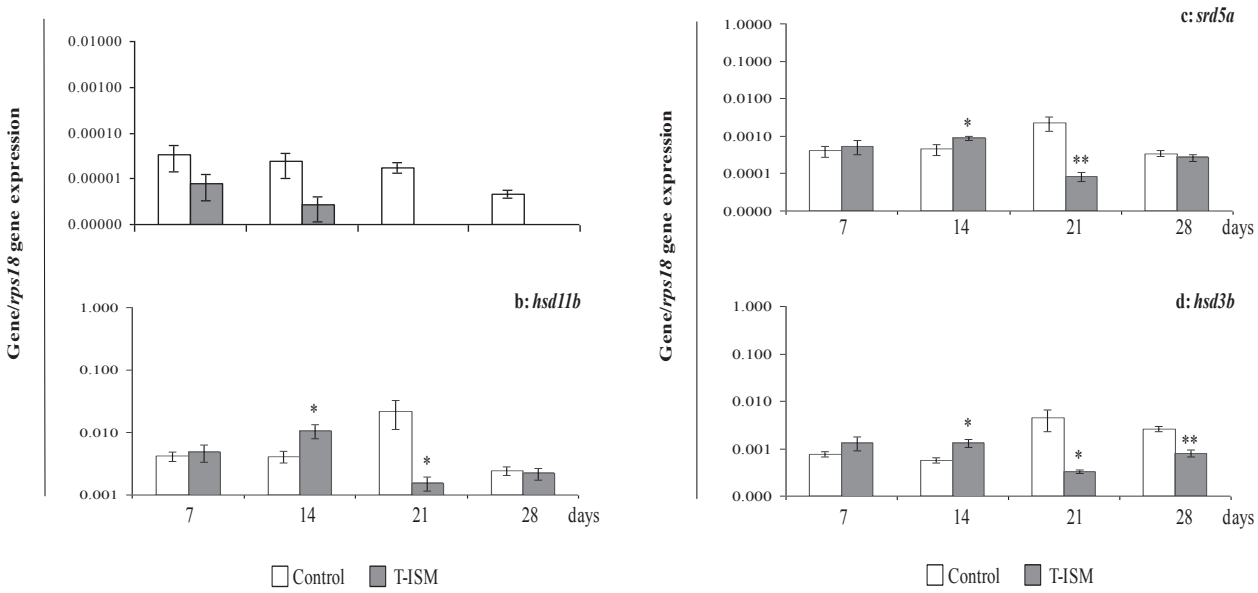

Figure 2. "Expression of genes that code for steroidogenic enzymes in the liver of gilthead seabream after T-ISM implants". The expression of *cyp19a1a* (a), *hsd11b* (b), *srd5a* (c), and *hsd3b* (d) genes of control (ISM implants without T) or T-ISM implanted specimens at 7, 14, 21 and 28 days post-implantation. The mRNA levels of all genes were studied by real-time qRT-PCR. Data represent means ± S.E.M. of duplicate samples corresponding to two independent pools obtained after mixing the same amount of mRNA from 3 fish (n=6 fish/group). Asterisks indicate significant differences compared with control group (*P ≤ 0.1, **P ≤ 0.05, ***P ≤ 0.01). ND, non detected.

No morphological differences were observed either in the testicular or in the ovarian areas between control (Fig. 3a-d) and T-ISM injected fish. The gonads showed the morphological characteristics described for the testicular involution stage in gilthead seabream [14]. In brief, the peripheral testicular area (Fig. 3a) showed some remaining post-meiotic cells (spermatocytes and spermatids, but not spermatozoa), while the internal testicular area (Fig. 3b,c) showed necrotic areas (Fig. 3b) and a well developed interstitial tissue (Fig. 3c). The spermatogenic tubules showed a very small lumen with no spermatozoa in some testicular areas or no lumen at all in other areas. Interestingly,

oocytes at the yolk vesicle stage were observed in the ovarian area of all fish (Fig. 3d). However, the proliferative activity ($Pcna^+$ cells) was completely blocked by the T-ISM treatment from day 7 onwards (Fig. 3e,f), while the apoptotic rates showed no differences between control and T-ISM injected fish (data not shown).

T-ISM implants trigger the influx of leukocytes into the gonad and modify the gene expression of several molecules involved in its trafficking. The number of $G7^+$ cells (AGs cells; Fig. 4a-c) was significantly higher in the testicular area of T-ISM implanted fish than in control fish at 21 dpi while IgM^+ cells (lymphocytes B; Fig. 4d-f) showed higher levels in T-ISM implanted fish than control fish at 14, 21 and 28 dpi. No immunostaining was observed when the first antiserum was omitted. The gonadal transcription of the chemokine gene, *ccl4*, (Fig. 5a) and the adhesion molecule E-selectin gene, *sele*, (Fig. 5b) was down-regulated at 14 and 21 dpi and at 14 and 28 dpi in T-ISM implanted fish, respectively.

Table 6: Correlation observed in liver between the sex hormone levels and the steroidogenic enzyme gene expressions analyzed in PBS-ISM (white squares) and T-ISM (gray squares) implanted fish groups. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference P. Written in bolds are the parameters that correlated.

liver	T	11KT	E_2	*cyp19a1a*	*hsd11b*	*srd5a*	*hsd3b*
T		-0.1 0.71	0.1 0.71	-0.23 0.39	-0.13 0.64	-0.12 0.67	-0.07 0.79
11KT	-0.19 0.49		-0.12 0.66	0.13 0.62	**0.58** **0.02**	0.41 0.12	0.00 1.00
E_2	-0.37 0.17	-0.30 0.28		-0.22 0.41	-0.06 0.83	-0.04 0.87	-0.04 0.89
cyp19a1a	0.4 0.14	-0.22 0.43	0.07 0.79		0.07 0.78	-0.09 0.73	-0.23 0.4
hsd11b	-0.14 0.6	-0.2 0.45	0.32 0.23	-0.06 0.83		**0.92** **0.00**	**0.52** **0.04**
srd5a	-0.16 0.56	-0.20 0.46	0.31 0.24	-0.13 0.62	**0.99** **0.00**		**0.80** **0.00**
hsd3b	-0.16 0.54	0.04 0.88	0.15 0.59	-0.26 0.34	**0.92** **0.00**	**0.91** **0.00**	

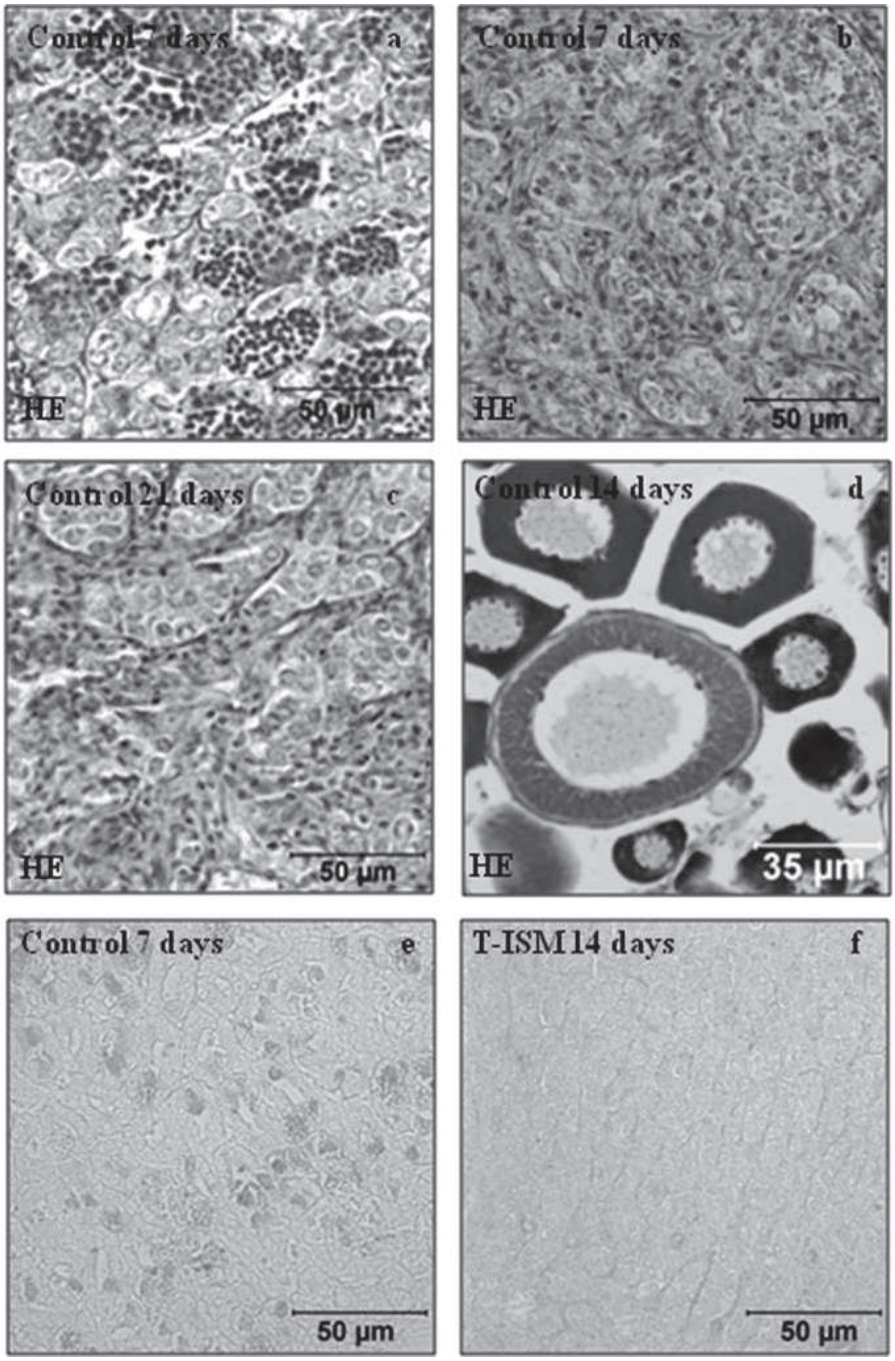

Figure 3. "Morphology of the gonad of gilthead seabream after T-ISM implants". Paraffin embedded sections of control (ISM implants without T) (a,b,c,d,e) or T-ISM implanted (f) specimens during 7 (a,b,e) or 14 (c,d,f) days post-implantation stained with hematoxylin-eosin (a-d) or immunostained with anti-proliferating cell nuclear antigen (Pcna) serum (e,f). The external testicular area (a) showed some remaining post-meiotic cells (spermatocytes and spermatids, but not spermatozoa), while the internal testicular area (b,c) showed necrotic areas delimited by dark lines (b) and a well develop interstitial tissue (arrows, c). Oocytes in the yolk vesicle stage were observed in the ovarian area of all fish (d). The proliferative activity was completely blocked by the T-ISM implants from day 7 onwards (e,f). Scale bar = 50 µm (a-f).

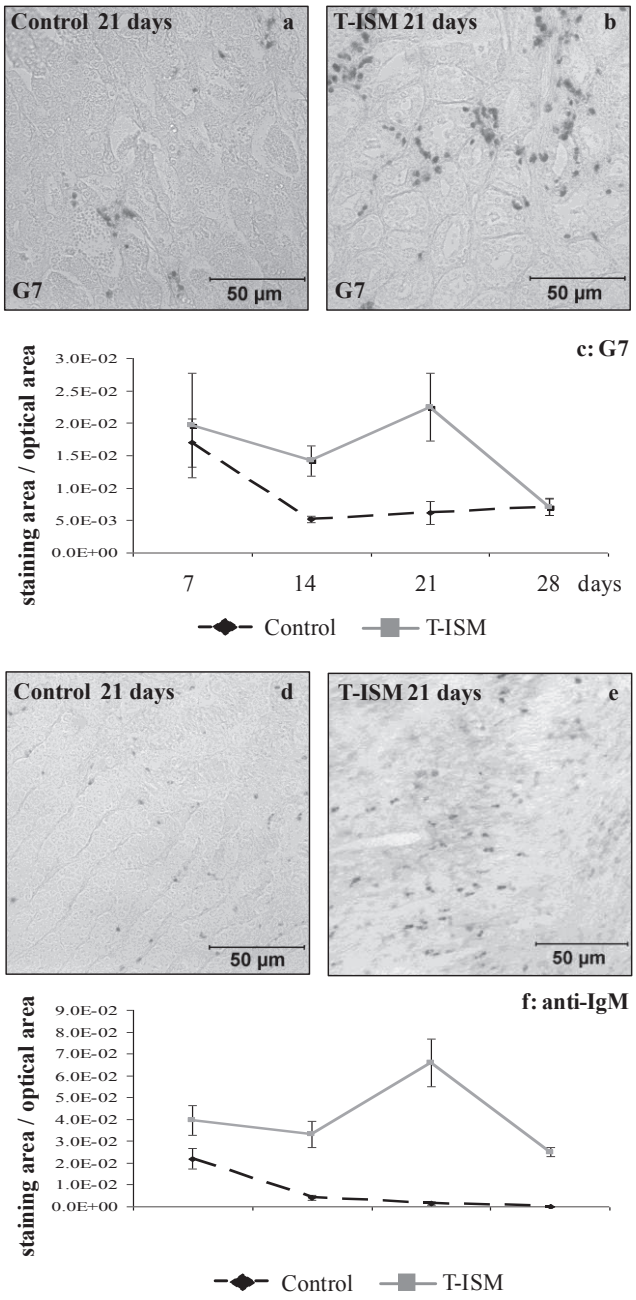

Figure 4. "Leukocytes present in the gonad of gilthead seabream after T-ISM implants". Paraffin embedded sections of the gonad of control (ISM implants without T) (a,d) or T-ISM implanted (b,e)specimens at 21 days (a,b,d,e) and subjected to immunostaining with the serum against gilthead seabream acidophilic granulocytes (G7) (a,b) or with the serum against gilthead seabream IgM (d,e). An increase in the G7 (a,b) and IgM (d,e) positive cells is observed in T-ISM implanted fish when compared with control fish. Scale bar = 50 µm (a,b,d,e). The mean of the staining area with G7 (c) or anti-IgM (f) per optical area from control and T-ISM implanted fish groups at 7, 14, 21 and 28 days post-implantation. 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 (*P ≤ 0.1, **P ≤ 0.05).

We next determined the gonadal transcript levels of some pro- and anti-inflammatory cytokines and toll-like receptors. The variations in several gene expressions observed in control fish throughout the experiment might be the normal variation observed during 28 days of development of the testicular involution stage in which a physiological inflammatory process strictly regulated occurs [14, 19]. Comparing the expression levels of control fish and T-ISM implanted fish in each time point of the experiment, we found that the expression of *il1b* had increased by 28 dpi (Fig. 5c) and *tgfb1* by 7 and 28 dpi (Fig. 5e), while the gonadal transcript levels of *tnfa* (Fig. 5d) were unchanged. However, the gonadal transcript levels of *tlr5s* (Fig. 5f) and *tlr9* (Fig. 5h) had increased by 7 and 14 dpi and decreased by 21 and 28 dpi, while the expression of *tlr5m* gene was seen to be sharply up-regulated at 28 dpi (Fig. 5g).

The levels of 11KT correlated with the expression of *ccl4*, *sele*, *tnfa* or *tlr9* genes in control fish but not in T-ISM implanted fish; the levels of E_2 only correlated with the expression of *tlr5s* in T-ISM implanted fish whereas the levels of T only correlated with transcript levels of *tlr5m* in control fish (Table 7). Moreover, there were both positive and negative correlations between the transcripts levels of the relevant immune molecules and those involved in the traffic of leukocyte analysed in control and T-ISM implanted fish (Table 7).

Table 7: Correlation observed in gonad between the sex hormone levels and several-relevant immune gene expressions analyzed in ISM (without T; white squares) and T-ISM (gray squares) implanted fish groups. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference P. Written in bolds are the parameters that correlated.

Gonad	T	11KT	E_2	*ccl4*	*sele*	*il1b*	*tnfa*	*tgfb1*	*tlr5s*	*tlr5m*	*tl9s*
T		-0.19	-0.31	-0.2	0.23	-0.03	0.39	0.28	-0.33	0.23	0.11
		0.51	0.26	0.45	0.39	0.91	0.14	0.29	0.21	0.4	0.69
11KT	-0.24		**0.81**	-0.29	0.02	0.03	-0.26	-0.34	0.92	-0.29	-0.08
	0.42		**0.00**	0.31	0.93	0.92	0.36	0.24	0	0.31	0.77
E_2	0.1	-0.22		-0.1	0.01	0.27	-0.31	-0.28	**0.70**	-0.29	0.08
	0.72	0.44		0.71	0.99	0.33	0.26	0.32	**0.00**	0.3	0.78
ccl4	0.33	**0.65**	0.12		0	0.32	0.16	**0.62**	0.01	**0.72**	**0.46**
	0.23	**0.01**	0.67		0.99	0.13	0.47	**0.00**	0.97	**0.00**	**0.02**
sele	-0.35	**0.81**	-0.01	**0.63**		0.22	-0.38	-0.11	-0.13	0.03	**0.57**
	0.21	**0.00**	0.98	**0.00**		0.3	0.07	0.61	0.53	0.89	**0.00**
il1b	0.06	-0.35	0.2	**-0.47**	**-0.6**		-0.34	0.14	0.08	-0.04	**0.77**
	0.83	0.23	0.46	**0.02**	**0.00**		0.11	0.5	0.72	0.85	**0.00**
tnfa	-0.31	**0.90**	-0.39	0.41	**0.61**	-0.36		**0.74**	-0.19	**0.59**	-0.34
	0.26	**0.00**	0.13	0.05	**0.00**	0.09		**0.00**	0.36	**0.00**	0.10
tgfb1	0.16	-0.36	0.35	-0.18	-0.19	**0.58**	-0.33		-0.1	**0.73**	0.15
	0.56	0.21	0.18	0.42	0.38	**0.00**	0.12		0.64	**0.00**	0.49
tlr5s	-0.41	**0.86**	-0.36	0.39	**0.66**	-0.39	**0.98**	-0.29		-0.16	-0.07
	0.13	**0.00**	0.17	0.07	**0.00**	0.07	**0.00**	0.17		0.47	0.74
tlr5m	**0.67**	-0.25	-0.08	0.14	-0.22	-0.13	-0.1	0.33	-0.21		0.34
	0.01	0.38	0.77	0.52	0.32	0.54	0.67	0.13	0.35		0.10
tlr9	-0.31	**0.96**	-0.29	**0.50**	**0.76**	-0.28	**0.93**	-0.26	**0.90**	-0.21	
	0.27	**0.00**	0.28	**0.01**	**0.00**	0.20	**0.00**	0.23	**0.00**	0.34	

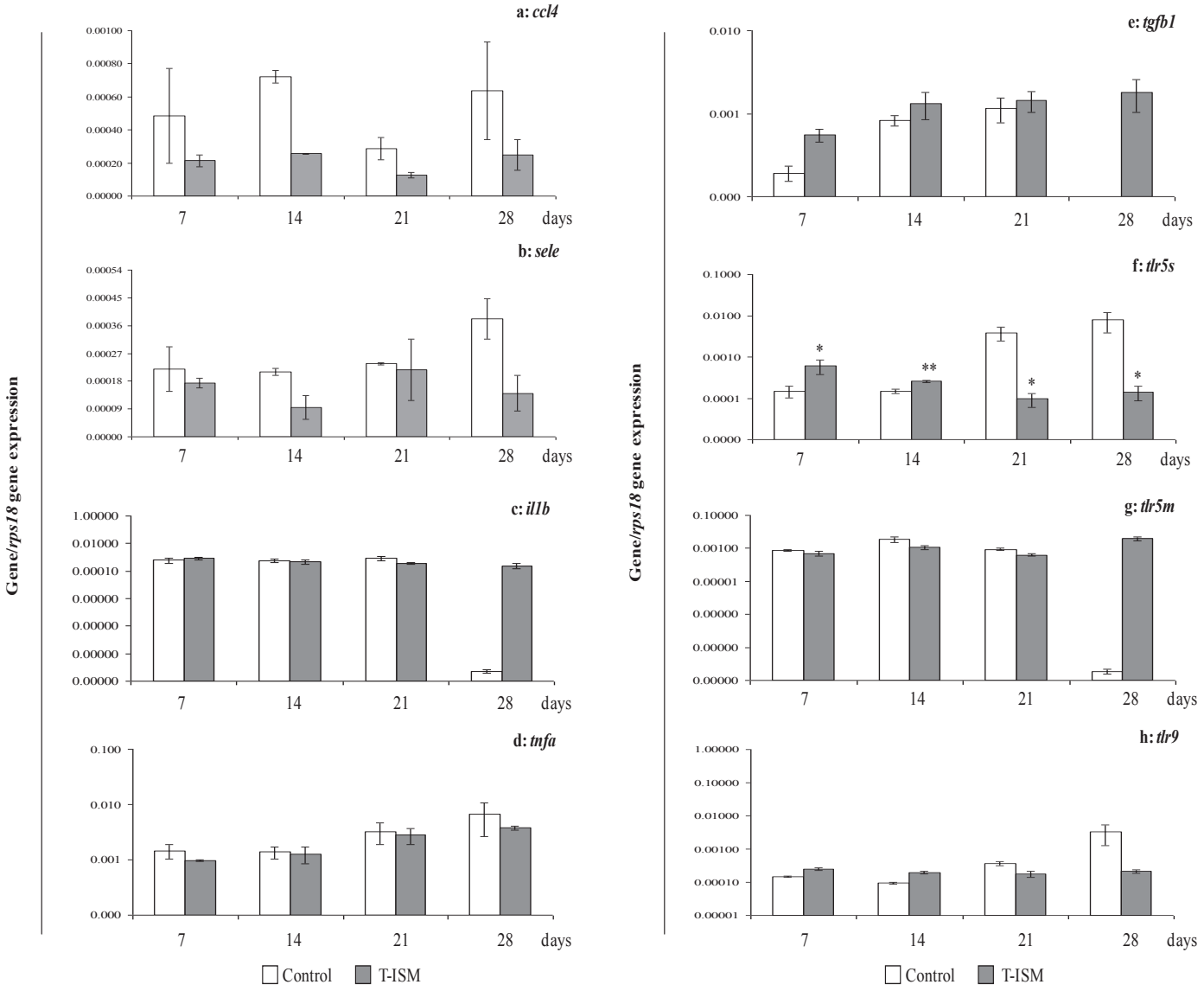

Figure 5. **"**Expression of genes that code for some immune-relevant molecules in the gonad of gilthead seabream after T-ISM implants". The expression of *ccl4* (a), *sele* (b) *il1b* (c), *tnfa* (d), *tgfb1* (e), *tlr5s* (f), *tlr5m* (g) and *tlr9* (h) of control (ISM implants without T) or T-ISM implanted specimens at 7, 14, 21 and 28 days post-implantation. The mRNA levels of all genes were studied by real-time qRT-PCR. Data represent means ± S.E.M. of duplicate samples corresponding to two independent pools obtained after mixing the same amount of mRNA from 3 fish (n=6 fish/group). Asterisks indicate significant differences compared with control group (*P ≤ 0.1, **P ≤ 0.05, ***P ≤ 0.01). ND, non detected.

