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"MODULACIÓN DE LA RESPUESTA INMUNITARIA POR HORMONAS Y FACTORES REPRODUCTORES EN TELEÓSTEOS"

Memoria que presenta
D. Sergio David Liarte Lastra
para optar al grado de Doctor con Mención Europea por la Universidad de Murcia.
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La presente Tesis Doctoral titulada "Modulación de la respuesta inmunitaria por hormonas y factores reproductores en teleósteos" realizada por D. Sergio David Liarte Lastra, es un compendio de publicaciones cuyas referencias completas son las siguientes:

Liarte S, Chaves-Pozo E, García-Alcazar A, Mulero V, Meseguer J, García-Ayala A (2007) 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, 20-35.

Liarte S, Cabas I, Chaves-Pozo E, Arizcum M, Meseguer J, Mulero V, García-Ayala A (2011a) Natural and synthetic estrogens modulate the inflammatory response in the gilthead seabream (*Sparus aurata* L.) through the activation of endothelial cells. Mol Immunol, En prensa.

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

Liarte S, Chaves-Pozo E, Abellán E, Meseguer J, Mulero V, Canario AV, García-Ayala A (2011c) Estrogen-responsive genes in macrophages of the bony fish gilthead seabream: A transcriptomic approach. Dev Comp Immunol 35, 840-849.

ÍNDICE DE CONTENIDOS

ABREVIATURAS

11-KT	11-cetotestosterona
143b1	Proteína 14-3-3 ß/α1
ADN-b	ADN-bacteriano
b2m	Microglobulina β-2
bcl7b	Proteína de la familia "célula B CLL/Linfoma 7", miembro B
bpi	Proteína bactericida incrementadora de la permeabilidad
ccl4	Proteína similar a la quimioquina C-C ligando 4
Células B	Linfocitos B
Células T	Linfocitos T
cox2	Ciclooxigenasa 2
dmrt1	Factor de transcripción relacionado con *doublesex* y *mab-3* tipo 1
E_2	17ß-estradiol
EE_2	17α-etinilestradiol
er	Receptor de estrógenos
friml	Isoforma hepática de la subunidad media de la ferritina
GO	Gene Onthologies Consortium
GTHs	Gonadotropinas
hells	Helicasa específica de tejido linfoide
igmh	Cadena pesada de la inmunoglobulina M
il	Interleuquina
KEGG	Kyoto Encyclopedia of Genes and Genomics
mcsfr	Receptor del factor estimulante de colonias de macrófagos
mhc1a	Proteína del complejo principal de histocompatibilidad 1α
mmp	Metaloproteasa de la matriz extracelular
mx	Proteína de resistencia de *myxovirus influenza*

NF-κB	Factor nuclear κB
nlrc3	Receptor similar a NOD C-3
ON	Óxido nítrico
PAMPs	Patrones moleculares asociados a patógenos
PCR	Reacción en cadena de la polimerasa
perf	Perforina 1
PRRs	Receptores de reconocimiento de patrones
psmd8	Subunidad 26s del proteasoma
RNIs	Intermediarios reactivos de nitrógeno
ROIs	Intermediarios reactivos de oxígeno
sele	Selectina-E
SSH	Supresión mediante hibridación substractiva
sumo	Proteína modificadora de bajo tamaño similar a ubiquitina 2
T	Testosterona
tcrb	Cadena β del receptor de células T
tgfb	Factor de crecimiento transformante ß
tgfbr	Receptor del factor de crecimiento transformante ß
tlr	Receptor similar a Toll
tnfa	Factor de necrosis tumoral α
ubiq	Ubiquitina
Uni-gen	Secuencia única

CAPÍTULO I: PUBLICACIONES DE QUE CONSTA LA TESIS

1. Testicular involution prior to sex change in gilthead seabream is characterized by a decrease in DMRT1 gene expression and by massive leukocyte infiltration

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Testicular involution prior to sex change in gilthead seabream is characterized by a decrease in DMRT1 gene expression and by massive leukocyte infiltration

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Abstract

Background: Leukocytes are found within the testis of most, if not all, mammals and are involved in immunological surveillance, physiological regulation and tissue remodelling. The testis of seasonal breeding fish undergoes a regression process. In the present study, the second reproductive cycle (RC) of the protandrous seasonal teleost fish, gilthead seabream, was investigated and the presence of leukocytes analysed. Special attention has been paid to the testicular degenerative process which is particularly active in the last stage of the second RC probably due to the immediacy of the sex change process.

Methods: Sexually mature specimens (n = 10–18 fish/month) were sampled during the second RC. Some specimens were intraperitoneally injected with bromodeoxyuridin (BrdU) before sampling. Light and electron microscopy was used to determine the different stages of gonadal development and the presence of leukocytes and PCR was used to analyse the gene expression of a testis-differentiating gene and of specific markers for macrophages and B and T lymphocytes. Immunocytochemistry and flow cytometry were performed using a specific antibody against acidophilic granulocytes from the gilthead seabream. Cell proliferation was detected by immunocytochemistry using an anti-BrdU antibody and apoptotic cells by in situ detection of DNA fragmentation.

Results: The fish in the western Mediterranean area developed as males during the first two RCs. The testis of all the specimens during the second RC underwent a degenerative process, which started at post-spawning and was enhanced during the testicular involution stage, when vitellogenic oocytes appeared in the ovary accompanied by a progressive increase in the ovarian index. However, only 40% of specimens were females in the third RC. Leukocytes (acidophilic granulocytes, macrophages and lymphocytes) were present in the gonad and acidophilic granulocyte infiltration occurred during the last two stages. At the same time DMRT1 gene expression decreased.

Conclusions: The results demonstrate that innate and adaptive immune cells are present in the gonads of gilthead seabream. Moreover, the whole fish population underwent a testicular degenerative process prior to sex change, characterized by high rates of apoptosis and necrosis and accompanied by an infiltration of acidophilic granulocytes and a decrease in DMRT1 levels.

Background

The testis is a dynamic tissue that is tightly controlled not only by hormones but also by local control mechanisms in which cell to cell interactions are involved. Leukocytes (macrophages, lymphocytes and mast cells) are found within the testes of most, if not all, mammals and are involved in immunological surveillance, physiological regulation and tissue remodelling [1-4]. Although the major focus of gonadal leukocyte research has been mammals, studies in other vertebrates may shed some light on the evolutionary mechanisms involved in the dysregulation of normal gonad physiology. Moreover, fish represent an attractive group of organisms for studying sex determination from the evolutionary point of view because they cover the complete range of sexuality, from hermaphroditism to gonochorism [5]. However, most of the fish models used to analyze the genes involved in sex determination and differentiation are gonochorism [6]. Unlike in mammals, sex-determining genes have not been described in fish, although some candidates have been proposed [6]. Thus, based on evolutionary conservation, it has been suggested that DMRT1 (double sex-and mab3-related transcription factor 1) may be involved in sex differentiation from invertebrates to human [6,7]. In trout, for example, DMRT1 has been described as being important in male differentiation but not in female differentiation. Moreover, its expression can be regulated by hormonal treatments that usually succeed in producing phenotypical sex change [8].

The gilthead seabream (*Sparus aurata* L.) is a protandrous hermaphroditic sparid fish with a heterosexual gonad that undergoes sex change during the second or third year of life, depending on the natural environment of the populations studied [9-11]. In most Mediterranean areas, the specimens undergo this sex change during the second year of life [12]. Several studies have dealt with the gilthead seabream sex change and its female physiology [13,14], but few studies have followed the male physiology throughout the reproductive cycle (RC). Our previous studies on the first RC of the gilthead seabream demonstrated that acidophilic granulocytes (produced in the head-kidney, the equivalent to mammalian bone marrow) infiltrate the testis under endocrine and paracrine regulation, display tissue specific functions and are involved in the testis degeneration that takes place during post-spawning [15-18].