4. Discussion.

A homogeneous population of gilthead seabream characterized by low levels of androgens and high levels of estrogens, as observed in the testicular involution stage in the second RC (Chaves-Pozo *et al.*, 2008a), was obtained by rearing spermiogenic active males separate from non-spermiogenic males for 30 days. At this stage, the gilthead seabream is an excellent model for studying the hormonal regulation of several processes such as the renewal of spermatogonia and Sertoli cells, the physiological migratory influx of leukocytes or the initial events that lead to sex change (Liarte *et al.*, 2007). All of these processes are thought to be triggered by estrogens, which remained higher than androgens during this stage (Chaves-Pozo *et al.*, 2007, 2008a; Cabas *et al.*, 2011a; Liarte *et al.*, 2011a; Condeca & Canario, 1999); however, the involvement of androgens in these processes has, until now, not been analysed.

Castillo-Briceño *et al.* (2013), demonstrated the feasibility of using ISM implants to deliver sex steroid hormones in gilthead seabream without promoting significant alterations in growth, survival, sex steroid plasma levels (T, 11KT and E_2) and some immune responses. Similarly, we corroborated that ISM implants, by themselves, scarcely modify sex steroid hormones levels and demonstrated that no changes are observed in the GSI, the HSI or in testicular morphology. In our study, when T-ISM implants were placed in specimens in which the testicular involution stage had been induced, T reached supra-physiological levels, while the E_2 levels did not reach physiological levels and the 11KT levels decreased by the end of the experiment (Chaves-Pozo *et al.*, 2008a). Since the synthesis of 11KT and E_2 depends on the availability of their precursor, T (Borg, 1994), changes in the plasma, gonad (organ which synthesizes *de novo* biologically active steroids (Stocco, 2001) and liver (mainly a steroid-metabolising organ (Arukwe, 2008) levels of both hormones were expected in implanted T-ISM fish. The T-ISM implants promoted a sharp increase in plasmatic T levels, while gonadal T levels remained almost steady preventing the changes in gonadal T levels observed in control specimens and enhancing the hepatic T levels, as expected for the main organ responsible for steroid processing and elimination (Arukwe, 2008). However, the fact that T-ISM implants lowered plasmatic and gonadal 11KT levels and that 11KT in liver fluctuated suggests different rates of 11KT elimination. Moreover, in the liver of T-ISM implanted fish, the levels of E_2 were similar to those of control fish.

In order to detect possible changes in the *de novo* synthesis of steroids and in the metabolism of T, we analysed the changes induced by T-ISM implants in the transcript levels of the genes that code for several enzymes involved in the synthesis and transformation of T. The decreased steroid levels in the gonads of T-ISM implanted fish suggest that *de novo* production in the gonad was not enhanced by the treatment as reflected by the down-regulation of *star* expression and the absence of modifications in the expression of *cyp11a1*, two enzymes that are synthesized rapidly in response to acute tropic hormone stimulation (Arukwe, 2008). However, the expression of *cyp11b1*, *srd5a*, *cyp19a1a*, and *hsd11b* genes was enhanced after 28 days of T-IMS treatment, probably, as a consequence of the higher levels of plasmatic T, suggesting that the transformation of T into OHT, DHT or E_2, and the transformation of OHT into 11KT was enhanced in T-IMS implanted fish, respectively. The up-regulation of *cyp19a1a* transcripts by androgens observed in the gilthead seabream gonad has also been described in the zebrafish brain and in mammals (Mouriec *et al.*, 2009). Interestingly, the transformation of DHT into βdiol was not enhanced in the gonad of T-IMS implanted fish as the transcript levels of *hsd3b* were not up-regulated at any sampling time.

Several studies have reported the expression of steroidogenic enzymes and proteins in fish hepatic tissues (Arukwe, 2008; Zhou et al, 2005), as was observed in the liver of the control specimens of gilthead seabream. In the liver, *cyp11b1* gene expression was not detected in the control or in T-ISM implanted fish; however, the gene expression of *hsd11b* was up-regulated at 14 days of T-ISM implantation and down-regulated at 21 and 28 days of T-ISM implantation, suggesting that the capacity of the liver to transform T into 11KT depends on the availability of T. The hepatic E_2 levels remained similar to those of control fish and the expression of *cyp19a1a* gene was down-regulated indicating that E_2 is not being produced in liver. Taking into account that in T-ISM implanted fish E_2 production is enhanced in the gonad, although the E_2 levels remain low in the blood and in the gonad, we can suggest that E_2 is being accumulated in the liver in T-ISM implanted fish. Furthermore, the expression of *srd5a* and *hsd3b* genes led to the decreased production of DHT and βdiol, respectively, in T-ISM treated fish at the latest sampling times of the experiment. Taken together, all these observations suggest that the βdiol production is not enhanced by T-ISM treatment and that there is no estrogenic effect derived from T transformation.

Our present knowledge on the role of androgens in testicular physiology in fish is mainly based on studies conducted in pre-pubertal individuals, when androgens stimulate the proliferation of germ cells (Campbell *et al.*, 2003; Cavaco *et al.*, 1998; Miura *et al.* 1991). However, in mammals, it is well known that the first (pubertal) wave of spermatogenesis differs from adult spermatogenesis in both regulation and timing (Handelsman *et al.*, 1999). A depletion of androgens, experimentally induced upon exogenous E_2 treatment, produces the down-regulation of testicular germ cell proliferation and an up-regulation of apoptosis in teleosts (Chaves-Pozo *et al.*, 2007; de Waal *et al.*, 2009). In contrast, the present data show that the depletion of E_2 induced by exogenous T treatment blocks germ cell proliferation, does not alter the apoptotic rate, and increases the gonadal transcription of *dmrt1*. In mammals, the depletion of *dmrt1* gene expression led to the loss of mitotic germ cells which had precociously entered meiosis (Don *et al.*, 2011). All these data explain why, even when germ cell proliferation was blocked, the testicular area was not deplete of germ cells after T-ISM implant since the maintenance of the *dmrt1* gene expression prevents germ cells from prematurely entering meiosis, as occurs upon estrogenic treatment (Cabas *et al.*, 2011a; Chaves-Pozo *et al.*, 2007). Although, further studies are needed to clearly determine why germ cell and Sertoli cell proliferation were blocked by T-ISM implant, we can venture that the depletion of estrogens is the main factor involved in blocking proliferation, since treatments of 18 days of duration with E_2 stimulate Sertoli cell proliferation [21]. All these data point to the androgen:estrogen ratio as the main factor that affects germ cell proliferation and testicular maintenance.

As regards the migratory influx of leukocytes into the gonad, previous results clearly pointed to E_2 as the main factor that triggered this influx by stimulating chemokines and adhesion molecules in the endothelial cells and macrophages (Chaves-Pozo *et al.*, 2007; Liarte *et al.*, 2011a; Liarte *et al.*, 2011b). However, our data show that, even when the plasmatic levels of E_2 were very low and the chemokine *ccl4* and the adhesion molecule *sele* gene expression were decreased in the T-ISM implanted group, migration of leukocytes into the gonad was enhanced. Our data also show that the production of βdiol is not enhanced by T-ISM implant, and that the migratory influx of leukocytes may also be regulated by androgens, probably through some pro-inflammatory cytokines, such as Il1b, whose gene expression is up-regulated. Interestingly, T-ISM implants down-regulated the gene expression of *tlr5s* and *tlr9,*

which may result in a partially impaired ability of the gonad to recognise and respond to pathogens, since *tlr5m* gene expression was conversely up-regulated and positively correlated with gonad T levels. It would be interesting to study the functional relevance of the opposite regulation of *tlr5s* and *tlr5m*, since it has been suggested that Tlr5s amplifies Tlr5m-mediated cellular responses in a positive feedback fashion (Tsujita *et al.*, 2004). Although androgens seem to depress the immune response of vertebrates (Cutulo *et al.*, 2005), our data in the gonad and previous data in the head-kidney of gilthead seabream, show that androgens enhance or diminish the immune function, depending on the activity analyzed (Castillo-Briceño *et al.*, 2013; Águila *et al.*, 2013).

5. Conclusion.

In conclusion, our experimental design allowed us to increase T levels to supra-physiological levels and reduce E_2 levels, while maintaining the main androgen of fish, 11KT, at physiological concentrations, without enhancing the transformation of T into βdiol, such conditions were then used to determine estrogen-dependent processes in male physiology. Moreover, analysis of the T, 11KT and E_2 levels in plasma, gonad and liver, together with the expression profiles of the main steroidogenetic enzymes involved in *de novo* synthesis and metabolism of T in gonad and liver provided a comprehensive explanation of the effect observed following T-ISM treatment. As regards gonad physiology, germ cell proliferation was totally blocked after T-ISM implant, although an increase in *dmrt1* gene expression may have prevented the complete depletion of germ cells of the gonad. The migratory influx of leukocytes into the testis, even when cytokine, chemokine and cell adhesion molecule gene expressions were down-regulated, points to a very complex regulation of this process. As far as the immune response is concerned, T in the gonad may partially impair the ability to recognize and respond to pathogens as a result of the down-regulation of several *tlr* genes.

CHAPTER II. *Identification of a constitutively active androgen receptor variant which is expressed in fish granulocytes and is modulated by testosterone and immune challenge.*

Abstract.

The biological activity of androgens is mediated by androgen receptor (AR), a ligand-activated transcription factor. In humans, alternative splicing of this receptor is usually implicated in process related with some diseases. However, the knowledge about the presence of AR variants in other species and its importance in immunity is scant. In this study, we report the identification of an AR variant lacking the ligand-binding domain (LBD), ARΔLBD, in the teleost fish gilthead seabream (*Sparus aurata* L.). ARΔLBD is expressed in the head-kidney (HK), the main hematopoietic and immune organ of fish, and its expression varied with the reproductive stage and is correlated with plasma testosterone (T) levels. Moreover, exogenous T increases the ARΔLBD expression in HK, whereas decreases those of the testis. In addition, ARΔLBD transcript is expressed in AGs, which are the functional equivalent of mammalian neutrophils, but not in MØs. Notably, the expression of ARΔLBD is modulated by both T and immune stimuli in AGs. Finally, we show using a transactivation assay in HEK-293 cells that the activity of ARΔLBD is constitutive and higher than those of wild type AR, which requires androgen stimulation for its activation. These results demonstrate for the first time the presence of a constitutively active variant of the AR generated by alternative splicing in vertebrate granulocytes and whose expression is modulated by androgen levels and immune challenges.

Keywords: Androgen receptor, alternative splicing, testis, testosterone, leukocytes, gilthead seabream.

1. Introduction.

A pivotal regulatory role for sex hormones, both estrogens and androgens, in cellular and humoral immune responses has been demonstrated (Cohn, 1979; Bouman *et al.*, 2005; Fairweather *et al.*, 2008; Namazi, 2009; Yeh and Chen, 2010; Lee & Chiang, 2012) , although the role of androgens has been less well investigated. The diverse roles of androgens in vertebrates, including teleosts, are mainly mediated by the nuclear androgen receptor (AR), although non-genomic pathways have also been described (Rahman & Christian, 2007). ARs has been reported in immune competent organs, such as the head-kidney, liver and spleen of sea bass (Blázquez & Piferrer, 2005) and zebrafish (de Waal *et al.*, 2008) suggesting that fish immune responses are sensitive to androgens and are susceptible to being directly and/or indirectly regulated by them.

The AR is a member of the steroid nuclear receptors transcription factor family (Dehm & Tindall, 2011) and is a ligand-activated transcription factor (Mangelsdorf *et al.*, 1995). The AR gene is composed of 8 exons (Hellwinkel *et al.*, 1999; Dehm & Tindall, 2011; Hörnberg *et al.*, 2011) and the AR protein is structurally organized into three domains: i) an amino-terminal domain (NTD) encoded by exon 1, ii) a DNA-binding domain (DBD) mainly encoded by exons 2 and 3, which includes two zinc-fingers, and iii) a ligand-binding domain (LBD) encoded by part of exon 4 and exons 5, 6, 7, and 8, where androgens and other androgen-like molecules are binding (Dehm & Tindall, 2011; Laurentino *et al.*, 2013). Exon 4 also encodes a hinge region, which acts as a flexible linker and contains the nuclear localization signal (Dehm & Tindall, 2011; Laurentino *et al.*, 2013; Zhou *et al.*, 1994). All these domains play an important functional role in the classical mode of AR activation by androgenic ligand (Dehm & Tindall, 2011).

Alternative splicing is a process for increasing functional diversity from a limited set of genes, by which several mRNA are generated from a single gene (Dehm & Tindall, 2011; Laurentino *et al.*, 2013). This mechanism is correlated with the complexity of organisms, and could play an important role in different processes (Foulkes *et al.*, 1992; Nilsen & Graveley, 2010; Laurentino *et al.*, 2013). The alternative spliced variants are commonly generated in steroid hormone receptors, and testis is the tissue where is most frequent (Hirata *et al.*, 2003; Yeo *et al.*, 2004; Elliott &

Grellscheid, 2006). In humans, many AR variants exist and are related with diseases, like androgen insensitivity syndrome (AIS), in which a loss of AR function is observed, or prostate cancer, in which an increased AR function is observed (Dehm & Tindall, 2011). Most of these variants lack one, mainly the LBD, or more domains. The LBD domain is not essential for transcriptional activity (Hörnberg *et al.*, 2011). In fact, some truncated AR variants lacking the LBD domain act as constitutively active receptors in prostate cancer cell lines (Hörnberg *et al.*, 2011; Dehm *et al.*, 2008; Dehm & Tindall, 2011). To date, the knowledge of the presence of splice AR variants is limited to humans, mainly in pathological conditions. Recently, some AR variants have been identified in healthy human tissues, and the presence of these variants in other vertebrates, such as fish, has been demonstrated (Laurentino *et al.*, 2013). Although the activity and functional relevance of fish AR variants have not been studies, it has been speculated that the conservation of AR splicing pattern in different tissues and in evolutionarily distant vertebrate species could indicate the functional importance of these AR forms (Laurentino *et al.*, 2013) .

The gilthead seabream, *Sparus aurata* L., is a seasonally breeding, marine, protandrous hermaphrodite teleost. Specimens are males in the Western Mediterranean area, at least during the two first reproductive cycles (RCs), in which the gonad has a functional testicular area and a non-functional ovarian area. The 1st male RC is divided into four stages: spermatogenesis (SG), spawning (S), post-spawning (PS), and resting (R). During the following RCs, resting is substituted by a testicular involution (TI) stage, which allowed sex change (Chaves-Pozo *et al.*, 2005a; Liarte *et al.*, 2007). The testis undergoes abrupt morphological changes, especially after spawning, including a massive infiltration of acidophilic granulocytes (AGs), an immune cell type essential for the normal physiology of the gonad, and whose presence in the gonad is actively regulated by tissue-specific factors and hormones (Chaves-Pozo *et al.*, 2003, 2005a, b; Liarte *et al.*, 2007). Moreover, plasma steroid, androgens (testosterone, T, and 11-ketotestosterone, 11KT) and estrogens (17β-estradiol), levels varied during the different stages of the RCs of gilthead seabream (Chaves-Pozo *et al.*, 2008a). We have also previously described that androgens modulate the immune response in gilthead seabream both in *in vivo* and *in vitro* (Castillo-Briceño *et al.*, 2013; Aguila *et al.*, 2013) and that both AGs and macrophages (MØ) expressed ARs and that these expression is modulated by androgens (Aguila *et al.*, 2013). In the present study, we report the

identification of a truncated form of the AR generated by alternative splicing that lacks the ligand-binding domain (ARΔLBD) in the gilthead seabream. head-kidney (HK) and in AGs and MØ, the main phagocytic cell types of the HK gilthead seabream, and its modulation by androgens and immune stimulus in order to demonstrate its mediation in the role of androgens in the immune response.

2. Material and Methods.

2.1 Animals and experimental design

Healthy mature gilthead seabream males (*Sparus aurata* L., Actinopterygii, Perciformes, Sparidae) with a mean body weight (bw) which varied from 270.3 ± 10.3 g (1st RC) to 716.8 ± 22.3 g (4th RC) were bred and kept at the Centro Oceanográfico de Murcia (IEO, Mazarrón, Murcia). The fish were kept in 12 and 6 m^3 tanks with the water temperature ranging from 13.6 to 26.5ºC, a flow-through circuit, a suitable aeration and filtration system and a natural photoperiod. The environmental parameters, mortality and food intake, were recorded daily.

The specimens were fed three times a day *ad libitum* with a commercial pellet diet (44% protein, 22% lipids, Skretting, Spain) and fasted for 24 h before sampling. Six fish from each sampling time (42 months) were anesthetized with 40 µl/l of clove oil and blood was obtained from the caudal peduncle using heparinized syringes and the plasma samples, obtained by centrifugation (10,000xg, 1 min, 4 ºC), were immediately frozen in liquid nitrogen and stored at -80 ºC until use. Then, specimens were decapitated and weighed, and the testis were removed and processed for light microscopy. HK and testis were embedded in TRizol and stored at -80ºC until processed for gene expression. 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 In situ forming microparticle (ISM) system

ISM implants have previously been used in gilthead seabream (Castillo-Briceño *et al.*, 2013; Sánchez-Hernández *et al.*, 2013). Briefly, polymer solutions were prepared by mixing poly-D,L-lactate-co-glycolate PLGA (P2066-1G, Sigma) with the solvent 2-pyrrolidone (Fluka Analytical) 20 % (w/w), in glass vials until a clear solution was

formed. T (Sigma) was dissolved in polymer solution 10% (w/w) and the T-ISM implants were prepared by emulsifying the steroid-containing polymer solution into peanut oil (external oil phase) at a polymer to oil phase ratio of 0.1:1 in a sonication bath (Ultrasons 3000512, P Selecta) for 10 minutes at room temperature. Pluronic F127 (Sigma) 1 % (w/w) based on the amount of the total formulation, and aluminum monostearate (Fluka Analytical) 2 % (w/w) based on the oil phase, were added to increase the stability of the emulsions. T-ISM implants were prepared with 1 mg of T/kg bw and the implants, both ISM (without T) and T-ISM, were placed by an intramuscular injection, approximately 2 cm below the dorsal fin, in order to achieve plasma concentrations above physiological levels (up to 2 ng/ml) in specimens in testicular involution of second RC. Six specimens from each group were sampled at 7 and 21 days post-implantation (dpi).

2.3 Cell culture

HK from 3 males were dissociated through a 100 µm nylon mesh and 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 mg/ml streptomycin (Biochrom). AGs were isolated by magnetic-activated cell sorting (MACS) as described previously (Sepulcre *et al*., 2002; Roca *et al*., 2006). Briefly, HK cell suspensions were incubated with a 1:10 optimal dilution of a mAb specific to gilthead seabream AGs (G7), washed twice with phosphate-buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA) (Sigma) and 5% charcoal/dextran-treated hormone-free fetal bovine serum (hf-FBS, Hyclone) and then incubated with cells micro-magnetic bead-conjugated anti-mouse IgG antibody (Miltenyi Biotec). After washing, G7+ (enriched in AGs) and G7− (depleted in AGs) cell fractions were collected by MACS following the manufacturer's instructions and their purity was analyzed by flow cytometry. MØs monolayers were obtained after overnight culture of total HK cells in serum-free medium (Roca *et al.*, 2006).