The aim of this study was to characterize the second RC, prior to sex change, of the gilthead seabream, focusing on cell renewal (proliferation, apoptosis and necrosis) and the presence of acidophilic granulocytes, macrophages and T and B lymphocytes in the testicular and ovarian area of the gonad. Moreover, since in the heterosexual gonad of sparids the mechanisms involved in the differentiation of one sex and those which block the development of the other might coexist, a study of the testis differentiating gene, DMRT1, in the gonads of gilthead seabream throughout the second RC was thought to be of interest.

Methods

Fish

Healthy specimens of sexually mature male gilthead seabream *Sparus aurata* L. (Sparidae, Perciform, Teleostei), with a body weight (bw) of 100 g, were obtained in November 2004, from CULMAMUR, S.L. (Águilas, Spain). The fish were kept at the Spanish Oceanographic Institute (Mazarrón, Murcia), in 14 m^3 running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/hour) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Trouvit, Burgos, Spain). Fish were fasted for 24 h before sampling. The fish with bw ranging from 230 to 1020 g were sampled from October 2005 to October 2006 (n = 10–18 fish/month). In order to determine the final sex ratio of the population, a final sampling was performed in November 2006 (n = 30 fish). At all sampling times the specimens were weighed, and the gonads and the head-kidneys were removed. Gonads were weighed and processed for light and electron microscopy, flow cytometry and gene expression studies, as described below. The head-kidneys were used as positive control in flow cytometry assays. Some specimens (n = 5/month) were weighed and injected intraperitoneally (i.p.) with 50 mg/kg bw of 5-bromo-2'-deoxyuridine (BrdU, Sigma) 2 h before sampling.

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

Light microscopy and immunocytochemical staining

The gonads were fixed in Bouin's solution or 4% paraformaldehyde solution, embedded in paraffin (Paraplast Plus; Sherwood Medical) and sectioned at 5μm. Some sections were stained with hematoxylin-eosin in order to determine the reproductive stage and the degree of development of each fish, whereas others were subjected to an indirect immunocytochemical method [19] using a monoclonal antibody (mAb) specific to gilthead seabream acidophilic granulocytes (G7) [20] and an anti-BrdU mAb (Caltag) to determine the presence of acidophilic granulocytes and proliferative cells, respectively, as has been previously described [16].

The sections were slightly counterstained with Maller hematoxylin. The specificity of the reactions was determined by omitting the first antiserum and in the case of BrdU detection, using gonad sections from fish that had

not been injected with BrdU. Slides were examined with an Axiolab (Zeiss) light microscope.

In situ detection of DNA fragmentation (TUNEL)

TUNEL was performed to identify apoptotic cells (*in situ* cell death detection kit; Roche), as described previously [18]. Slides were examined with an Axiolab (Zeiss) light microscope.

Electron microscopy

Samples were fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 4–5 h at 4°C, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at 4°C, and then embedded in Epoxi resins. Ultrathin sections were obtained with a Reichert-Jung ultramicrotome, contrasted with uranyl acetate and lead citrate, and examined with a Zeiss EM 10C electron microscope.

Cell suspensions

The gonad and head-kidney cell suspensions were obtained as described previously [15].

Flow cytometry

Aliquots of 5 × 10^6 cells were washed in flow cytometry (FC) buffer [PBS containing 2% fetal calf serum (FCS) and 0.05% sodium azide] and incubated for 30 min on ice with 100µl of G7, at the optimal dilution of 1:100 in FC buffer. After being washed, cell suspensions were incubated for 30 min on ice with 50µl of fluorescein isothiocyanate (FITC) labelled anti-mouse $F(ab')_2$ fragments of goat antibody (Caltag) at the optimal dilution of 1:1000 in FC buffer. Cells were then washed twice and data were collected in the form of two parameter forward-scatter (FSC) and side-scatter (SSC) dot plots and green fluorescence (FL1) histograms by using a fluorescence-activated cell sorter (Becton Dickinson). Each G7 staining was carried out in duplicate.