AGs from fish in TI stage of the 3rd RC were incubated during 3 and 16 h in sRPMI medium supplemented with 5% hf-FBS alone (control) or containing 0, 0.1, 1, 10 ng/ml of T or 11KT. After the androgen treatment, cells were processed for gene expression analysis. Furthermore, AGs and MØs from specimens at SG to PS of the 3rd RC were incubated in sRPMI medium supplemented with 5% hf-FBS alone (control) or

stimulated with 50 µg/ml of *Vibrio anguillarum* genomic DNA (VaDNA) as immune stimuli for 3 and 16 h. After the stimulation, cells were processed for gene expression analysis.

2.4 Light microscopy

The testis were fixed in 4% paraformaldehyde solution, embedded in paraffin (Paraplast Plus; Sherwood Medical), and sectioned at 5 µm. After dewaxing and rehydratation, some sections were stained with hematoxylin-eosin in order to determine the degree of development of each specimen.

2.5 Analysis of gene expression

Total RNA was extracted from testis, HK or HK-enriched cell fractions with TRIzol reagent (Cayman) following the manufacturer's instructions. RNA was treated with RQ1 RNAse-free DNAse, amplification grade (1 unit/µg RNA, Promega). BioScript Reverse Transcriptase (Bioline) was used to synthesize first strand cDNA with oligo-dT18 primer from 1 µg of total RNA, at 42 ºC for 30 min.

The ARΔLBD variant was isolated from gilthead seabream testis using primers F3/R4 (Table 1), sequenced using an ABI PRISM 377 sequencer (Applied Biosystems) and deposited in the European Nucleotide Archive (ENA) under accession number

The presence of ARΔLBD transcripts in the testis of specimens from 1st and 2nd RCs was analyzed by conventional RT-PCR using the primers pairs indicated in Table 1. The reaction mixtures were incubated for 2 min 95ºC, followed by 35 cycles of 45s at 95ºC, 45s at annealing temperature for each primers pairs and 45s at 72ºC, and finally 10 min at 72ºC. As a RT-PCR control, the expression β-actin was used. The expression of wild type AR and ARΔLBD transcripts was also analyzed by real time RT-PCR (RT-qPCR) performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) as previously described (Chaves-Pozo *et al.*, 2008b). 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. The amplification efficiency of primers for AR and ARΔLBD were 2.0 and 2.1, respectively.

Table 1: Gene accession numbers and primer sequences used for gene expression analysis.

Gene	Accession number	Name	Sequence (5'-3')	Use
AR	JN412131.1	F3	CTATGCGCAAGCAAAAATGA	Conventional PCR
		R4	GGAGCTTCTTCACCGTCATC	
		F1	AAGATTGGGCAACAGAAGAAC	Real time PCR
		R2	CAGCATCCTGCCGTTGAC	
ARΔLBD	JN412131.1	F6	AGGATTGCCAGACACCGGATGCA	Conventional PCR
		R4c	TGTAGGTGAGACGACACTCGTC	
		F6	AGGATTGCCAGACACCGGATGCA	Real time PCR
		R4	GGAGCTTCTTCACCGTCATC	
RPS18	AM490061	F	AGGGTGTTGGCAGACGTTAC	Real time PCR
		R	CTTCTGCCTGTTGAGGAACC	

2.6 Enzyme-linked immunosorbent assay (ELISA)

Plasma levels of T, 11KT and E_2 were quantified by ELISA following the method previously used in gilthead seabream (Chaves-Pozo *et al.*, 2008a). Steroids were extracted from 20 µl of plasma in 1.3 ml of methanol (Panreac). Then, the methanol were evaporated at 37º C and the steroids were resuspended in 400 µl of reaction buffer [0,1 M phosphate buffer with 1mM EDTA (Sigma), 0.4 M NaCl (Sigma), 1,5 mM NaN_3 (Sigma) and 0.1% albumin from bovine serum (Sigma)] and 50 µl were used in each well so that 5 µl of plasma in each well for all the assays. T, 11KT and E_2 standards, 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 x 10^{-4} to 2.5 ng/ml (0.03-125 pg/well), a blank and a non-specific binding control (negative control) were established in all the assays.

Standards and extracted plasma were run in duplicate and all the measures were corrected with the blank and negative control. The lower limit of detection for all the assays was 12.2 pg/ml. The intra-assay coefficients of variation (calculated from sample duplicates) were 13.8 ± 2.7% for T, 15.4 ± 3.1% for 11KT and 14.0 ± 3.6% for E_2. Details on cross-reactivity for specific antibodies were provided by the supplier (2.2% of anti-T reacts with 11KT; 0.01% of anti-11KT reacts with T; 0.1% of anti-E_2 reacts with T).

2.7 Transactivation assay.

The wild type an AR and ARΔLBD were synthesized and cloned in pUC57 (GenScript) and subcloned in the with *Eco*RI and *Bam*HI restriction sites of pcDNA6-V5-His(A). The reporter plasmid contained the MMTV-LTR promoter, coupled to the luciferase gene (MMTV-LUC) (de Ruiter *et al.*, 1995).

Human embryonic kidney 293 (HEK-293) cells were seeded in Petri's plate (Nunc) at a density of 1.6 x 10^6 cells, in phenol-red free Dulbecco's modified Eagle's medium supplemented with 10% hf-FBS, and 100 mg/ml penicillin and 100 mg/ml streptomycin. Cells were co-transfected with 4 µg plasmid MMTV-LUC and 4 µg AR plasmids, using LyoVec (InvivoGen). Twenty-four hours after co-transfection, the cells were seed 24-well cell culture plates, and cultured with different concentrations of T or 11KT. Twenty-four hours after, cell were lysed with 100 µl Passive Lysis Buffer (Dual-Luciferase Reporter Assay System, Promega) and the luciferase activity in 20 µ1 lysate was measured in a OPTOCOMP II luminometer (MGM Instruments, Inc.) after automatic injection of 100 µ1 of Luciferase Assay Reagent (Dual-Luciferase Reporter Assay System, Promega). The expression of the proteins was confirmed by western blot using a mAb to the V5 epitope (Invitrogen).

2.8 Statistical analysis.

All data were analyzed by one-way ANOVA and a *post hoc* test (Tukey Honestly Significant Difference) to determine differences between groups ($P \leq 0.1$). Normality of the data was previously assessed using a Shapiro-Wilk test and homogeneity of variance was also verified using the Levene test. Non-normally distributed data were log-transformed prior to analysis and a non-parametric Kruskal–Wallis test, followed by a multiple comparison test, was used when data did not meet

parametric assumptions. In addition, non-parametric Pearson correlation tests were applied to test correlations among hormonal levels and gene expression levels. Statistical analyses were conducted using Statgraphics 15.0 (StatPoint, Inc). All data are presented as mean of standard error to the mean (SEM). Significance level (P) was fixed at 0.1 ($P < 0.01$*; $P < 0.01$**; $P < 0.001$**).

3. Results.

3.1 Identification of an AR alternative spliced transcript in gilthead seabream

Two AR fragments were amplified by RT-PCR using the primer pair F3/R4 for AR (Table 1) and gilthead seabream testis cDNA as template (Figure 1a). BLASTN and BLASTX searches confirmed that the largest sequence was the full length AR and the smallest ones was an AR alternative spliced transcript generated by skipping of exons 5, 6 and 7, resulting in a premature stop codon and an AR protein lacking the ligand-binding domain (ARΔLBD) (Figure 1b).

Table 2. Correlation observed between testosterone (T), 11-ketotestosterone (11KT) and 17β-estradiol (E_2) and the ARdeltaLBD/ARfl transcripts relation in testis and HK of gilthead seabream males from the post-spawning stage of the 2nd reproductive cycle (RC) to the spawning stage of 4th RC. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference P. Written in bolds are the parameters that correlated

	T	11KT	E_2
ARΔLBD/AR in HK	**0.40**	0.14	0.19
	0.03	0.49	0.34
ARΔLBD/AR in testis	**-0.34**	**-0.40**	0.28
	0.04	**0.02**	0.10

3.2 The expression of the ARΔLBD in head-kidney correlates with plasma testosterone levels.

We designed specific primers (Table 1) to analyze the expression of ARΔLBD and AR by RT-qPCR in testis (Figure 2a) and HK (Figure 2b) from PS of the 2nd RC to S of the 4th RC. We observed that the ratio between the transcript levels of ARΔLBD and AR was different in the testis and HK. Thus, this ratio increased in testis at PS and TI stages, especially in the 2nd RC (Figure 2a), while it was higher in the HK from the SG stage of both RCs (Figure 2b).

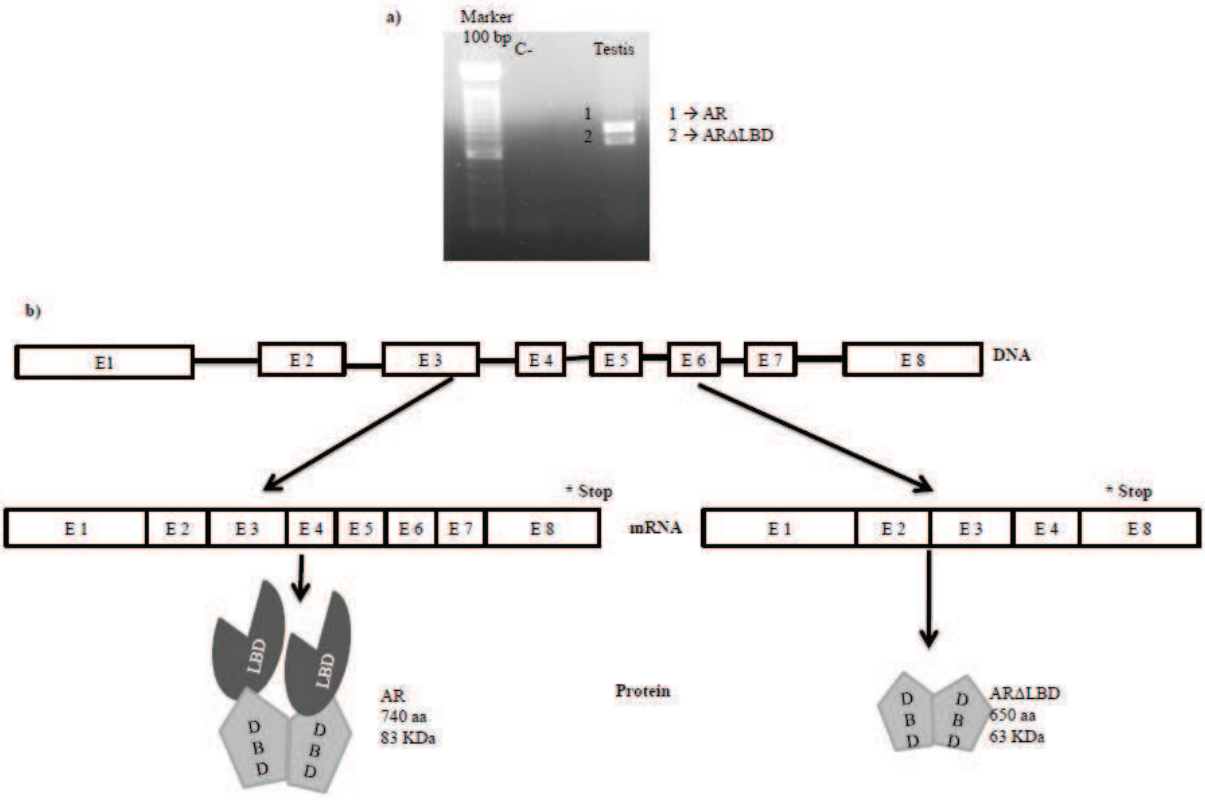

Figure 1. Representative RT-PCR using testis cDNA showing the amplicons corresponding to AR and ARΔLBD variants. A negative control (C-) without template gave no amplification. (b) Scheme of full-length AR and alternatively spliced ARΔLBD variant at mRNA and protein levels. The ARΔLBD shows a premature stop codon that results in a truncated protein lacking the LBD.

In order to study if there was a correlation between the expression of the two AR transcripts and the plasma sexual steroid levels, we analyzed the plasma level of T, 11KT and E_2 throughout the RCs (Figure 2c). While androgens levels (T and 11KT) increased during SG and S stages, the E_2 levels decreased in S, as expected. However, in PS and TI stages the androgen levels significantly declined while E_2 had a slight increase (Figure 2c). Interestingly, a positive correlation between ARΔLBD/AR expression ratio in the HK and the plasma T levels was observed, while a negative correlation between ARΔLBD/AR expression and both androgens was observed in the testis (Table 2).

3.3 Exogenous testosterone modifies the expression of the AR variants.

We have previously demonstrated that the administration of exogenous T *in vivo* to gilthead seabream resulted in increased plasma T levels in the first week after the implantation, with a decrease in the following weeks, although the levels remained higher than in control fish (Sánchez-Hernández *et al.*, 2013). Therefore, we analyzed the expression of ARΔLBD and AR in testis (Figure 3a) and in HK (Figure 3b) and found that while T was able to decrease the ARΔLBD/AR expression ratio in the testis, it promoted a significant increased expression ratio in the HK at 7 dpi (Figure 3a), curiously when the T plasma levels peaked (Sánchez-Hernández *et al.*, 2013). However, no differences were observed at 21 dpi (Figure 3b) when the T plasma levels decreased (Sánchez-Hernández *et al.*, 2013).

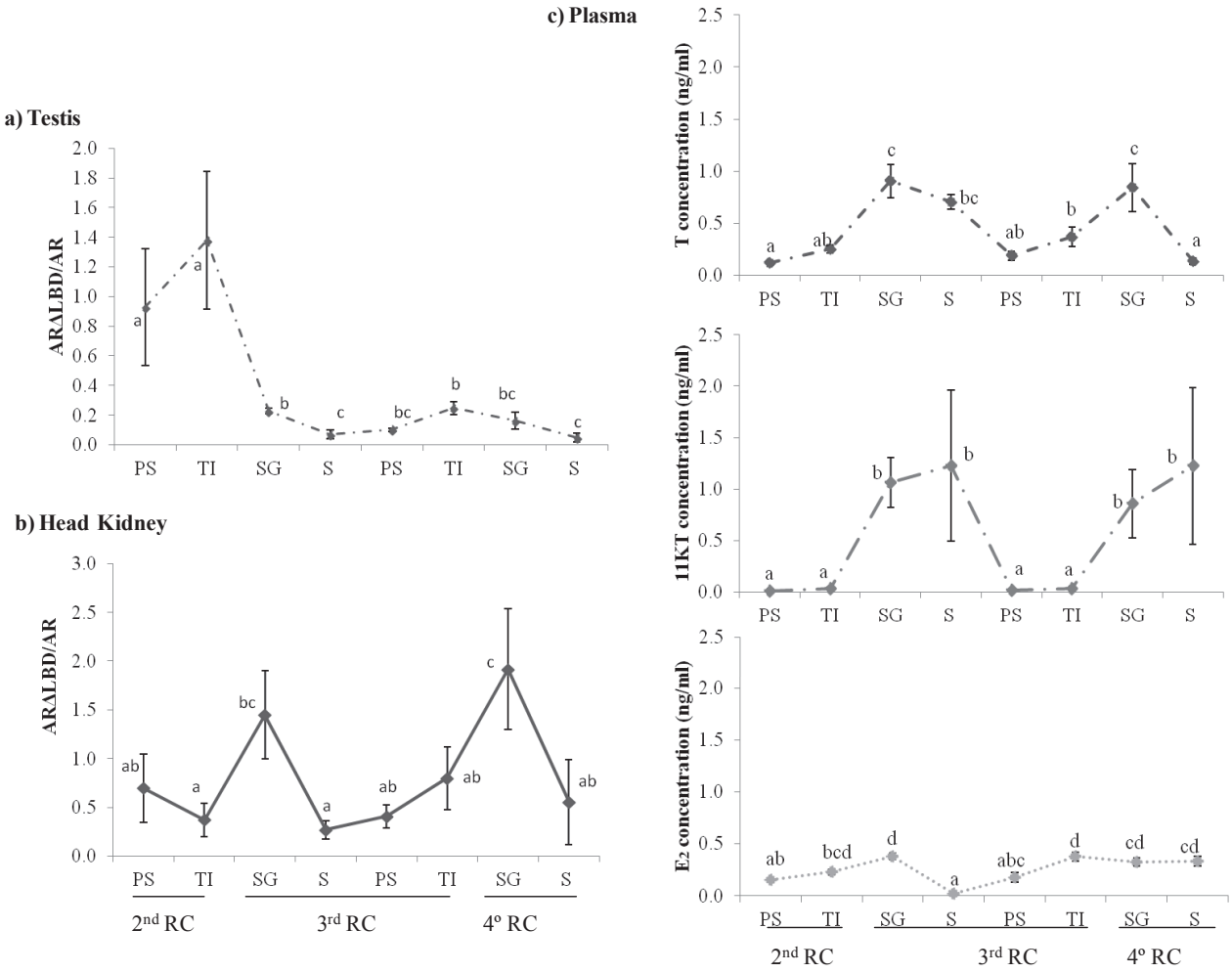

Figure 2. ARΔLBD/AR expression ratio in testis (a) and head-kidney (b) obtained by RT-qPCR, and sex steroid hormone levels in the plasma (c) of gilthead seabream males in different reproductive stages (RC). The mRNA levels of all genes were analyzed by RT-qPCR. Data represent means ± S.E.M. of duplicate samples from 5 fish/group. The hormone levels were obtained by ELISA from 5 fish/group and represent the means ± S.E.M. of duplicate samples. Different letters indicate significant differences among groups ($P < 0.001$). SG, spermatogenesis; S, spawning; PS, post-spawning; R, resting; TI, testicular involution.

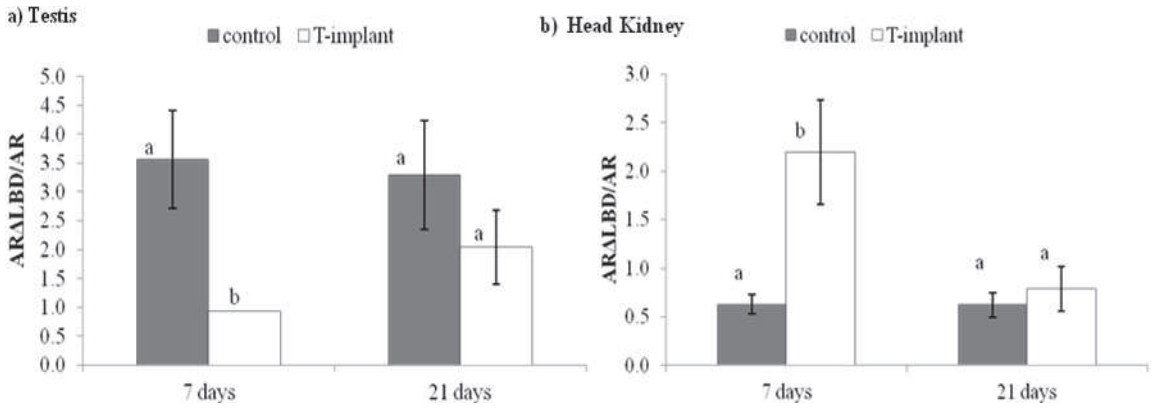

Figure 3. ARΔLBD/AR expression ratio in testis (a) and head-kidney (b) of gilthead seabream males after *in situ* forming microparticle implants with testosterone (T-ISM). The mRNA levels of all genes were analyzed by RT-qPCR. Data represent means ± S.E.M. of duplicate samples corresponding to two independent pools obtained after mixing the same amount of mRNA from 3 fish (n=6 fish/group). Different letters denote significant differences among groups ($P< 0.001$).

3.4 The ARΔLBD transcript is expressed in AGs, but not in MØs, and its expression is modulated by testosterone and immune insults.

We analyzed the expression of the AR and ARΔLBD transcripts in the two professional phagocytic cell types of the gilthead seabream, namely AGs and MØs. Interestingly, we observed the expression of both forms in AGs, whereas MØs only expressed wild type AR (data not shown). We next analyzed the expression of the two AR variants in AGs from specimens at SG, S and PS stages of the 3rd RC when the levels of T showed the highest and smallest levels, respectively (Figure 2c). It was found that the ARΔLBD/AR expression ratio varied in these reproductive stages following the same pattern as the plasma T levels (Figure 4a). In fact, a statistically significant positive correlation was observed between them, while no correlation was found with 11KT and E_2 (Table 3). More importantly, the ARΔLBD/AR ratio increased in a dose- and time-dependent manner in AGs obtained from the HK of TI stage specimens exposed to increasing T concentrations for 3 and 16 h (Figure 4b). Of note was also the fact that stimulation with bacterial DNA (VaDNA), a powerful pathogen-associated molecular pattern (PAMP) for these cells (Sepulcre *et al.*, 2007; Sepulcre *et al.*, 2011), of AGs differentially regulated the ARΔLBD/AR ratio depending on the

plasma T levels (Figure 4c). Thus, VaDNA-stimulation decreased ARΔLBD/AR ratio at SG, when the plasma T levels peaked, while it increased this ratio at S stage, when the T levels were low (Figures 4a and 4c).

Table 3. Correlation observed between the sex hormone levels and the relation ARdeltaLBD/ARfl in AGs in males from spermatogenesis to post-spawning of 3rd reproductive cycle.. The first number corresponds to Pearson coefficient of correlation and the second to the significant difference P. Written in bolds are the parameters that correlated.

Correlations			
	T	**11KT**	**E_2**
ARΔLBD/AR	**0.70**	0.53	0.33
	0.02	0.08	0.29

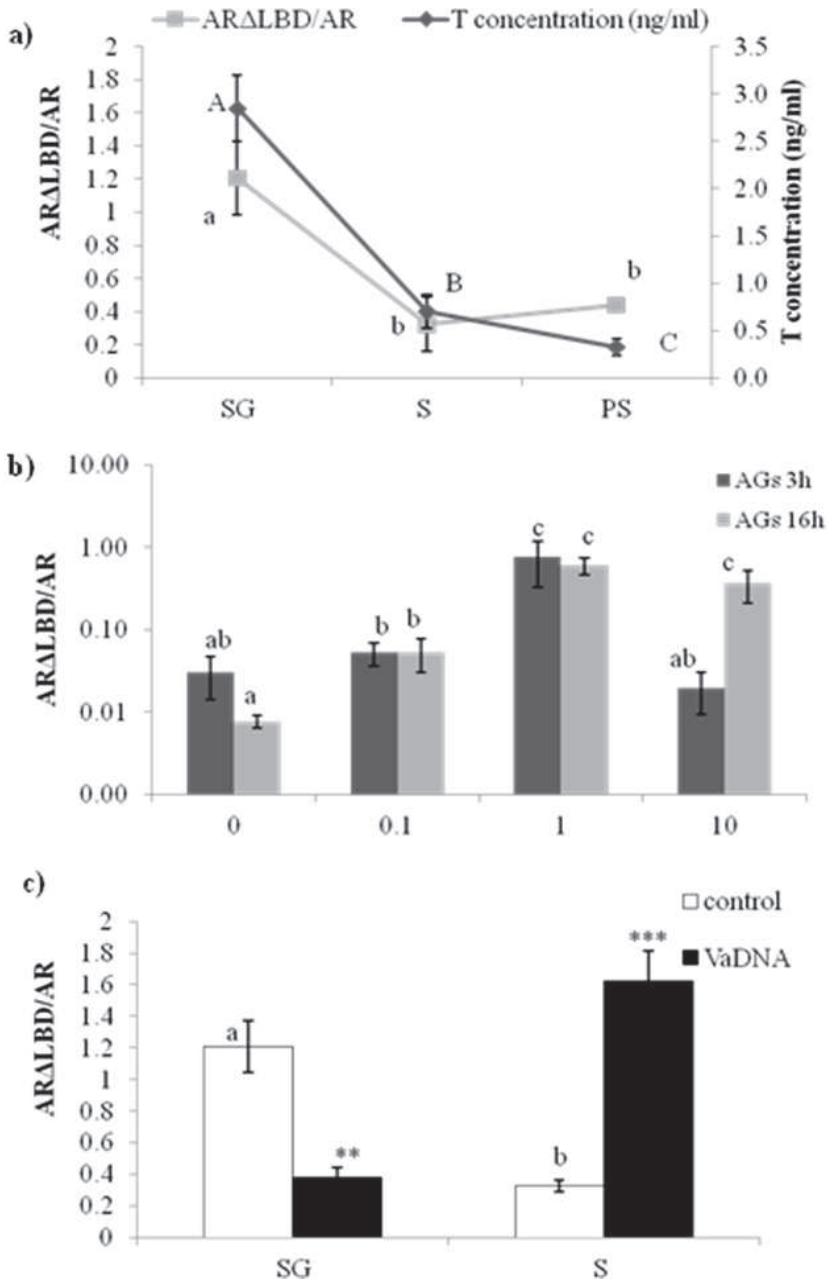

Figure 4. ARΔLBD/AR expression ratio in acidophilic granulocytes (AGs) of gilthead seabream males (a) at spermatogenesis (SG), spawning (S) and post-spawning (PS) of the 3rd reproductive cycle (RC) related with T plasma levels at the same reproductive stages. (b) ARΔLBD/AR expression ratio in AGs at testicular involution stage treated with different T concentrations for 3 and 16 h. (c) ARΔLBD/AR expression ratio in AGs activated or not with 50 µg/ml genomic DNA from *Vibrio anguillarum* (VaDNA). The mRNA levels of all genes were analyzed by RT-qPCR. Data represent means ± S.E.M. of triplicate samples. Different letters denote significant differences between groups ($P < 0.01$). Asterisks indicate significant differences between groups (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

3.5 The ARΔLBD is constitutively active

The above results prompted us to investigate the activity of both ARΔLBD and wild type AR in a transcription activation assay using HEK-293 cells (Figure 5). Strikingly, ARΔLBD was constitutively active, resulted in more than a 10-fold increased transactivation activity compared to non-stimulated AR and no differences in its activity was found upon incubation with T and 11KT. In sharp contrast, although the activity of wild type AR increased in a dose-dependent manner with physiological concentrations of both androgens, the strongest stimulation was observed with 11KT.

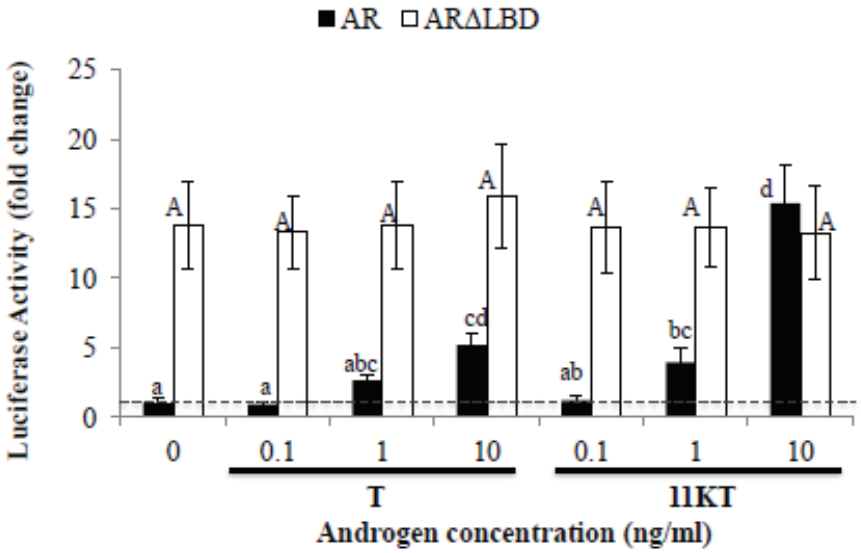

Figure 5. Transactivation assay in HEK-293 cells transfected with the reporter construct MMTV-LUC in combination with the expression constructs of full-length AR (AR) or truncated AR (ARΔLBD). Twenty-four hours after transfection, cells were incubated with the indicated concentration of testosterone (T) or 11-ketotestosterone (11KT) for 24 h and the luciferase activity measured as indicated in Materials and Methods. Data represent means ± S.E.M. of triplicate samples and are representative of two independent experiments. Different letters denote significant differences between groups ($P < 0.001$).

4. Discussion.

It is known that sex steroid hormones can regulate the immune response in vertebrates (Cohn, 1979; Bouman *et al*., 2005; Fairweather *et al*., 2008; Namazi, 2009; Yeh & Chen, 2010; Lee & Chiang, 2012). The modulation that estrogens exert on the

immune competence of fish has been extensively studied, and their ability to affect the maturation and function of leukocytes has been demonstrated (Yamaguchi *et al.*, 2001; Watanuki *et al.*, 2002; Cabas *et al.*, 2012). We have previously described in gilthead seabream a fine modulation of T of the innate immune response (Castillo-Briceño *et al.*, 2013) and a regulation of T and 11KT, with a competitive effect, on the sensitivity of phagocytes to pathogens and damage signals, as both AGs and MØ express the AR (Aguila *et al.*, 2013).

AR variants in humans are related with some diseases, like AIS or prostate cancer, in which a loss or an increase of AR function is observed, respectively (Dehm & Tindall, 2011). Most AR variants lack a functional domain, mainly the LBD, which is not essential for transcriptional activity (Hörnberg *et al.*, 2011). Some truncated AR variants lacking the LBD domain act as constitutively active receptors in prostate cancer cells (Dehm *et al.*, 2008; Dehm & Tindall, 2011; Hörnberg *et al.*, 2011). The knowledge about the presence of alternative splice ARs is scarce in other species. A recent report has described the presence of some variants in the testis of healthy humans and truncated forms in the testis of other species, including the gilthead seabream (Laurentino *et al.*, 2013). The alternatively spliced AR found in gilthead seabream testis encodes an AR protein only with the NTD ($AR\Delta2^{stop}$). This variant was described for first time in patient with AIS (Hellwinkel *et al.*, 1999) and displayed no ligand or transcriptional activities (Dehm & Tindall, 2011). By the contrast, the alternative spliced that we have found in the present study (ARΔLBD) shows the NTD and DBD, but lack the LBD, and it is constitutively active and ligand-independent. This variant shows similarity to the transcript $AR^{v5,6,7es}$ which arise through skipping of exons 5-7 (Sun *et al.*, 2010) and also lack the LBD. This transcript is present in patient with metastatic castration-resistant prostate cancer (CRPC) (Dehm & Tindall, 2011) and, interestingly, interacts with the wild type AR enhancing ligand-dependent and ligand-independent activities (Dehm & Tindall, 2011).

Our gene expression profile study suggests that the AR and ARΔLBD would play different roles in the testis, since they showed different expression profiles throughout the RCs. Importantly, the ARΔLBD/AR expression ratio variant in the testis shows a statistically significant negative correlation with plasma T and 11KT levels throughout the RCs. This result, together with the ability of exogenous T to decrease the ARΔLBD/AR expression ratio in the testis, demonstrates that androgens would regulate

testicular physiology through not only AR engagement but also via modulation of the expression level of the constitutively active ARΔLBD variant.

We also found that both AR and ARΔLBD are expressed in HK, the main hematopoietic and immune competent organ in fish, and its expression varied from that of the testis and depended on the reproductive stages of the animals. It is known that exogenous T can modify the expression of wild type AR in HK (Castillo-Briceño *et al.*, 2013) and we extend this previous observation to the ARΔLBD variant. Curiously, and in contrast to the testis, we observed a positive correlation of the ARΔLBD/AR expression ratio in the HK and exogenous T also increased this ratio, suggesting a role of androgens in the regulation of fish immunocompetence through several mechanisms. This is not surprising since alternative splicing allows the immune system a high degree of diversity and to rapidly adapt and respond to changing environmental conditions (Lynch, 2004).

One of the most interesting results of this study is that AGs, but no MØs, expressed the ARΔLBD variant. In addition, ARΔLBD/AR expression ratio in AGs oscillated in the different months of the RCs, and there is a positive correlation with T plasma levels, probably reflecting the results obtained in the HK. More importantly, T added *in vitro* was able to increase the ARΔLBD/AR expression ratio in purified AGs. Furthermore, stimulation of AGs with bacterial DNA, a powerful immune stimulus for gilthead seabream AGs (Sepulcre *et al.*, 2007, 2011) also regulated the alternative splicing of the AR, but depending of the reproductive stage of the fish, suggesting a crosstalk between endocrine and immune stimuli in the regulation of AR alternative splicing and AG functions. These data are in agreement with studies in mammals that suggest that gene splicing can be altered in response to antigen stimulation (Lynch, 2004). Although further experiments are required to illuminate the signaling pathway involved in regulation of the alternative splicing of the AR in AGs, our results demonstrate for the first time, to the best of our knowledge, that an alternative spliced variant of the AR is expressed by granulocytes. In view of the essential role of AR in human and mouse granulopoiesis and survival (Chuang *et al.*, 2009), our results have uncovered a new mechanism for the regulation of neutrophil biology and may explained the conflicting results showing that androgens are less important than AR in neutrophil homeostasis (Chuang *et al.*, 2009).

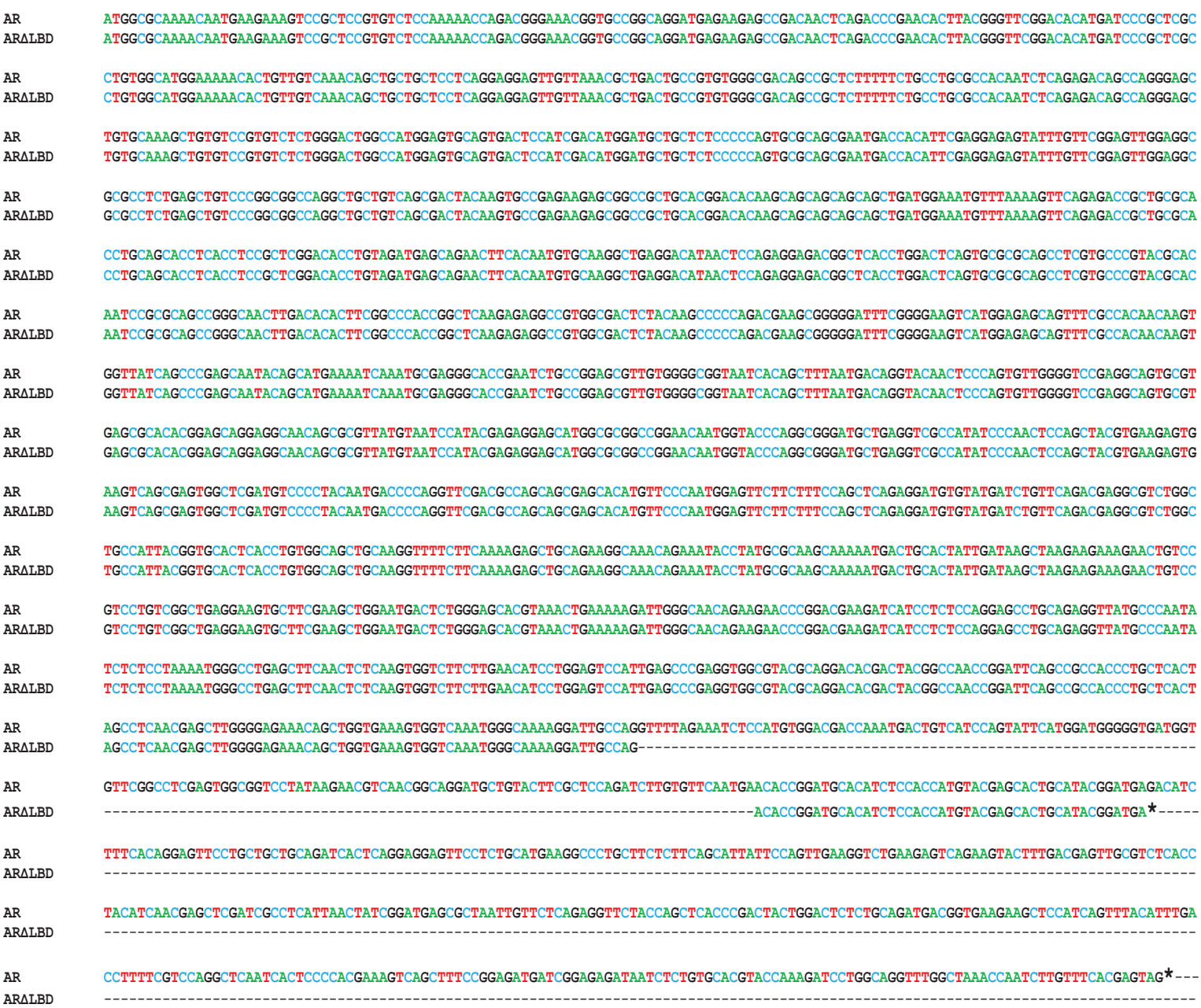

Figure S1. Sequence alignment of AR and ARΔLBD using BioEdit v7.0.5. Only the coding sequences are shown for clarity. The stop codons for both variants are indicated with asterisks

III. CONCLUSIONS

1. Testosterone implants modify the plasma sex steroid hormones balance; increasing testosterone levels and decreasing 17β-estradiol levels.

2. Testosterone implants do not affect *de novo* synthesis of sex steroid hormones. However, they induce testosterone transformation into androgenic compounds.

3. Testosterone implants block the germ cell proliferation, enhances the migratory influx of leukocytes into the testis through increasing the pro-inflammatory cytokine levels, and impair its ability to recognize and respond to pathogens.

4. A truncated form of the AR lacking the ligand-binding domain, ARΔLBD, is expresses is testis and head kidney. This variant is produced by alternative splicing of the AR gene.

5. ARΔLBD is expressed in the head kidney and its expression varied with the reproductive stage and is correlated with plasma testosterone levels. Moreover, exogenous testosterone increases the ARΔLBD expression in head kidney, whereas decreases those of the testis.

6. ARΔLBD is expressed in acidophilic granulocytes but not in macrophages. Furthermore its expression can be modified by testosterone and immune stimuli.

7. The ARΔLBD has ligand-independent activity, which is higher than that of wild type androgen receptor.

REFERENCES

Abbas, A.K., Lichtman, A.H., Pober, J.S. Citoquinas. En: Abbas AK, Lichtman AH, Pober JS (eds) Inmunología Celular y Molecular. McGraw-Hill – Interamericana de España, Madrid (2001).

Abruzzini, A.F., Ingram, L.O., Clem, L.W. Temperature-mediated processes in teleost immunity: Homeoviscous adaptation in teleost lymphocytes. Proc. Soc. Exp. Biol. Med. 169 (1982) 12-18.

Adori, M., Kiss, E., Barad, Z., Barabás, K., Kiszely, E., Schneider, A., Kövesdi, D., Sziksz, E., Abrahám, I.M., Matkó, J., Sármay, G. Estrogen augments the T celldependent but not the T-independent immune response. Cell Mol. Life Sci. 67 (2010) 1661–1674.

Aderem, A., Ulevithc, R.J. Toll-like receptors in the induction of innate immune response. Nature 406 (2000) 782-787.

Águila, S., Castillo-Briceño, P., Sánchez-Hernández, M., Cabas, I., García-Alcazar, A., Meseguer, J., Mulero, V., García-Ayala, A. Specific and non-overlapping functions of testosterone and 11-ketotestosterone in the regulation of professional phagocyte responses in the teleost fish gilthead seabream. Mol. Immunol. 53 (2013) 218-226.

Ahrens-Fath, I., Politz, O., Geserick, C., Haendler, B. Androgen receptor function is modulated by the tissuespecific AR45 variant. FEBS J. 272 (2005) 74–84.

Alexander, J.B., Ingram, G.A. Non cellular nonspecific defense mechanisms of fish. Ann. Rev. Fish Dis. 2 (1992) 249-279.

Akira, S., Takeda, K. Toll-like receptor signaling. Nat. Rev. Immunol. 4 (2004) 499-511.

Alexander, W. H., Stimson, W. H. Sex hormones and the course of parasitic infection. Parasitol. Today 4 (1988) 189–1936.

Amer, M.A., Miura, T., Miura, C., Yamauchi, K. Involvement of sex steroid hormones in the early stages of spermatogenesis in Japanese huchen *(Hucho perryi*). Biol. Reprod. 65 (2001) 1057-1066.

Anderson, D.P. Immunostimulants, adjuvants, and vaccine carriers in fish: Applications to aquaculture. Ann. Rev. Fish. Dis. 2 (1992) 281-307.

Arukwe, A. Steroidogenic acute regulatory (StAR) protein and cholesterol side-chain cleavage (P450scc)-regulated steroidogenesis as an organ-specific molecular and cellular target for endocrine disrupting chemicals in fish. Cell Biol. Toxicol. 24 (2008) 527-540.

Avtalion, R.R. Environmental control of the immune response in fish. Crit. Rev. Environ. Control 11 (1981) 163-188.

Bauer M.P., Bridgham, J.T., Langenau, D.M., Johnson, A.L., Goetz, F.W. Conservation of steroidogenic acute regulatory (StAR) protein structure and expression in vertebrates. Mol. Cell Endocrinol. 168 (2000) 119-125.

Beato, M. Gene regulation by steroid hormones. Cell. 56 (1989) 335–344.

Benten, W. P. M., Bettenhaeuser, U., Wunderlich, F., Van Vliet, E., Mossmann, H. Testosterone induced abrogation of self-healing of *Plasmodium chabaudi* malaria in B10 mice: mediation by spleen cells. Infect. Immun. 59 (1991) 4486–4490.

Besseau, L., Faliex, E. Resorption of unemitted gametes in *Lithognathus mormyrus* (Sparidae, Teleostei): A possible synergic action of somatic and immune cells. Cell. Tissue Res. 276 (1994) 123-132.

Billar, R. A quantitative analysis of spermatogenesis in the trout, *Salmo trutta fario.* Cell Tissue Res. 230 (1983) 495-502.