Analysis of gene expression

Total RNA was extracted from gonad fragments (n = 4–5 gonads/month) with TRIzol Reagent (Invitrogen) following the manufacturer's instructions and treated with DNase I, amplification grade (1 unit/µg RNA, Invitrogen). The SuperScript III RNase H- Reverse Transcriptase (Invitrogen) was used to synthesize first strand cDNA with oligo-dT_{18} primer from 1µg of total RNA, at 50°C for 60 min. Total mRNA were obtained after mixing the same amount of mRNA from 4–5 fish/month.

The mRNA levels of the testis differentiating gene, DMRT1, were analyzed by real-time PCR with an ABI PRISM 7700 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures were incubated for 10 min at 95°C, followed by 40 cycles of 15s at 95°C, 1 min at 60°C, and finally 15s at 95°C, 1 min 60°C and, 15s at 95°C. For each mRNA, gene expression was corrected by the ribosomal protein S18 content in each sample, and in all cases, each PCR was performed with triplicate samples. The primers used are shown in Table 1.

The mRNA levels of macrophage colony stimulating factor receptor (M-CSFR), T cell receptor β chain (TCR-β) and immunoglobulin M heavy chain (IgM-H) genes, as markers for macrophages and T and B lymphocytes, respectively, were analyzed by semi-quantitative PCR with an Eppendorf Mastercycle Gradient Instrument (Eppendorf). Reaction mixtures were incubated for 2 min at 95°C, followed by 35 cycles of 45s at 95°C, 45s at the specific annealing temperature for each gene (see Table 1), 1 min at 72°C, and finally 10 min at 72°C. As a RT-PCR control expression *β-actin* was used.

Analysis of the reproductive stage

As an index of the reproductive stage, we calculated the gonadosomatic index (GSI) as 100 × [W_G/W_B] (%), where

Table 1: Primers used for gene expression analysis by RT-PCR. Gene name abbreviation, accession number, primer sequence (forward and reverse) and annealing temperature used for gene expression analysis.

Gene	Accession Number	Annealing temperature	Name	Sequence (5'-3')
β-actin	X89920	55	F	ATCGTGGGGCGCCCCAGGCAC
β-actin			R	CTCCTTAATGTCACGCACGATT
S18	AM490061	60	F	AGGGTGTTGGCAGACGTTAC
S18			R	CTTCTGCCTGTTGAGGAACC
DMRT1	AM493678	60	F	GATGGACAATCCCTGACACC
DMRT1			R	GGGTAGCGTGAAGGTTGGTA
M-CSFR	AM050293	60	F3	CTGCCCTACAATGACAAG
M-CSFR			R4	TCAGACATCAGAGCTTCC
TCR-β	AM490435	60	F1	GCTTCTTCAATGGGACAGGA
TCR-β			R1	CCGTAGACACAGCCCTTGAT
IgM-H	AM493677	60	F1	CAGCCTCGAGAAGTGGAAAC
IgM-H			R1	GAGGTTGACCAGGTTGGTGT

W_G is gonad weight (in grams) and W_B is body weight (in grams).

As an index of ovarian development, the ovarian ratio, calculated as ovarian area (mm^2)/total gonad area (mm^2) × 100 (%) was measured, taking longitudinal sections (n = 5–14) stained with hematoxilin-eosin from the middle part of the gonad (n = 3/month) and in all cases corresponding to approximately 30% of the total volume of the organ. The ovarian area included the ovigerous lamellae and the ovarian cavity, and was drawn manually over the digital image. The total area of the gonad covered the ovarian area, the spermatogenetic tubules and the efferent duct, and was measured using an image analysis threshold method employed to differentiate borders. The ratio between these two areas was calculated from measurements of gonad tissue images obtained with an Olympus SZ11 overhead projector, a Sony DXC 151 AP video camera, and the software MIP 4.5 Consulting Image Digital (CID, Barcelona).