Billard, R., Fostier, A., Weil, C., Breton, B. Endocrine control of spermatogenesis in teleost fish. Can. J. Fish. Aquat. Sci. 39 (1982) 65-79.

Blázquez, M., Piferrer F. Sea bass (Dicentrarchus labrax) androgen receptor: cDNA cloning, tissue-specific expression and mRNA levels during early development and sex differentiation. Mol. Cell. Endocrinol. 15 (2005) 23737–23748.

Blencow, BJ. Alternative splicing: new insights from global analyses. Cell 126 (2006) 37-47.

Borg, B. Androgens in teleost fishes. Comp. Biochem. Physiol. 109 C (1994) 219-245.

Bouman, A., Heineman, M.J., Faas, M.M. Sex hormones and the immune response in humans. Hum. Reprod. Update 11 (2005) 411–423.

Boutz, P.L., Stoilov, P., Li, Q., Lin, C.H., Chawla, G., Ostrow, K., Shiue, L., Ares, M. Jr., Black, D.L. A post-transcriptional regulatory switch in polypyrimidine tract-binding proteins reprograms alternative splicing in developing neurons. Genes Dev. 21 (2007) 1636–1652Brinkmann, A.O. Molecular basis of androgen insensitivity. Mol. Cell. Endocrinol. 179 (2001) 105–109.

Bruslé-Sicard, S., Fourcault, B. Recognition of sex-inverting protandric *Sparus aurata*: Ultrastructural aspects. J. Fish. Biol. 50 (1997) 1094-1103.

Cabas, I., Chaves-Pozo, E., García-Alcázar, A., Meseguer, J., Mulero, V., García-Ayala, A. Dietary intake of 17alpha-ethinylestradiol promotes leukocytes infiltration in the gonad of the hermaphrodite gilthead seabream. Mol. Immunol. 48 (2011) 2079-2086.

Cabas, I., Liarte, S., García-Alcázar, A., Meseguer, J., Mulero, V., García Ayala, A. 17α-Ethynylestradiol alters the immune response of the teleost gilthead seabream (Sparus aurata L.) both in vivo and in vitro. Dev. Comp. Immunol. 36 (2012) 547-556.

Callard, G.V. Spermatogenesis. In: Vertebrate endocrinology: fundamentals and biomedical implications, volume 4 (1991) pp 303-341. Eds Pang PKT y Schreibman MP New York: Academic Press.

Campbell, B., Dickey, J.T., Swanson, P. Endocrine changes during onset of puberty in male spring Chinook salmon, *Oncorhynchus tshawytscha*. Biol. Reprod. 69 (2003) 2109-2117.

Castillo-Briceño, P., Sepulcre, M.P., Chaves-Pozo, E., Meseguer, J., García-Ayala, A., Mulero, V. Collagen regulates the activation of professional phagocytes of the teleost fish gilthead seabream. Mol. Immunol. 46 (2009) 1409–1415.

Castillo-Briceño, P., Cabas, I., Arizcun, M., Meseguer, J., Mulero, V., García-Ayala, A. Identification of a β1 integrin isoform with restricted tissue expression in a teleost fish. Reprod. Fertil. Dev. 23 (2011) 654–664.

Castillo-Briceño, P., Águila-Martínez, S. Liarte, S., García-Alcazar, A., Meseguer, J., Mulero, V., García-Ayala, A. In situ forming microparticle implants for delivery of sex steroids in fish: Modulation of the immune response of gilthead seabream by testosterone. Steroids 78 (2013) 26-33.

Cavaco, J.E., Vilrokx, C., Trudeau, V.L., Schulz, R.W., Goos, H.J. Sex steroids and the initiation of puberty in male African catfish (*Clarias gariepinus*). Am. J. Physiol. 275 (1998) R1793-1802.

Chaves-Pozo, E., Pelegrín, P., Mulero, V., Meseguer, J., García-Ayala, A. A role for acidophilic granulocytes in the testis of the gilthead seabream (*Sparus aurata* L., Teleostei), J. Endocrinol. 179 (2003) 165-174.

Chaves-Pozo, E., Pelegrín, P., García-Castillo, J., García-Ayala, A., Mulero, V., Meseguer, J. Acidophilic granulocytes of the marine fish gilthead seabream (*Sparus aurata* L.) produce interleukin-1beta following infection with *Vibrio anguillarum*. Cell. Tissue Res. 316 (2004) 189-195.

Chaves-Pozo, E., Mulero, V., Meseguer, J., García-Ayala, A. An overview of cell renewal in the testis throughout the reproductive cycle of a seasonal breeding teleost, the gilthead seabream (*Sparus aurata* L). Biol. Reprod. 72 (2005a) 593-601.

Chaves-Pozo, E., Mulero, V., Meseguer, J., García-Ayala, A. Professional phagocytic granulocytes of the bony fish gilthead seabream display functional adaptation to testicular microenvironment. J. Leukoc. Biol. 78 (2005b) 345-351.

Chaves-Pozo, E., Liarte, S., Vargas-Chacoff, L., García-López, A., Mulero, V., Meseguer, J., Mancera, J.M., García-Ayala, A. 17Beta-estradiol triggers postspawning in spermatogenically active gilthead seabream (*Sparus aurata* L.) males. Biol. Reprod. 76 (2007) 142-148.

Chaves-Pozo, E., Arjona, F.J., García-López, A., García-Alcázar, A., Meseguer, J., García-Ayala, A. Sex steroids and metabolic parameter levels in a seasonal breeding fish (*Sparus aurata* L.). Gen. Comp. Endocrinol. 156 (2008a) 531-536.

Chaves-Pozo, E., Liarte, S., Fernández-Alacid, L., Abellán, E., Meseguer, J., Mulero, V., García-Ayala, A. Pattern of expression of immune-relevant genes in the gonad of a teleost, the gilthead seabream (*Sparus aurata* L.). Mol. Immunol. 45 (2008b) 2998-3011.

Chaves-Pozo, E., Liarte, S., García-Ayala, A. Immune and reproductive interaction: An essential clue for understanding gonad functions in gilthead seabream. In: García-Ayala A, Meseguer J, Chaves-Pozo E (eds) Recent Advances in Fish Reproductive Biology. Research Signpost, Kerala (2010) pp. 127-139.

Chaves-Pozo, E., Cabas, I., García-Ayala, A. Sex steroids modulate fish immune response. In: S.M. Kahn (ed.), Sex steroids, InTech, Rijeka (2012) pp. 199-220.

Chawla, A., Repa, J.J., Evans, R.M., Mangelsdorf, D.J. Nuclear receptors and lipid physiology: Opening the X-files. Science 294 (2001) 1866-1870.

Chuang, K-H., Altuwaijri, S., Li, G., Lai, J-J., Chu, C-Y., Lai, K-P., Lin, H-Y., Hsu, J-W., Keng, P., Wu, M-C., Chang, C. Neutropenia with impaired host defense against microbial infection in mice lacking androgen receptor. J. Exp. Med. 206 (2009) 1181-1199.

Clem, L.W., Faulmann, E., Miller, N.W., Ellsaesser, C., Lobb, C.J., Cuchens, M.A. Temperature-mediated processes in teleosts immunity: Differential effects of *in vitro* and *in vivo* temperatures on mitogenic responses of channel catfish lymphocytes. Dev. Comp. Immunol. 8 (1984) 313-322.

Clem, L.W., Sizemore, R.C., Fellsaesser, C.F., Miller, N.W. Monocytes as accessory cells in fish immune responses. Dev. Comp. Immunol. 9 (1985) 803-809.

Clem, L.W., Miller, N.W., Bly, J.E. Evolution of lymphocyte subpopulations, their interactions and temperature sensitivities. In: Warr GW, Cohen N (eds) Phylogenesis of Immune Functions. CRC Press, Boca Raton, Florida (1991) pp. 191-213.

Cohn, D.A. High sensitivity to androgen as a contributing factor in sex differences in the immune response. Arthritis Rheum. 22 (1979) 1217–1233.

Collet, B., Secombes, C.J. Type I-interferon signalling in fish. Fish Shellfish Immun. 12 (2002) 389-397.

Condeca, J.B., Canario, A.V. The effect of estrogen on the gonads and on *in vitro* conversion of androstenedione to testosterone, 11-ketotestosterone, and estradiol-17beta in *Sparus aurata* (Teleostei, Sparidae). Gen. Comp. Endocrinol. 116 (1999) 59-72.

Congleton, J., Sun, B.L. Interferon-like activity produced by anterior kidney leucocytes of rainbow trout stimulated *in vitro* by infectious hematopoietic virus or poly I:C. Dis. Aquatic. Org. 25 (1996) 185-190 .

Cuchens, M.A., Clem, L.W. Phylogeny of lymphocyte heterogeneity. II. Differential effects of temperature on fish T-like and B-like cells. Cell. Immunol. 34 (1977) 219-230.

Cuesta, A., Vargas-Chacoff, L., García-López, A., Arjona, F.J., Martínez-Rodríguez, G., Meseguer, J., Mancera, J.M., Esteban, M.A. Effect of sex-steroid hormones, testosterone and estradiol, on humoral immune parameters of gilthead seabream. Fish Shellfish Immun. 23 (2007) 693–700.

Cutolo, M., Capellino, S., Montagna, P., Ghiorzo, P., Sulli, A., Villaggio, B. Sex hormone modulation of cell growth and apoptosis of the human monocytic/macrophage cell line. Arthritis Res. Ther. 7 (2005) R1124-1132.

Cutress, M.L., Whitaker, H.C., Mills, I.G., Stewart, M., Neal, D.E. Structural basis for the nuclear import of the human androgen receptor. J. Cell Sci. 121 (2008) 957–968.

D'Ancona, U. Ermfroditismo ed intersessualita nei Teleost-tei. Experientia 5 (1949) 381-389.

Danilova, N., Bussmann, J., Jekosch, K., Steiner, L.A. The immunoglobulin heavychain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. Nat. Immunol. 6 (2005) 295-302

De Gendt, K., Swinnen, J.V., Saunders, P.T., Schoonjans, L., Dewerchin, M., Devos, A., Tan, K., Atanassova, N., Claessens, F., Lecureuil, C., Heyns, W., Carmeliet, P., Guillou, F., Sharpe, R.M., Verhoeven, G. A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. PROC. NATL. ACAD. SCI 101 (2004) 1327–1332.

Dehm, S.M., Tindall, D.J. Alternatively spliced androgen receptor variants. Rev. Endocr.-Relat. Cancer 18 (2011) R183–R196.

Dehm, S.M., Schmidt, L.J., Heemers, H.V., Vessella, R.L., Tindall, D.J. Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. Cancer Res. 68 (2008) 5469–5477.

Deluca, D., Wilson, M., Warr, G.W. Lymphocyte heterogeneity in the trout, *Salmo gairdneri*, defined with monoclonal antibodies to IgM. Eur. J. Immunol. 13 (1983) 546-551.

de Ruiter, P. E., Teuwen, R., Trapman, J., Dijkema, R ., Brinkrnann A.O. Synergism between androgens and protein kinase-C on androgen-regulated gene expression. Mol. and Cell. Endocrinol. 110 (1995) R1-R6.

de Waal, P.P., Wang, D.S., Nijenhuis, W.A., Schulz, R.W., Bogerd, J. Functional characterization and expression analysis of the androgen receptor in zebrafish (Danio rerio) testis. Reproduction 136 (2008) 225–234.

de Waal, P.P. , Leal, M.C., García-López, A., Liarte, S., de Jonge, H., Hinfray, N., Brion, F., Schulz, R.W., Bogerd, J. Oestrogen-induced androgen insufficiency results in a reduction of proliferation and differentiation of spermatogonia in the zebrafish testis, J. Endocrinol. 202 (2009) 287-297.

Devlin, R.H., Nagahama, Y. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208 (2002) 191–364.

Don, J., Nir, U., Breitbart, H. DMRT1 at the border between mitosis and meiosis. Asian J. Androl. 13 (2011) 189-190.

Douard ,V., Brunet, F., Boussau, B., Ahrens, I., Vlaeminck-Guillem, V., Haendler, B., Laudet, V., Guiguen, Y. The fate of the duplicated androgen receptor in fishes: a late neofunctionalization event? BMC Evol. Biol. 8 (2008) 336.

Ekman, D.R., Villeneuve, D.L., Teng, Q., Ralston-Hooper, K.J., Martinovic-Weigelt, D., Kahl, M.D., Jensen, K.M., Durhan, E.J., Makynen, E.A., Ankley, G.T., Collette, T.W. Use of gene expression, biochemical and metabolite profiles to enhance exposure and effects assessment of the model androgen 17beta-trenbolone in fish. Environ. Toxicol. Chem. 30 (2011) 319-329.

Elliott, D.J., Grellscheid, S.N. Alternative RNA splicing regulation in the testis. Reproduction 132 (2006) 811–819.

Ellis, A.E. Antigen-trapping in the spleen and kidney of the plaice *Pleuronectes platessa* L. J. Fish Dis. 3 (1980) 413-426.

Fairweather, D., Frisancho-Kiss, S. Rose, N.R. Sex differences in autoinmune disease from a pathological perspective. Am. J. Pathol. 173 (2008) 600–609.

FAO Fisheries and Aquaculture Department. The state of world fisheries and aquaculture. FAO communication division. Viale delle Terme di Caracalla, Rome, Italy (2012).

Feldmann, M. Cell cooperation in the antibody response. In: Roitt IM, Brostoff J, Male DK (eds) Immunology Mosby, Londres (1996)

Filipin Mdel, V., Caetano, L.C., Brazão, V., Santello, F.H., Toldo, M.P., do Prado Jr., J.C. DHEA and testosterone therapies in Trypanosoma cruzi-infected rats are associated with thymic changes. Res. Vet. Sci. 89 (2010) 98–103.

Foradori, C.D., Weiser, M.J., Handa, R.J. Non-genomic actions of androgens. Front. Neuroendocrinol. 29 (2008) 169–181.

Foulkes, N.S., Mellstrom, B., Benusiglio, E., Sassone-Corsi, P. Developmental switch of CREM function during spermatogenesis: from antagonist to activator. Nature 355 (1992). 80–84.

Friedman, A.E. Testosterone may speed up the growth of prostate cancer due to aromatase activity. BJU Int. 106 (2010) 434.

Fujiki, K., Shin, D.H., Nakao, M., Yano, T. Molecular cloning of carp (*Cyprinus carpio*) CC chemokine, CXC chemokine receptors, allograft inflammatory factor-1, and natural killer cell enhancing factor by use of suppression subtractive hybridization. Immunogenetics 49 (1999) 909-914.

García-Ayala, A., Villaplana, M., García Hernandez, M.P., Chaves Pozo, E., Agulleiro, B. FSH-, LH- and TSH-expressing cells during develpment of *Sparus aurata* L. (Teleostei). An immunocytochemical study. Gen. Comp. Endocrinol. 134 (2003) 72-79.

García-Ayala, A., Liarte, S., Cabas, I., Castillo-Briceño, P., Mulero, I., Chaves-Pozo, E. Inmunoendocrinología en peces. In: I. Mulero, A. García-Ayala, J. Meseguer (eds), Acuariología y Cultivo de peces:Inmunopatología, Aulas del Mar (2010) pp 137-148.

García-Ayala, A., Chaves-Pozo, E. Immunocytochemical tools reveal a new research field between the boundaries of immunology and reproductive biology in teleosts. In: H. Dehghani (eds.), Applications of immunocytochemistry, InTech, Rijeka (2012) pp. 135-158.

Gillgrass, A., Chege, D., Bhavanam, S., Kaushic, C. Estradiol limits viral replication following intravaginal immunization leading to diminished mucosal IgG response and non-sterile protection against genital herpes challenge. Am. J. Reprod. Immunol. 63 (2010) 299–309.

Greiner, S., Stefanski, V., Dehnhard, M., Voigt, C.C., 2010. Plasma testosterone levels decrease after activation of skin immune system in a free-ranging mammal. Gen. Comp. Endocrinol. 168, 466–473.

Grier, H.J. Cellular organisation of the testis and spermatogenesis in fishes. Am. Zool. 21 (1981) 345-357.

Gorelick, D.A., Watson, W., Halpern, M.E. Androgen receptor gene expression in the developing and adult zebrafish brain. Dev. Dyn. 237 (2008) 2987–2995.

Grondel, J.L., Harmsen, E.G.M. Phylogeny of interleukins: growth factors produced by leucocytes of the cyprinid fish, *Cyprinus carpio* L. Immunology 52 (1984) 477-482.

Grossman, C.J. Interactions between the gonadal steroids and the immune system. Science 227 (1985) 257-261.

Haire, R.N., Rast, J.P., Litman, R.T., Litman, G.W. Characterization of three isotypes of immunoglobulin light chains and T-cell antigen receptor alpha in zebrafish. Immunogenetics 51 (2000) 915-923.

Handelsman, D.J., Spaliviero, J.A., Simpson, J.M., Allan, C.M., Singh, J. Spermatogenesis without gonadotropins: maintenance has a lower testosterone threshold than initiation. Endocrinology 140 (1999) 3938-3946.

Hannema, S.E., Hughes, I.A. Regulation of Wolffian duct development. Horm. Res. 67 (2007) 142–151.

Hansen, J.D., La Patra S Induction of the rainbow trout MHC class I pathway during acute IHNV infection. Immunogenetics 54 (2002) 654-661.

Hansen, J.D., Landis, E.D., Phillips, R.B. Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. Proc. Natl. Acad. Sci. U S A 102 (2005) 6919-6924.

Harbott, L.K., Burmeister, S.S., White, R.B., Vagell, M., Fernald, R.D. Androgen receptors in a cichlid fish, *Astatotilapia burtoni*: structure, localization, and expression levels. J. Comp. Neurol. 504 (2007) 57–73.

Harms, C.A., Kennedy-Stoskopf, S., Horne, W.A., Fuller, F.J., Tompkins, W.A.F. Cloning and sequencing hybrid striped bass (*Morone saxatilis*×*M. chrisops*) transforming growth factor-β (TGF-β), and development of a reverse transcription quantitative competitive polymerase chain reaction (RT-qcPCR) assay to measure TGF-β mRNA of teleost fish. Fish Shellfish Immun. 10 (2000) 61- 85.

Hasselquist, D., Marsh, J. A., Sherman, P. W. & Wingfield, J. C. Is avian humoral immunocompetence suppressed by testosterone? Behav. Ecol. Sociobiol. 45 (1999) 167‑175.

Hayashi, F., Means, TK., Luster, A.D. Toll-like receptor stimulate human neutrophil function. Blood 102 (2003) 2660-2669.

Hedger, M.P. Testicular leukocytes: What are they doing? Rev. Reprod. 2 (1997) 38-47

Heinlein, C.A., Chang, C. Androgen receptor (AR) coregulators: an overview. Endocr. Rev. 23 (2002) 175–200.

Hellwinkel, O. JC., Bull, K., Holterhus, P.M., Homburg, N., Struve, D., Hiort, O. Complete androgen insensitivity caused by a splice donor site mutation in intron 2 of the human androgen receptor gene resulting in an exon 2-lacking transcript with premature stop-codon and reduced expression. J. Steroid Biochem. Mol. Biol. 68 (1999) 1-9.

Henderson, N.E. The annual cycle in the testis of eastern brook trout *Salvelinus fontinalis* (Mitchill). Can. J. Zool. 40 (1962) 631-641.

Hess, R.A. Estrogen in the adult male reproductive tract: A review. Reprod. Biol. Endocrinol. 1 (2003) 1-5.

Hirata, S., Shoda, T., Kato, J., Hoshi, K. Isoform/variant mRNAs for sex steroid hormone receptors in humans. Trends Endocrinol. Metab. 14 (2003) 124–129.

Hoeger, B., Hitzfeld, B., Köllner, B., Dietrich, D.R., van den Heuvel, M.R. Sex and low-level sampling stress modify the impacts of sewage effluent on the rainbow trout (Oncorhynchus mykiss) immune system. Aquat. Toxicol. 73 (2005) 79–90.

Hörnberg, E., Bovinder Ylitalo, E., Crnalic, S., Antti, H., Stattin, P., Widmark, A., Dergh, A., Wikström, P. Expression of androgen receptor splice variants in prostate cancer bone metastases is associated with castration-resistance and short survival. PLoS One (2011) 6(4):e19059.

Jang, S.I., Mulero, V., Hardie, L.J. Secombes CJ Inhibition of rainbow trout phagocyte responsiveness to human tumor necrosis factor α (hTNFα) with monoclonal

antibodies to the hTNFα 55 kDa receptor. Fish Shellfish Immun. 5 (1995a) 61-69.

Jault, C., Pichon, L., Chluba, J. Toll-like receptor gene family and TIR-domain adapters in *Danio rerio*. Mol. Immunol. 40 (2004) 759-771.

Jang, S.I., Hardie, L.J., Secombes, C.J. Elevation of rainbow trout (*Oncorhynchus mykiss*) macrophage respiratory burst activity with macrophage-derived supernatants. J. Leukoc. Biol. 57 (1995b) 943-947.

Kime, D.E. Classical and non classical reproductive steroids in fish. Rev. Fish. Biol. 3 (1993) 160-180.

Klein, S.L. The effects of hormones on sex differences in infection: from genes to behavior. Neurosci. Biobehav. Rev. 24 (2000) 627-638.

Kuiper, G.G., Shughrue, P.J., Merchenthaler, I., Gustafsson, J.A. The estrogen receptor beta subtype: a novel mediator of estrogen action in neuroendocrine systems. Front. Neuroendocrinol. 19 (1998) 253-286.

Kurtz, J., Kalbe, M., Langefors, A., Mayer, I., Milinski, M., Hasselquisti, D. An experimental test of the immunocompetence handicap hypothesis in a teleost fish: 11-ketotestosterone suppresses innate immunity in three-spined sticklebacks. Am. Nat. 170 (2007) 509–519.

Kusakabe, M., Todo, T., McQuillan, H.J., Goetz, F.W., Young, G. Characterization and expression of steroidogenic acute regulatory protein and MLN64 cDNAs in trout. Endocrinology 143 (2002) 2062–2070.

Laing, K.J., Zou, J.J., Wang, T., Bols, N., Hirono, I., Aoki, T., Secombes, C.J. Identification and analysis of an interleukin 8-like molecule in rainbow trout (*Oncorhynchus mykiss*). Dev. Comp. Immunol. 26 (2002) 433-444.

Lamas, J., Ellis, A.E. Atlantic salmon (*Salmo salar*) neutrophil responses to *Aeromonas salmonicida*. Fish Shellfish Immun. 4 (1994) 201-219.

Laurentino, S., S. Pinto, P. I., Tomás, J., Cavaco, J. E., Sousa, M., Barros, A., Power, D. M., Canário, A.V., Socorro, S. Identification of androgen receptor variants in testis from humans and other vertebrates. Andrologia. 45 (2013) 187-194.

Law, W.-Y., Chen, W.-H., Song, Y.-L., Dufour, S., Chang, C.-F. Differential in vitro suppressive effects of steroids on leukocyte phagocytosis in two teleosts, tilapia and common carp. General and Comparative Endocrinology 121 (2001) 163-172.

Lawrence, D.A. Transforming growth factor-β: A general review. Eur. Cytokine Netw. 7 (1996) 363-374.

Le Gac, F., Loir, M. Male reproductive system fish. In: Knobil E, Neil JD (eds) Encyclopedia of Reproduction. Academic Press New York 3 (1999) pp. 20-30.

Lee, T.P., Chiang, B.L. Sex differences in spontaneous versus induced animal models of autoimmunity. Autoimmun. Rev. 11 (2012). 422–429.

Leet, J.K., Gall, H.E., Sepulveda, M.S. A review of studies on androgen and estrogen exposure in fish early life stages: effects on gene and hormonal control of sexual differentiation. J. Appl. Toxicol. 31 (2011) 379-398.

Liarte, S., Chaves-Pozo, E., García-Alcázar, A., Mulero, V., Meseguer, J., García-Ayala, A. Testicular involution prior to sex change in gilthead seabream is characterized by a decrease in DMRT1 gene expression and by massive leukocyte infiltration. Reprod. Biol. Endocrinol. 5 (2007) 20-35.

Liarte, S., Chaves-Pozo, E., Abellán, E., Meseguer, J., Mulero, V., García-Ayala, A. 17beta-Estradiol regulates gilthead seabream professional phagocyte responses through macrophage activation. Dev. Comp. Immunol. 35 (2011a) 19-27.

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. 48 (2011b) 1917-1925.

Lo Nostro, F.L., Antoneli, F.N., Quagio-Grassiotto, I., Guerrero, G.A. Testicular interstitial cells, and steroidogenic detection in the protogynous fish,

Synbranchus marmoratus (Teleostei, Synbranchidae). Tissue Cell. 36 (2004) 221-231.

Lobb, C.J., Clem, L.W. Fish lymphocytes differ in the expression of surface immunoglobulin. Dev. Comp. Immunol. 6 (1982) 473-479.

Loir, M., Sourdaine, P., Mendis-Handagama, S.M., Jegou, B. Cell-cell interactions in the testis of teleosts and elasmobranchs. Microsc. Res. Techniq. 32 (1995) 533-552.

Lynch, KW. Consequences of regulated pre-mRNA splicing in the immune system. Nature Reviews. Immunology 4 (2004) 931–940.

Makeyev, E.V., Zhang, J., Carrasco, M.A., Maniatis, T. The microRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. Mol. Cell. 27 (2007) 435–448.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., Evans, RM. The nuclear receptor superfamily: the second decade. Cell 83 (1995) 835–839.

Marsden, M.J., Hamdani, S.H., Secombes, C.J. Proliferative responses of rainbow trout, *Oncorhynchus mykiss*, T and B cells to antigens of *Aeromonas salmonicida.* Fish Shellfish Immun. 5 (1995) 199-210.

Margiotta-Casaluci, L., Sumpter, J.P. 5alpha-Dihydrotestosterone is a potent androgen in the fathead minnow (*Pimephales promelas*), Gen. Comp. Endocrinol. 171 (2011) 309-318.

McCruden, A.B., and Stimson, W.H. Sex hormones and immune function. In: Psychoneuroimmunology, 2nd Ed. (1991) pp.475–493, Academic Press, New York.

Medzhitov, R., Janeway, C.A. Innate immune recognition: mechanism and pathways- Immunol. Rev. 173 (2000) 89-97.

Micale, V., Perdichizzi, F., Santangelo, G. The gonadal cycle of captive white bream, *Diplodus sargus* (L.). J. Fish Biol. 31 (1987) 435-440.

Miller, W.L. Molecular biology of steroidogenesis. Endoc. Rev. 9 (1988) 295-317.

Miller, W.L. Regulation of mRNAs for human steroidogenic enzymes. Endocr. Res. 15 (1989) 1-16.

Miller, N.W., Deuter, A., Clem, L.W. Phylogeny of lymphocyte heterogeneity: The cellular requirements for the mixed leukocyte reaction with channel catfish. Immunology 59 (1986) 123-128.

Miller, W.L. Minireview: Regulation of steroidogenesis by electron transfer. Endocrinology 146 (2005) 2544-2550.

Miura, T. Spermatogenic cycle in fish. In: Knobil E, Neil JD (eds) Encyclopedia of Reproduction. Academic Press New York, 4 (1999) pp. 571-578.

Miura, T., Yamauchi, K., Takahashi, H , Nagahama, Y. Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (*Anguilla japonica*), Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 5774-5778.

Miura, T., Miura, C., Ohta, T., Nader, M.R., Todo, T., Yamauchi, K. Estradiol-17beta stimulates the renewal of spermatogonial stem cells in males. Biochem. Biophys. Res. Commun. 264 (1999) 230-234.

Miura, T., Miura, C. Japanese eel: A model for analysis of spermatogenesis. *Zoological* Science 18 **(**2001)1055-1063.

Miura, T., Ando, N., Miura, C., Yamauchi, K. Comparative studies between *in viv*o and *in vitro* spermatogenesis of Japanese eel (*Anguilla japonica*). Zool. Sci. 19 (2002) 321-329.

Molero, L., García-Durán, M., Díaz-Recasens, J., Rico, L., Casado, S., López-Farre, A. Expression of estrogen receptor subtypes and neuronal nitric oxide synthase in neutrophils from women and men: Regulation by estrogen. Cardiovasc Res 56 (2002) 43-51.

Mortensen, A.S., Arukwe, A. Effects of 17alpha-ethynylestradiol on hormonal responses and xenobiotic biotransformation system of Atlantic salmon (*Salmo salar*). Aquat. Toxicol. 85 (2007) 113–23.

Mouriec, K., Gueguen, M.M., Manuel, C., Percevault, F., Thieulant, M.L., Pakdel, F., Kah, O. Androgens upregulate cyp19a1b (aromatase B) gene expression in the

brain of zebrafish (*Danio rerio*) through estrogen receptors, Biol. Reprod. 80 (2009) 889-896.

Nagahama, Y. The functional morphology of teleost gonads. En: Fish physiology, volume IXA (1983) pp 223-264. Eds Hoar WS, Randall DJ & Donaldson EM. New York: Academic Press.

Nam, B.H., Hirono, I., Aoki, T. The four TCR genes of teleost fish: The cDNA and genomic DNA analysis of Japanese flounder (*Paralichthys olivaceus*) TCR alpha-,beta-, gamma-, and delta-chains. J. Immunol. 170 (2003) 3081-3090

Namazi, M.R. The Th1-promoting effects of dehydroepiandrosterone can provide an explanation for the stronger Th1-immune response of women. Iran J. Allergy Asthma Immunol. 8 (2009) 65–69.

Nash, J.P., Kime, D.E., Van der Ven, L.T., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P., Tyler, C.R. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. Environ. Health Perspect. 112 (2004) 1725-1733.

Navarro, V., Quesada, J., Abad, M.E., Taverne, N., Rombout, J.H.W.M. Immuno (cyto) chemical characterization of monoclonal antibodies to gilthead seabream (*Sparus aurata*) immunoglobulin. Fish Shellfish Immun. 31 (1993) 67-177.

Nes, W.D., Lukyanenko, Y.O., Jia, Z.H., Quideau, S., Howald, W.N., Pratum, T.K., West, R.R., Hutson, J.C. Identification of the lipophilic factor produced by macrophages that stimulates steroidogenesis. Endocrinology 141 (2000) 953-958.

Nilsen, T.W., Graveley, B.R. Expansion of the eukaryotic proteome by alternative splicing. Nature 463 (2010) 457–463.

Olsen, N. J., Kovacs, W. J. Gonadal steroids and immunity. Endocr. Rev. 17 (1996) 369–384.

Oshiumi, H., Tsujita, T., Shida, K., Matsumoto, M., Ikeo, K., Seya, T. Prediction of the prototype of the human Toll-like receptor gene family for the pufferfish, Fugu rubripes, genome. Immunogenetics 54 (2003) 791-800.

Pascuali, A. Contributo allo studio dell'ermafroditismo e del differenziamento della gonade nell'orata (*Sparus auratus* L.) Publicazioni della Stazione Zoologica di Napoli 18 (1941) 282-312.

Paavonen, T. Hormonal regulation of immune responses. Ann. Med. 26 (1994) 255–258.

Pinto, P.I., Passos, A.L., Martins, R.S., Power, D.M., Canário A.V. Characterization of estrogen receptor beta b in sea bream (Sparus auratus): phylogeny, ligand-binding, and comparative analysis of expression. Gen. Comp. Endocrinol. 145 (2006) 197–207.

Posma, E., Moes, H., Heineman, M.J., Faas, M.M. The effect of testosterone on cytokine production in the specific and non-specific immune response. Am. J. Reprod. Immunol. 52 (2004) 237–243.

Quigley, C.A., De Bellis, A., Marschke, K.B., el-Awady, M.K., Wilson, E.M., French, F.S. Androgen receptor defects: historical, clinical, and molecular perspectives. Endocr. Rev. 16 (1995) 271–321.

Rahman, F., Christian H.C. Non-classical actions of testosterone: an update. Trends Endocrinol. Metab. 18 (2007) 371–378.

Rettew, J.A., Huet-Hudson, Y.M., Marriott, I. Testosterone reduces MØ expression in the mouse of toll-like receptor 4, a trigger for inflammation and innate immunity. Biol. Reprod. 78 (2008) 432–437.

Rifkind D, Frey JA. Sex differences in antibody response of CFW mice to Candida albicans. Infect. Immun. 5 (1972) 695-698.

Roberts, C. W., Satoskar, A., Alexander, J. Sex steroids, pregnancy-associated hormones and immunity to parasitic infection. Parasitol. Today 12 (1996) 382–388.

Roca, F.J., Sepulcre, M.P., López-Castejón, G., Meseguer, J., Mulero, V. The colony-stimulating factor-1 receptor is a specific marker of MØs from the bony fish gilthead seabream. Mol. Immunol. 43 (2006) 1418–1423.

Romano, A., Rubolini, D., Caprioli, M., Boncoraglio, G., Ambrosini, R., Saino, N. Sex-related effects of an immune challenge on growth and begging behavior of barn swallow nestlings. PLoS ONE 6 (2011) e22805.

Ros, A.F.H., Groothuis, T.G.G., Apanius, V. The relation among gonadal steroids, immunocompetence, body mass, and behavior in young black-headed gulls (*Larus ridibundus*). Am. Nat. 150 (1997) 201–219.

Roy, A.K., Lavrovsky, Y., Song, C.S., Chen, S., Jung, M.H., Velu, N.K., Bi, B.Y., Chatterjee, B. Regulation of androgen action. Vitam. Horm. 55 (1999) 309–352.

Sadovy, Y., Shapiro, D.Y. Criteria for the diagnosis of hermaphroditism in fishes. Copeia 1 (1987) 136-156.

Saha, N. R., Usami, T. & Suzuki, Y. A double staining flow sytometric assay for the detection of steroid induced apoptotic leucocytes in common carp (Cyprinus carpio). Dev. Comp. Immunol.27 (2003) 351‑363.

Sánchez, L.. Sex-determining mechanisms in insects. Int. J. Dev. Biol. 52 (2008) 837–856.

Sánchez-Hernández, M., Chaves-Pozo, E., Cabas, I., Mulero, V., García-Ayala, A., García-Alcázar A. Testosterone implants modify the steroid hormone balance and the gonadal physiology of gilthead seabream (Sparus aurata L.) males. J. Steroid Biochem. 138 (2013) 183-194.

Sato, T., Matsumoto, T., Kawano, H., Watanabe, T., Uematsu, Y., Sekine, K., Fukuda, T., Aihara, K., Krust, A., Yamada, T., Nakamichi, Y., Yamamoto, Y., Nakamura, T., Yoshimura, K., Yoshizawa, .T, Metzger, D., Chambon, P., Kato, S. Brain masculinization requires androgen receptor function. Proc. Natl. Acad. Sci. 101 (2004) 1673–1678.

Schulz, R.W., Vischer, H.F., Cavaco, J.E., Santos, E.M., Tyler, C.R., Goos, H.J., Bogerd, J. Gonadotropins, their receptors, and the regulation of testicular functions in fish. Comp. Biochem. Phys. B 129 (2001) 407-417.

Schuurs, A. H. W. M., Verheul, H. A. M. Effects of gender and sex steroids on the immune response. J. Steroid Biochem. 35 (1990) 157–172 .

Scott, A.P. Reproductive endocrinology of fish. In: Comparative vertebrate endocrinology (1987) pp 223-256. Eds Chester-Jones I, Ingleton PM y Philips JG. New York: Plenum Pres.

Scott, A.P., Sumpter, J.P. Seasonal variations in testicular germ cell stages and in plasma concentrations of sex steroids in male rainbow trout (*Salmo gairdneri*) maturing at 2 years old. Gen. Comp. Endocrinol. 73 (1989) 46-58.

Senthilkumaran, B., Yoshikuni, M., Nagahama, Y. A shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation. Mol. Cell. Endocinol. 215 (2004) 11-18.

Sepulcre, M.P., Pelegrín, P., Mulero, V., Meseguer, J. Characterisation of gilthead seabream acidophilic granulocytes by a monoclonal antibody unequivocally points to their involvement in fish phagocytic response. Cell. Tissue Res. 308 (2002) 97-102.

Sepulcre, M.P., López-Castejón, G., Meseguer, J., Mulero, V. The activation of gilthead seabream professional phagocytes by differnt PAMPS underlines the behavioural diversity of the main innate immune cells of bony fish. Mol. Immunol. 44 (2007) 2009-2016.

Sepulcre, M.P., López-Muñoz, A., Angosto, D., García-Alcázar, A., Meseguer, J., Mulero, V. TLR agonists extend the functional lifespan of professional phagocytic granulocytes in the bony fish gilthead seabream and direct precursor differentiation towards the production of granulocytes. Mol. Immunol. 48 (2011) 846-859.

Sharp, G.J., Secombes, C.J. The role of reactive oxygen species in the killing of the bacterial fish pathogen *Aeromonas salmonicida* by rainbow trout macrophages. Fish Shellfish Immun. 3 (1993) 119-129.

Shin, C., Manley, J.L. Cell signalling and the control of pre-mRNA splicing. Nature Rev. Mol. Cell Biol. 5 (2004) 727–738.

Shiina, H., Matsumoto, T., Sato, T., Igarashi, K., Miyamoto, J., Takemasa, S., Sakari, M., Takada, I., Nakamura, T., Metzger, D. Chambon, P., Kanno, J., Yoshikawa, H., Kato, S. Premature ovarian failure in androgen receptor-deficient mice. Proc. Natl. Acad. Sci 103 (2006) 224–229.

Shrestha, T.K., Khanna, S.S. Histology and seasonal changes in the testes of a hillstream fish *Schizothorax plagiostomus*. Z. Mikrosk. Anat. Forsch. 90 (1976) 749-761.

Sizemore, R.C., Miller, N.W., Cuchens, M.A., Lobb, C.J., Clem, L.W. Phylogeny of lymphocytes heterogeneity: The cellular requirements for *in vitro* mitogenic responses of channel catfish leukocytes. J. Immunol. 133 (1984) 2920-2924.

Skarmeta, M.A., Bandin, I., Santos, Y., Toranzo, A.E. *In vitro* killing of *Pasteurella piscicida* by fish macrophages. Dis. Aquat. Org. 23 (1995) 51-57.

Slater, C.H., Fitzpatrick, M.S., Schreck, C.B. Characterization of an androgen receptor in salmonid lymphocytes: possible link to androgen-induced immunosuppression. Gen. Comp. Endocrinol. 100 (1995) 218–225.

Soma, K.K. Testosterone and agression: Berthold, birds and beyond. Journal of Neuroendocrinology 18 (2006) 543–551

Sperry, T.S., Thomas, P. Identification of two nuclear androgen receptors in kelp bass (Paralabrax clathratus) and their binding affinities for xenobiotics: comparison with Atlantic croaker (Micropogonias undulatus) androgen receptors. Biol. Reprod. 61 (1999a) 1152–1161.

Sperry, T.S., Thomas, P. Characterization of two nuclear androgen receptors in Atlantic croaker: comparison of their biochemical properties and binding specificities. Endocrinology 140 (1999b) 1602–1611.

Sperry, T.S., Thomas, P. Androgen binding profiles of two distinct nuclear androgen receptors in Atlantic croaker (*Micropogonias undulatus*). J. Steroid. Biochem. 73 (2000) 93–103.

Stocco, D.M. StAR protein and the regulation of steroid hormone biosynthesis. Annu Rev Physiol 63 (2001) 193-213Straub, R.H. The complex role of estrogens in inflammation. Endocr. Rev. 28 (2007) 521-574.

Sumathy, K., Desai, K.V., Kondaiah, P. Isolation of transforming growth factor-β 2 cDNA from a fish, *Cyprinus carpio* by RT-PCR. Gene 191 (1997) 103-107.

Sun, S., Sprenger, C.C., Vessella, R.L., Haugk, K., Soriano, K., Mostaghel, E.A., Page, S.T., Coleman, I.M., Nguyen, H.M., Sun, H., Nelson, P.S., Plymate, S.R. Castration resistance in human prostate cancer is conferred by a frequently occurring androgen receptor splice variant. J. Clin Invest. 120 (2010) 2715–2730.

Svensson, E.I., McAdam, A.G., Sinervo, B. Intralocus sexual conflict over immune defense, gender load, and sex-specific signaling in a natural lizard population. Evolution 63 (2009) 3124–3135

Tsujita, T., Tsukada, H., Nakao, M., Oshiumi, H., Matsumoto, M., Seya, T. Sensing bacterial flagellin by membrane and soluble orthologs of Toll-like receptor 5 in rainbow trout (*Onchorhynchus mikiss*). J. Biol. Chem. 279 (2004) 48588-48597.

Vainikka, A., Joikinen, E. I., Kortet, R., Paukku, S., Pirhonen, J., Rantala, M. J., Taskinen. J. Effects of testosterone and b-glucan on immune functions in tench. J. Fish. Biol. 66 (2005) 348‑361.

Vallejo, A.N., Miller, N.W., Clem, L.W. Antigen processing and presentation in teleost immune responses. Ann. Rev. Fish Dis. 2 (1992) 73-89.

Van Muiswinkel WB The piscine immune system: Innate and acquired immunity. In: Woo P (eds) Fish Diseases and Disorders. CAB International, Oxon, 1995 pp. 729-750.

Villeneuve, D.L., Blake, L.S., Brodin, J.D., Greene, K.J., Knoebl, I., Miracle, A.L., Martinovic, D., Ankley, G.T. Transcription of key genes regulating gonadal steroidogenesis in control and ketoconazole- or vinclozolin-exposed fathead minnows. Toxicol. Sci. 98 (2007) 395-407.

Von Eschen, K.B., Rudbach, J.A. Inactivation of endotoxin by serum: A phylogenetic study. J Infect. Dis. 129 (1974) 21-27.

Wahli, W., Martinez, E. Superfamily of steroid nuclear receptors: Positive and negative regulators of gene expression. Faseb. J. 5 (1991) 2243-2249.

Wang, T., Ward, M., Grabowski, P., Secombes, C.J. Molecular cloning, gene organization and expression of rainbow trout (*Oncorhynchus mykiss*) inducible nitric oxide synthase (iNOS) gene. Biochem. J. 358 (2001) 747-755.

Watanuki, H., Yamaguchi, T., Sakai, M. Suppression in function of phagocytic cells in common carp *Cyprinus carpio* L. injected with estradiol, progesterone or 11-ketotestosterone. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 132 (2002) 407–413.

Weltzien, F.A., Andersson, E., Andersen, O., Shalchian-Tabrizi, K., Norberg, B. The brain–pituitary–gonad axis in male teleosts, with special emphasis on flatfishv(Pleuroneciformes). Comp Biochem Physiol A: Mol. Integr. Physiol. 137 (2004) 447-477.

Wermenstam, N.E., Pilstrom, L. T-cell antigen receptors in Atlantic cod (*Gadus morhua* L.): Structure, organisation and expression of TCR alpha and beta genes. Dev. Comp. Immunol. 25 (2001)117-135.

Xie J, Black DL. A CaMK IV responsive RNA element mediates depolarization-induced alternative splicing of ion channels. Nature. 2001; 410:936–939.

Yada, T, Nakanishi, T. Interaction between endocrine and immune system in fish. Int. Rev. Cytol.220 (2002) 35-92.

Yamaguchi, T., Watanuki, H., Sakai M. Effects of estradiol, progesterone and testosterone on the function of carp, Cyprinus carpio, phagocytes in vitro. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 129 (2001) 49–55.

Yeh, S.H., Chen, P.J. Gender disparity of hepatocellular carcinoma: the roles of sex hormones. Oncology 78 (2010) 172–179.

Yeo, G., Holste, D., Kreiman, G., Burge, CB. Variation in alternative splicing across human tissues. Genome Biol. 5 (2004) R74.

Young, G., Kusakabe, M., Nakamura, I., Lokman, P.M., Goetz, F.W. Gonadal steroidogenesis in teleost fish. In: Melamed P and Sherwood N (eds)

''Hormones and their Receptors in Fish Reproduction'' World Scientific, New Jersey (2004) pp. 155–223.

Zapata, A.G., Chibá, A., Varas, A. Cells and tissues of the immune system of fish. In: Iwama G. & Nakanishi T. (eds) The Fish Immune System. Organism, Pathogen and Environment. Academic Press, San Diego (1996) pp. 1-62.

Zhang, Y.A., Salinas, I., Li, J., Parra, D., Bjork, S., Xu, Z., LaPatra, S.E., Bartholomew, J., Sunyer, J.O. IgT, a primitive immunoglobulin class specialized in mucosal immunity. Nat. Immunol. 11 (2010) 827-835.

Zhou, Z.X., Sar, M., Simental, J.A., Lane, M.V., Wilson, E.M. A ligand-dependent bipartite nuclear targeting signal in the human androgen receptor. Requirement for the DNAbinding domain and modulation by NH2-terminal and carboxyl-terminal sequences. J. Biol. Chem. 269 (1994) 13115–13123.

Zhou, L.Y., Wang, D.S., Senthilkumaran, B., Yoshikuni, M., Shibata, Y., Kobayashi, T., Sudhakumari, C.C., Nagahama, Y. Cloning, expression and characterization of three types of 17beta-hydroxysteroid dehydrogenases from the Nile tilapia, *Oreochromis niloticus*. J. Mol. Endocrinol. 35 (2005) 103-116.

Zohar, Y., Abraham, M., Gordin, H. The gonadal cycle of the captivity-reared hermaphroditic teleost *Sparus aurata* (L.) during the first two years of life. Annales de biologie animale, biochimie, biophysique 18 (1978) 877-882.

Zou, J., Grabowski, P.S., Cunningham, C., Secombes, C.J. Molecular cloning of interleukin 1β from rainbow trout (*Oncorhynchus mykiss*) reveals no evidence of an ICE cut site. Cytokine 11 (1999a) 552-560.

Zou, J., Cunningham, C., Secombes, C.J. The rainbow trout (*Oncorhynchus mykiss*) interleukin-1β gene has a differ organization to mammals and undergoes incomplete splicing. Eur. J. Biochem. 259 (1999b) 901-908.

RESUMEN EN CASTELLANO

ABREVIATURAS

11KT	11-cetotestosterone
ADN	Ácido desoxirribonucléico
ADNc	ADN complementario
AGs	Granulocitos acidófilos
AR	Receptor andrógenos
ARβ	Receptor andrógenos isoforma beta
ARΔLBD	Receptor andrógenos con ausencia del dominio de unión al ligando
ARN	Ácido ribonucléico
ARNm	Ácido ribonucléico mensajero
βdiol	5α-androstae-3β-17βdiol
bp	Pares de bases
ccl4	CC quimioquina ligando 4
CR	Ciclo reproductor
CP	Cáncer de próstata
cyp	Enzimas del citocromo P450
DHT	Dihidrotestosterona
Dmrt1	Doble sexo y mab3 relacionados con factor de transcripción 1
dpi	Días post-implante
E_2	17β-estradiol
EDCs	Compuestos de disrupción endocrina
EE_2	17α-etinilestradiol
ER	Receptor de estrógenos
ES	Espermatogénesis
FSH	Hormona folículo estimulante
FSH-R	Receptor de la hormona folículo estimulante
IGS	Índice gonadosomático
IHS	Índice hepatosomático

IT	Involución testicular
HK	Riñón cefálico
hsds	Deshidrogenasas hydroxysteroideas
IFN	Interferón
il	Interleucina
ISM	Implante de micropartículas *in situ*
LH	Hormona luteinizante
LH-R	Receptor de la hormona luteinizante
Ly	Linfocitos
MØs	Macrófagos
OHT	11β-hidroxitestosterona
PRRs	Receptores de reconocimiento de patrones
PP	Post-puesta
Q	Quiescencia
ROS	Radicales libres de oxígeno
P	Puesta
Sele	E-Selectina
SIA	Síndrome de insensibilidad a los andrógenos
star	Proteina de regulación aguda de la esteroidogénesis
T	Testosterona
tgfβ	Factor transformante de crecimiento β
tlr	Receptores similares a Toll

1. Resumen.

En la siguiente Tesis Doctoral hemos analizado la influencia de la testosterona (T) exógena sobre el metabolismo de esteroides sexuales y la fisiología de la gónada de la dorada (*Sparus aurata* L.). Además hemos descrito la presencia de un mecanismo de procesamiento alternativo del ARN, para el receptor de andrógenos (AR), cuya expresión varía con el estado reproductor y se correlaciona con el nivel de T. Esta forma truncada del AR está presente en los granulocitos acidófilos (GAs), descubriéndonos así nuevos mecanismos de regulación en la biología de los neutrófilos de vertebrados.

El uso de la dorada como ejemplar de estudio se debe a que es una especie de alto interés comercial en el Mar Mediterráneo. La dorada es un teleósteo marino que presenta un hermafroditismo protándrico, siendo los ejemplares machos durante sus dos primeros años de vida, al menos en las condiciones ambientales del Sureste Mediterráneo, y pudiendo cambiar de sexo a partir del segundo año. El ciclo reproductor (CR) de los ejemplares macho se caracteriza por presentar cuatro etapas secuenciales: espermatogénesis (ES), puesta (P), post-puesta (PP) y quiesciencia (Q) durante el primer CR. La etapa de Q es sustituida por una etapa de involución testicular (IT), en el segundo CR que permite el cambio de sexo. Tras la puesta, el testículo suele sufrir una serie de cambios morfológicos, entre los que se encuentra una infiltración masiva de leucocitos, la cual es mayor en la etapa de IT, antes del cambio de sexo, que ha puesto de manifiesto la importancia fisiológica de la regulación inmunitaria en la gónada.

En primer lugar, quisimos comprobar la capacidad de la T exógena para modificar el balance de las hormonas sexuales esteroideas y la fisiología de la gónada (proliferación celular, apoptosis, migración de leucocitos, estado inmunitario). Con este fin, incorporamos la T en el cuerpo de las doradas utilizando el sistema de micropartículas *in situ* (ISM) (T-ISM), el cual libera la hormona sin producir ninguna alteración fisiológica en los ejemplares, como había sido previamente descrito en nuestro grupo de investigación. La T de los T-ISM promueve un incremento supra-fisiológico en los niveles plasmáticos de T lo que, a su vez, provoca una disminución de los niveles de 17β-estardiol (E_2). Sin embargo, no se vieron alterados los niveles de 11-cetotestosterona (11KT). El perfil de expresión génica en gónada e hígado de algunos de los enzimas implicados en el metabolismo esteroidogénico nos mostró que la T

liberada de los T-ISM no era transformada en compuestos estrogénicos. La T, además, bloquea la proliferación de las células germinales del testículo aunque el incremento en la expresión del gen que codifica para dmrt1 podría prevenir la eliminación completa de las células germinales. Por otra parte, comprobamos que los andrógenos, al igual que había sido demostrado para los estrógenos, están implicados en la migración de leucocitos hacia la gónada, aunque los niveles de expresión de algunas citoquinas, quimioquinas y moléculas de adhesión celular sufrieron una disminución. Además, la T de los T-ISM promueve una disminución de la expresión de los genes que codifican para los receptores similares a Toll (*tlrs*), lo que podría provocar una inhibición de la respuesta inmunitaria en la gónada al quedar inhibida la capacidad de reconocer y responder a patógenos.

Posteriormente, identificamos una variante, constitutivamente activa, del AR que carece de dominio de unión al ligando (ARΔLBD) en testículo y riñón cefálico (RC) de dorada y su expresión varía con el estado reproductor y con los niveles plasmáticos de T. Esta variante se forma por un procesamiento alternativo del ARNm del AR. Nuestros datos sugieren que este procesamiento alternativo es capaz de modificar la fisiología del testículo, debido a que los andrógenos son capaces de modular el procesamiento alternativo del AR. Además, los andrógenos, concretamente la T, es capaz de regular la respuesta inmunitaria a través de la variante ARΔLBD. Otro dato interesante es que sólo los AGs expresan esta forma truncada mientras que los macrófagos (MØs) no la expresan. Además, el procesamiento alternativo del AR no sólo se ve modulado por T, sino también por estímulos inmunitarios, aunque esta modulación depende del estado reproductor de los individuos.

Todos estos datos nos indican una interacción entre los estímulos endocrinos e inmunitarios en la regulación del procesamiento alternativo del AR así como de las funciones de los AGs. Además, la T podría estar implicada en los cambios fisiológicos de la gónada, entre los que se encuentra el aumento de leucocitos y la disminución de la capacidad de reconocer patógenos de la gónada, a través de la regulación del ARΔLBD.

2. Introducción.

Los peces son uno de los grupos más grandes de vertebrados. Está compuesto aproximadamente de 24.600 especies entre las que unas 23.700 son teleósteos. Esta gran diversidad se pone de manifiesto en diversos aspectos de la biología de los mismos, como es el caso de las estrategias reproductoras, la fisiología y su forma de vida. Por otro lado, en los últimos años la demanda de pescado y marisco por parte de la población mundial se ha incrementado exponencialmente, sometiendo al medio marino a una gran presión por parte de las pesquerías comprometiendo así su capacidad de renovación. La pesca extractiva representa apenas el 60% del volumen anual de producción mundial de pescado (FAO, 2012), por lo que la acuicultura se presenta como la única forma de satisfacer la demanda en el futuro próximo. Por lo tanto, la FAO estima que en 2030 más del 65% de los alimentos acuáticos procederá de la acuicultura. Por otra parte, España es el tercer productor de pescado cultivado de la Unión Europea ; la acuicultura en nuestro país tiene un gran potencial de riqueza y empleo y un futuro prometedor como actividad suministradora de alimentos sostenibles y de alta calidad para los consumidores.

La gran diversidad existente entre los peces teleósteos conlleva a su vez una gran diversidad en su biología, especialmente en estrategias reproductoras y en los ciclos reproductores (CRs). Hay dos estrategias reproductivas básicas: gonocoristas, los cuales desarrollan un único sexo funcional y hermafroditas, cuando desarrollan dos sexos funcionales a lo largo de su vida. A su vez los hermafroditas pueden ser protándricos o protóginos, cuando desarrollan primero el sexo masculino o femenino, o ambisexuales cuando desarrollan ambas gónadas simultáneamente (Devlin y Nagahama, 2002). Entre los hermafroditas, los espáridos se caracterizan por la peculiar morfología que tiene su gónada bisexual. En este grupo, se habla de un ovotestículo de tipo delimitado que consiste en un área ovárica, en la porción mediodorsal de la gónada, y un área testicular, en la porción latero-ventral, delimitadas ambas por una pared de tejido conectivo (Sadovy y Shapiro, 1987; Besseau y Faliex, 1994; Bruslé-Sicard y Fourcault, 1997).

En este contexto, la dorada (*Sparus aurata* L.) es un pez teleósteo, marino, hermafrodita protándrico de puesta es estacional que presenta una gónada bisexual. Los individuos de esta especie se desarrollan como machos durante, al menos, los dos primeros CRs en el sureste mediterráneo, pudiendo cambiar de sexo a partir del tercero dependiendo de las condiciones ambientales (Zohar *y col.,* 1978; Liarte *y col.,* 2007). Su

CR consta de cuatro etapas: espermatogénesis (ES), puesta (P), post-puesta (PP) y quiescencia (Q) durante el primer CR, siendo esta última sustituida por una etapa de involución testicular (IT) durante el segundo CR (Chaves-Pozo *y col.*, 2005a; Liarte *y col.*, 2007). Tras la puesta, el testículo sufre una serie de cambios morfológicos bruscos, que provocan una remodelación tisular y entre los que se incluyen una infiltración masiva de granulocitos acidófilos (AGs), células fagocíticas esenciales para la fisiología normal de la gónada (Chaves-Pozo *y col.*, 2003, 2005a, b; Liarte *y col*., 2007). Estos procesos se correlacionan con un aumento importante en los niveles séricos de 17β-estradiol (E_2) y una disminución de los andrógenos, testosterona (T) y 11-cetotestosterona (11KT), principal andrógeno en esta especie (Chaves-Pozo *y col.*, 2008b).

Al igual que en mamíferos, en peces, la síntesis de hormonas esteroideas tiene lugar en la gónada, en un proceso conocido como esteroidogénesis (Young *y col.*, 2004). Este proceso conlleva una señal de transducción intracelular, la cual es provocada por las hormonas hipofisarias (hormona folículo estimulante, FSH, y hormona leuteinizante, LH), la síntesis de colesterol, el transporte del mismo hacia la mitocondria y las consiguientes conversiones enzimáticas desde el colesterol hasta los productos finales como la T, dihidrotestosterona (DHT) o el E_2. El E_2 es considerada la principal hormona femenina aunque hay estudios que sugieren que también es importante para la reproducción en los machos (Miura *y col.*., 1999b, 2002, 2003; Amer *y col.*, 2001; Hess, 2003). Por otra parte, la T y la 11KT son las principales hormonas masculinas en peces (Miura *y col.*, 1999b, 2002, 2003; Amer *y col.*, 2001; Hess, 2003). Mientras que la 11 KT es considerada el principal andrógeno es peces (Nagahama, 1983; Kime, 1993; Borg, 1994), la T, es un andrógeno aromatizable que puede transformarse en otros compuestos como DHT, 11β-hidroxitestosterona (OHT) o E_2 (Mouriec *y col.*, 2009).

Los peces constituyen el primer grupo animal que posee un sistema inmunitario innato y adaptativo bien estructurados, 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 diferentes especies de vertebrados. Una de las diferencias más importantes es que en peces existe un predominio de la respuesta inmunitaria innata frente los vertebrados superiores en

los cuales predomina la respuesta adaptativa (Anderson 1992). La respuesta innata es la primera en actuar, protegiendo al individuo a través de barreras como la piel y las mucosas (estás últimas además contienen enzimas bactericidas).Una vez que el patógeno se encuentra dentro de los individuos, las células fagocíticas los reconocen mediante receptores de reconocimiento de patrones (PRRs), los cuales en su mayoría son receptores semejantes a Toll (tlrs) (Akira y Takeda, 2004). Este reconocimiento, a su vez, provoca una serie de señales intracelulares que llevan a la producción de citoquinas y radicales libres de oxígeno (ROS). Las citoquinas son moléculas de pequeño peso molecular que pueden regular procesos biológicos como la inflamación, crecimiento celular, reparación tisular, fibrosis, etc. En peces se agrupan en varias categorías: i) factores de crecimiento (Grondel y Harmsen, 1984; Lawrence, 1996; Yin *y col.*, 1997), ii) citoquinas pro-inflamatoriascomo la interleucina 1β (il1β) (Jang *y col.*, 1995a, b; Zou *y col.*, 1999a, b; Fujiki *y col.*, 2000), citoquinas inmunosupresoras o anti-inflamatoriascomo el factor de crecimiento transformante β (tgfβ) (Sumathy *y col.*, 1997; Laing *y col.*, 1999; Harms *y col.*, 2000), iii) quimioquinascomo CCl4 (Daniels *y col.*, 1999; Fujiki *y col.*, 1999; Laing *y col.*, 2002) y los interferones (IFN) (Congleton y Sun, 1996; Collet y Secombes, 2002; Hansen y La Patra, 2002).

En los últimos años se ha demostrado que las hormonas esteroideas sexuales modulan diferentes actividades en la fisiología de los peces. El interés de estos estudios se debe, especialmente, al aumento en los niveles de compuestos de disrupción endocrina (EDC), entre los cuales se incluyen hormonas xenobióticas, que pueden encontrarse en ambientes acuáticos. De hecho, se han detectado los EDCs en estos ambientes en concentraciones capaces de alterar la reproducción y el comportamiento de los peces (Nash *y col.*, 2004; Mortensen y Arukwe, 2007). Numerosos estudios han puesto de manifiesto la complejidad de las respuestas del organismo a estrógenos; sin embargo, los efectos de los andrógenos han sido menos estudiados y, la mayoría, se han centrado en alteraciones de la reproducción. Por otra parte, se sabe que los andrógenos modulan su actividad por medio del receptor de andrógenos (AR), un receptor de membrana que actúa como factor de transcripción. Sin embargo, los efectos de los mismos son poco conocidos y, en algunos casos, contradictorios, lo que podría ser indicativo de la complejidad en su regulación. La unión de los andrógenos al AR regula la transcripción de ciertos genes aunque en los últimos años también se han descrito otras vías de actuación alternativas para los andrógenos (Rahman y Christian, 2007).

Los andrógenos están implicados en muchos eventos fisiológicos, principalmente reproductores como la diferenciación sexual, el comportamiento reproductor o la aparición de caracteres sexuales (Sato *y col.*, 2004; Soma 2006). No obstante, el AR ha mostrado un patrón de expresión generalizado en diversos tejidos lo que sugiere que podría estar implicado en la regulación de diversas actividades biológicas (de Waal *y col.*, 2008). Entre estas actividades que pueden estar reguladas por los andrógenos y el AR se halla la respuesta inmunitaria. Se ha visto que el AR se expresa en leucocitos y en tejidos inmunitarios en distintas especies de peces entre las que se incluye la dorada (Slater *y col.*, 1995; Blázquez y Piferrer, 2005; de Waal *y col.*, 2008; Castillo-Briceño *y col.*, 2013; Águila *y col.*, 2013). En general, se considera que la testosterona tiene efectos inmunosupresores al disminuir la expresión de tlrs (Rettew *y col.*, 2008) o modificar la producción de citoquinas (Posma *y col.*, 2004) pero también se ha visto que pueden aumentar la respuesta humoral en algunas especies de pájaros (Hasselquist *y col.,* 1999) o aumentar el número de anticuerpos producidos por la infección del virus Seoul en machos de rata (Klein *y col.*, 2000). Por otro lado, también se ha visto que una respuesta inmunitaria puede disminuir los niveles plasmáticos de T (Greiner *y col.*, 2010). En peces, además, los dos principales andrógenos parecen tener efectos diferentes sobre la respuesta inmunitaria. Así mientras que la T parece aumentar diferentes parámetros inmunitarios en dorada (Cuesta *y col.*, 2007), la 11KT parece tener un efecto inmunosupresor en la carpa (Yamaguchi *y col.*, 2001; Watanaki *y col.*, 2002; Kurtz *y col.*, 2007), aunque también se ha visto que la 11KT es capaz de aumentar la acumulación de pro-interleucina 1β en fagocitos de dorada (Chaves-Pozo *y col*., 2003). Por otra parte recientes estudios señalan la incapacidad de los andrógenos para regular la explosión respiratoria de los leucocitos de dorada, aunque si son capaces de regular fuertemente la expresión de genes inmunitarios (Águila *y col*., 2013). Además, en dorada, la T induce la expresión de la citoquina pro-inflamatoria il1β en AGs, mientras que la 11KT es capaz de inhibir los genes inmunitarios en AGs, al contrario que ocurre con MØ (Águila *y col*., 2013)

Estos datos contradictorios sobre los efectos de los andrógenos en el sistema inmunitario, junto con la necesidad del sistema inmunitario de adaptarse rápidamente a los cambios ambientales (Lynch, 2004), nos llevan a pensar en los procesamientos alternativos del ARNm, los cuales consisten en que un mismo gen da varios transcritos distintos con funciones distintas (Dehm y Tindal, 2011; Laurentino *y col.*, 2013). Los

tejidos donde se producen estos procesos con mayor frecuencia son los reproductores y los inmunitarios. En los primeros porque en adultos se dan una gran cantidad de procesos de desarrollo en las células germinales durante toda la vida y se necesitan varias vías para la coordinación, división y diferenciación de tan alto número de células (Elliot y Grellscheid, 2006), y en los segundos debido a que, como hemos mencionado antes, requieren gran diversidad y una alta capacidad para adaptarse rápidamente a cambios ambientales (Lynch, 2004). En este contexto, los mecanismos de procesamiento alternativo del AR son variados y se conocen básicamente en humanos. Las principales variantes que producen estos procesamientos alternativos están relacionadas con enfermedades, especialmente con el síndrome de insensibilidad a los andrógenos (SIA), en el que los nuevos transcritos del AR carecen de actividad, o con el cáncer de próstata (CP), en el que la mayoría de las variantes son constitutivamente activas (Dehm y Tindall, 2011). Estudios recientes han mostrado la presencia de algunas de las formas variantes en otras especies como la rata, la rana o la dorada (Laurentino *y col.*, 2013). La conservación de estas variantes del AR en organismos evolutivamente distantes nos muestra que podrían tener importancia a nivel funcional (Laurentino *y col.*, 2013).

Con todos estos antecedentes, la presente Tesis Doctoral se centra en determinar el efecto de la T, suministrada mediante un implante, sobre el balance de las principales hormonas sexuales esteroideas en peces así como sobre la fisiología de la gónada de la dorada. Además, tras identificar una variante truncada del AR, constitutivamente activa, queremos determinar su mediación en el papel de los andrógenos en la respuesta inmunitaria analizando su nivel de expresión en distintos momentos del CR y la modulación de su actividad.

3. Objetivos.

1. Evaluar el efecto de implantes de testosterona sobre el balance de hormonas sexuales esteroideas y la morfología de la gónada en ejemplares macho de dorada.

2. Caracterizar molecular y funcionalmente una variante truncada del receptor de andrógenos constitutivamente activa.

4. Resultados y discusión

Capítulo I. *Los implantes de testosterona modifican el balance de hormonas esteroideas sexuales y la fisiología de la gónada en ejemplares macho de dorada.*

La primera parte de esta Tesis Doctoral consistió en ver si la T, colocada en implantes ISM, podría modificar el balance de las hormonas sexuales esteroideas y la fisiología de la gónada en los machos de dorada, centrándonos en los eventos de proliferación celular, apoptosis, y migración de leucocitos, entre otros. Para ello, separamos y utilizamos un lote de ejemplares macho en espermatogénesis activa y, tras un periodo de aclimatación de 30 días, se les inyectó un implante intramuscular basado en el sistema ISM, previamente utilizado en dorada (Castillo-Briceño *y col.*, 2013), con 1 mg de T/Kg de masa corporal. Los peces se muestrearon semanalmente durante un mes.

En primer lugar, corroboramos los resultados de Castillo-Briceño *y col*. 2013 sobre que el implante ISM sin T no provoca variaciones en los niveles plasmáticos de las hormonas sexuales esteroideas ni en el índice gonadosomático (IGS) así como tampoco lo hace sobre la morfología de la gónada. Sin embargo, el implante con T provocó un aumento del IGS y del índice hepatosomático (IHS) a partir del día 14 y del día 7 hasta el día 21, respectivamente. Además, el implante de T promovió un aumento importante de los niveles de T plasmáticos, alcanzando niveles supra-fisiológicos mientras que los niveles de E_2 descendieron por debajo de los fisiológicos y los niveles de 11KT disminuyeron sólo al final del experimento (Chaves-Pozo *y col.*, 2008a). Por otra parte, cuando observamos los niveles de hormonas esteroideas en gónada -principal órgano donde se sintetizan (Borg, 1994)- e hígado-órgano en el cual se metabolizan (Arukwe, 2008)-, pudimos comprobar que en gónada el nivel de T se mantuvo constante mientras que en el hígado aumentó como era de esperar en un órgano encargado de procesar y eliminar estos compuestos (Arukwe, 2008). Sin embargo, la disminución de los niveles de 11KT, tanto en plasma como en gónada, así como las fluctuaciones en hígado nos sugiere que las tasas de eliminación de la misma son diferentes.

A continuación, nos centramos en el análisis de la expresión de genes que codifican algunas enzimas involucradas en la esteroidogénesis tanto en la síntesis *de novo* como en la transformación de la T, teniendo en cuenta los niveles de T, E_2 y 11KT

observados en plasma, gónada e hígado. Así, observamos que los niveles de expresión de ARNm de la enzima *star* no se vieron inducidos e, incluso, disminuyeron en la última semana de muestreo. Tampoco observamos ninguna variación en los niveles del transcrito de *cyp11a*. Al no encontrar variación en el nivel de expresión de estas dos enzimas, que se sintetizan rápido en respuesta a un aumento agudo de hormonas (Arukwe, 2008), sugerimos que la síntesis *de novo* de esteroides no se vio afectada por el implante de T. Por otro lado, el aumento en los niveles de ARNm de enzimas encargadas de la trasformación de T, como *cyp11b1*, *srd5a*, *cyp19a1a* y *hsd11b*, nos reveló la transformación de T en OHT, DHT y E_2. Este aumento en los niveles de ARNm también se ha observado en el cerebro del pez cebra y en mamíferos (Mouriec *y col.*, 2009). Sin embargo no hubo transformación de DHT a βdiol, como muestran los niveles de transcritos de *hsd3b*, los cuales apenas se modificaron a lo largo del experimento. Por otra parte, el hecho de que los niveles de E_2 en hígado se mantuvieran similares en peces con el implante de T y peces control unido a la baja expresión del transcrito de *cyp19a1a,* nos indicó que esta hormona no era producida en hígado.

A continuación, y conociendo los efectos que los estrógenos tienen en la fisiología de la gónada, nos dispusimos a analizar si los andrógenos tienen algún efecto relevante en eventos relacionados con la proliferación celular, apoptosis y migración de leucocitos en la gónada. Así, pudimos observar que el aumento de T en plasma, que a su vez provocó una disminución de E_2, produjo un bloqueo de la proliferación celular que no tuvo sin embargo ningún efecto en la tasa de apoptosis. Estos datos contrastan con lo observado cuando hay una aportación exógena de E_2 la cual provoca, a su vez, una caída de los niveles de andrógenos y una reducción en la tasa de la proliferación celular aunque aumenta la de apoptosis (Chaves-Pozo *y col.*, 2007; de Waal *y col.*, 2009). No obstante, la expresión elevada del ARNm del gen que codifica dmrt1 al final del experimento impide se agoten las células germinales a pesar del bloqueo de su proliferación (Chaves-Pozo *y col.,* 2007; Cabas *y col.,* 2011a),. Estudios previos indican que el E_2 es el factor principal que desencadena la migración de leucocitos hacia la gónada por influencia de quimioquinas y moléculas de adhesión en las células endoteliales y MØs (Chaves-Pozo *y col.,* 2007; Liarte *y col.* 2011a, b). El implante de T-ISM provocó un aumento de leucocitos, principalmente AGs y linfocitos (Ly), en el testículo aunque hubo una disminución de los genes que codifican para quimioquinas como *ccl4* y moléculas de adhesión como la *sele*. Nuestros datos sugieren que la

migración de leucocitos que tiene lugar en la gónada tras el T-ISM también puede ser regulada por andrógenos, probablemente, por medio de algunas citoquinas pro-inflamatorias como la *il1b*. Además, observamos que los implantes de T-ISM provocaron una disminución en la expresión génica de *tlr5s* y *tlr9*, que afectan a la capacidad de la gónada para reconocer y responder a los patógenos, a través de los Tlrs. Aunque los andrógenos parecen deprimir la respuesta inmunitaria de los vertebrados (Cutolo *y col.* 2005), nuestros datos en gónada de dorada, y los datos anteriores en riñón cefálico, parecen determinar que, en esta especie, los andrógenos aumentan o disminuyen la respuesta inmunitaria en función de la actividad analizada (Castillo-Briceño *y col.* 2013; Águila *y col.* 2013).

En conclusión, el diseño experimental utilizado permitió aumentar los niveles plasmáticos de T, a dosis supra-fisiológicas, provocando al mismo tiempo una disminución de los niveles de E_2 y el mantenimiento de los niveles de 11KT en concentraciones fisiológicas. En conjunto, podemos concluir que los andrógenos también juegan un papel importante en el mantenimiento de la morfología de la gónada. La migración de leucocitos hacia el testículo indica una compleja regulación de este proceso ya que se produce un aumento del número de AGs y Ly aún a pesar de existir una disminución de la expresión de genes que codifican para citoquinas, quimioquinas y moléculas de adhesión celular.

Capítulo II. *Identificación de una variante truncada del receptor de andrógenos constitutivamente activa que es expresada en granulocitos y modulada por andrógenos y estímulos inmunitarios*.

La interacción inmuno-reproductora y la regulación por parte de las hormonas sexuales esteroideas de procesos implicados en la respuesta inmunitaria en vertebrados es conocida desde hace varias décadas (Cohn, 1979; Bouman *y col.*, 2005; Fairweather *y col.*, 2008; Namazi, 2009; Yeh y Chen, 2010; Lee y Chiang, 2012). Diferentes estudios han demostrado en peces, incluyendo la dorada, que los estrógenos, tanto naturales como sintéticos, modulan la respuesta inmunitaria (Yamaguchi *y col.*, 2001; Watanuki *y col.*, 2002; Liarte *y col*., 2007. 2011a, b; Cabas *y col.*, 2012). Además, los estrógenos modulan la maduración y la función de los leucocitos (Yamaguchi *y col.*, 2001; Watanuki *y col.*, 2002; Cabas *y col.*, 2012). Sin embargo, son más escasos, y a veces

con resultados contradictorios, los estudios que han puesto de manifiesto la participación de los andrógenos en esta regulación (Yamaguchi *y col*., 2001; Watanaki y col, 2002; Chaves-Pozo *y col*., 2003; Cuesta y *col.*, 2007: Kurtz *y col*., 2007; Castillo-Briceño *y col.*, 2013; Águila *y col*., 2013. En dorada, estudios previos en nuestro laboratorio indican que la T ejerce una regulación de la respuesta inmunitaria innata y que la T y la 11KT ejercen una regulación competitiva sobre la sensibilidad de de los fagocitos a patógenos y señales de daño, ya que tanto los AGs como los MØs expresan el AR (Castillo-Briceño *y col.*, 2013; Águila *y col.*, 2013). Por este motivo, en este capítulo nos propusimos profundizar en el conocimiento del papel del ARs en la respuesta inmunitaria y, más concretamente, en la identificación de una variante truncada nueva, constitutivamente activa, del AR en leucocitos y su modulación por parte de los andrógenos y de estímulos inmunitarios.

En primer lugar, identificamos a partir de un ADN complementario (ADNc), procedente de testículo, dos fragmentos del AR. Las búsquedas BLASTN y BLASTX nos confirmaron que el fragmento mayor correspondía al AR y el fragmento menor correspondía a una forma truncada del AR producida por un procesamiento alternativo del ARNm, en el cual se eliminaban los exones 5 al 7 y el codón de stop se adelantaba, dando lugar a la proteína del AR que carecía de dominio de unión a ligando (LBD), denominado ARΔLBD. ARΔLBD se expresa en RC, además de en testículo.

El conocimiento actual sobre variantes del AR está centrado en humanos, siendo sin embargo en otras especies bastante escaso. En humanos estas variantes se expresan, principalmente, en casos de enfermedad como el síndrome de insensibilidad a los andrógenos (SIA) y el cáncer de próstata (CP), en las cuales se observa una pérdida o un incremento de la función del AR. Sin embargo, estudios recientes han mostrado la presencia de estas variantes en humanos sanos y en otras especies como la rata, la rana o la dorada (Laurentino *y col*., 2013), lo que podría indicar una complejidad en la regulación de los efectos de los andrógenos. En dorada se ha descrito previamente en testículo la presencia de una variante que tiene únicamente el dominio amino terminal (NTD), la cual se produce por la eliminación del exón 2 que a su vez provoca que se adelante el codón de stop dando lugar a una proteína truncada con un único dominio conocida como $AR\Delta 2^{stop}$ (Laurentino *y col.*, 2013). Esta variante fue descrita por primera vez en pacientes con SIA (Hellwinkel *y col.*, 1999). Nuestra variante, sin

embargo, es similar a la variante descrita por Sun *y col.*, $AR^{v5,6,7es}$, que es producida por la eliminación de los exones 5-7 (Sun *y col.*, 2010).

El perfil de expresión en testículo del AR y del ARΔLBD nos sugirió que ambas formas tienen distintas funciones según el CR. Además cabe señalar que el ratio de expresión de las dos variantes, ARΔLBD/AR, en testículo se correlacionó negativamente con los niveles plasmáticos de los principales andrógenos, T y 11KT. Estos datos junto con la habilidad de la T exógena de disminuir el ratio de expresión en testículo nos sugirieron que los andrógenos no sólo regulan la fisiología de la gónada a través del AR sino que también a través de la modulación de los niveles de expresión de la variante truncada, ARΔLBD. Por otra parte, encontramos expresión de la variante truncada en RC, la cual también variaba en función del CR, aunque de forma distinta a la que se mostró en testículo. Es conocido que la T exógena puede modificar la expresión del AR (Castillo-Briceño *y col*. 2013), lo cual también vimos que ocurría con ARΔLBD . Curiosamente, la correlación que mostró el ratio de expresión de las dos variantes en RC con los niveles plásmaticos de T fue positiva, al contrario que en testículo. Además la T exógena también fue capaz de incrementar el ratio de expresión de las dos variantes. Los resultados obtenidos en RC no son sorprendentes ya que los mecanismos de procesamiento alternativo permiten que el sistema inmunitario presente un alto grado de diversidad para ser capaz de adaptarse y responder rápidamente a las cambios ambientales (Lynch, 2004).

A continuación, quisimos comprobar que tipos celulares del RC expresaban el ARΔLBD una vez que sabíamos que GAs y MØs expresaban el AR (Águila *y col*., 2013). Curiosamente sólo los AGs presentaron la forma truncada. Los AGs son las principales células fagocíticas que infiltran el testículo tras la S, proceso que está regulado por hormonas esteroideas, produciéndose un aumento de la migración cuando aumenta el E_2, ya sea exógeno o endógeno, o cuando existe un aumento de compuestos estrogénicos (Chaves-Pozo *y col.*, 2008a, b, 2012; Liarte *y col.*, 2007; Cabas *y col.*, 2011). Curiosamente, la ratio de expresión de las dos variantes, ARΔLBD/AR, en los AGs aumenta cuando aumentan los niveles plasmáticos de T, resultado que se confirma, cuando en cultivos *in vitro*, también incrementa la expresión de la ratio con el aumento de la concentración de T. Por otra parte, también hemos observado que la estimulación de los AGs mediante ADN bacteriano (Sepulcre *y col.*, 2007, 2011), provocó un aumento considerable de la ratio de expresión ARΔLBD/AR durante la P, sugiriendo

una interacción cruzada entre los estímulos endocrinos e inmunitarios que regulan los mecanismos de procesamiento alternativo y las funciones de los AGs. Todos estos datos están en concordancia con estudios en mamíferos que sugieren que el procesamiento alternativo del ARNm de genes puede ser alterado en respuesta a la estimulación de antígenos (Lynch, 2004).

Por último, quisimos conocer cuál era la actividad del ARΔBD. Para ello, construimos un plásmido con el AR y otro con la variante ARΔLBD. Posteriormente, cada plásmido fue co-transfectado con el plásmido de luciferasa MMTV-LUC (de Ruiter *y col.*, 1995) en células HEK-293. Finalmente, las células fueron tratadas con distintas concentraciones de andrógenos, T o 11KT. La actividad de la variante ARΔLBD fue constitutivamente activa y mucho mayor que la del AR. Mientras que la variante ARΔLBD no mostró diferencias significativas cuando se incubó con las distintas concentraciones de andrógenos, el AR mostró una actividad dependiente de los andrógenos, aunque la actividad fue mucho mayor con la 11KT.

Por lo tanto, aunque se requieren más estudios para esclarecer la vía de señalización implicada en la regulación de este procesamiento alternativo del AR en AGS, nuestros resultados demuestran, por primera vez, que una variante debida a un procesamiento alternativo del AR se expresa en granulocitos. Además, teniendo en cuenta la importancia del AR en la granulopoiesis y supervivencia de granulocitos humanos y de ratón (Chuang *y col.*, 2009), nuestros resultados muestran un mecanismo nuevo en los mecanismos de regulación de la biología de los neutrófilos y podría explicar los resultados contradictorios que muestran que los andrógenos parecen ser menos importantes que el AR en la homeostasis de neutrófilos (Chuang *y col.*, 2009).

5. Conclusiones.

1. El implante de testosterona modifica el balance plasmático de las hormonas esteroideas sexuales. En concreto, aumenta la testosterona y disminuye el 17β-estradiol.

2. El implante de testosterona no influye en la síntesis *de novo* de las hormonas esteroideas sexuales, aunque induce la transformación de testosterona en otros compuestos androgénicos.

3. El implante de testosterona bloquea la proliferación de células germinales e induce la infiltración de leucocitos hacia la gónada a través de un aumento de expresión del ARNm de citoquinas pro-inflamatorias. No obstante, se ve disminuida la capacidad de la gónada de reconocer y responder a los patógenos.

4. Existe una variante truncada del receptor de andrógenos, ARΔLBD, en testículo y riñón cefálico que carece de dominio de unión al ligando y que es producida por un procesamiento alternativo del ARNm del receptor de andrógenos.

5. La expresión del ARΔLBD en riñón cefálico varía con la etapa reproductora y se correlaciona con los niveles plasmáticos de testosterona. Por otra parte, la testosterona exógena aumenta la expresión ARΔLBD en riñón cefálico, mientras que disminuye la de testículo.

6. ARΔLBD se expresa en granulocitos acidófilos, pero no en macrófagos, y su expresión es modulada por testosterona y por estímulos inmunitarios.

7. La actividad de ARΔLBD es independiente del ligando y mucho más elevada que la del AR.

FIGURES

TABLES

ANNEXES

Publications related with thesis.

Águila S, Castillo-Briseño P, **Sánchez M**, Cabas I, García-Alcázar A, Meseguer J, Mulero V, García-Ayala A. Specific and non-overlapping functions of testosterone and 11-ketotestosterone in the regulation of professional phagocyte responses in the teleost fish gilthead seabream. Mol Immunol. 2013 53(3):218-26.

Sánchez-Hernández M, Chaves-Pozo E, Cabas I, Mulero V, García-Ayala A, García-Alcázar A. Testosterone implants modify the steroid hormone balance and the gonadal physiology of gilthead seabream (*Sparus aurata* L.) males. J. Steroid. Biochem. 2013 138:183-194.

Sánchez-Hernández M, Arizcun M, García-Alcázar, A, Sarropoulou, E, Mulero, V, García-Ayala, A. Identification of a constitutively active androgen receptor variant which is expressed in fish granulocytes and is modulated by testosterone and immune challenge (under review).

Participation in (inter) national conferences.

- **Sánchez-Hernández, M.,** Castillo-Briceño, P., Abellán, E., García-Alcázar, A., García Ayala, A., Mulero, V. "Seasonal changes in the expression of immune related molecules in the teleost fish gilthead seabream." 9th International Congress on the Biology of Fish. Barcelona, Cataluña, España 05/07/2010. Poster.
- Águila, S., **Sánchez-Hernández, M.,** Castillo-Briceño, P., García-Alcázar, A., Meseguer, J., Mulero, V., García Ayala, A. "Androgens modulates in vitro the activation of professional phagocytes from the bony fish gilthead seabream." GIA. International Symposium Genomics in Aquaculture. Heraclion, Kriti, Grecia. 14/09/2011. Poster.
- **Sánchez-Hernández, M.,** Cabas, I., Chaves Pozo, E., García Alcázar, A., Meseguer, J., Mulero, V., García Ayala, A. "Testosterone implants alters the reproductive performance of the gilthead seabream." GIA. Intenational Symposium Genomics in Aquaculture. Heraclion, Kriti, Grecia 14/09/2011. Poster.
- **Sánchez-Hernández M.,** García Moreno, D., García Alcázar, A., Meseguer, J., Mulero, V., García Ayala, A. "A constitutively active variant of the androgen receptor is expressed in acidophilic granulocytes of the gilthead seabream". First International Conference of Fish and Shellfish Immunology. Vigo, Galicia, España 28/06/2013. Poster.
- **Sánchez-Hernández, M.,** García-Ayala, A., Mulero, V., Sarropoulou, E., García-Alcázar, A. "*Expression of a variant of the androgen receptor in different tissues of sea bream (Sparus aurata, L.)*." XIV Congreso Nacional de Acuicultura.