In order to determine oocyte growth, oocyte nuclear and cell diameters were drawn manually and measured by image analysis using an Axiolab (Zeiss) light microscope, a CoolSNAP digital camera (RS Photometrics) and SPOT Advance 3.3 software (Diagnostic Instruments, Inc.).

Calculations and statistics

FC assays were performed with cells from at least three different fish. A quantitative study of the FC results was made by using the statistical option of the Lysis Software Package (Becton Dickinson). The number of oocytes measured (n = 111–269) was always higher than the number obtained by the formula (standard deviation · 0.83/mean · 0.05)^2. All data were analyzed by ANOVA and a Waller-Duncan multiple range test to determine differences among groups ($P \leq 0.05$).

Results

Morphology, cell proliferation and apoptosis in the testicular area of the gonad

All the specimens during the second RC were male. The testicular area was composed of tubules consisting of spermatogonia stem cells and cysts (a cohort of synchronically developed germ cells enclosed by a cohort of Sertoli cells) of primary spermatogonia, A and B spermatogonia, spermatocyte, and spermatids and free spermatozoa. Based on the morphological changes observed in the testicular area, the second RC can be divided into four stages: spermatogenesis, spawning, post-spawning and testicular involution. During spermatogenesis (from October to January, Fig. 1a), spawning (February, Fig. 1b) and post-spawning (March, Fig. 1c) the testis showed a similar morphology to that described in the first RC of the gilthead seabream [16]. Interestingly, after post-spawning, during the testicular involutive stage (from April to July) the involutive process which started at post-spawning became more apparent. As regards morphology during the testicular involution stage, the testicular area could be divided into two areas (Fig. 1d): (i) the testicular peripheral area located at the edge of the gonad and formed by a dense tissue with no tubular lumen and a germinal compartment composed of spermatogonia stem cells and some primary spermatogonia cysts, similar to that observed at post-spawning (Fig. 1d,e), and (ii) the testicular internal area located next to the efferent duct and the ovary and formed by wide necrotic areas (Fig. 1d, f) composed of cell debris and surrounded by well developed interstitial tissue with large clusters of eosinophilic cells (Fig. 1f).

The immunodetection of BrdU and the *in situ* detection of DNA fragmentation, in the testicular area, were associated with the second RC (Fig. 2). Thus, the proliferative cell types and their proliferation rates during spermatogenesis (Fig. 2a), spawning (data not shown) and post-spawning (Fig. 2b) were similar to that observed during the same stages of the first RC [16]. During testicular involution (Fig. 2c) many BrdU positive spermatogonia stem cells and primary spermatogonia cysts could be seen randomly distributed throughout the testis.

Apoptosis is one of the most important mechanisms of cell death and is involved in several physiological processes related with tissue renewal. In the testicular area of the gonad, apoptosis was only detected during post-spawning (Fig. 2d) and testicular involution (Fig. 2e, f). Surprisingly, the apoptotic cells in the peripheral testicular area (Fig. 2e) were more numerous than in the internal testicular area during the testicular involution stage (Fig. 2f). In both stages, apoptotic cells had the features of primary spermatogonia, that is, they were set in the germinal compartment, isolated from each other, and possessed large and round nuclei.

Morphology, cell proliferation and apoptosis in the ovarian area of the gonad

The ovary was formed by folds of the germinal epithelium, named ovigerous lamellae that surrounded an ovarian cavity (Fig. 3a). These ovigerous lamellae contained the different types of germ cells embedded in a smooth connective tissue and delimited by epithelial cells (Fig. 3b). Interestingly, the testicular and ovarian areas of the gonad developed independently. Thus, the ovarian area from October to March was composed of nests of oogonia and immature oocytes (pre-perinucleolar and perinucleolar), while the testicular area was developing through its spermatogenesis, spawning and post-spawning stages. The ovarian area started to develop with an asynchronous pattern at the end of March, coinciding with the testicular post-spawning stage, when vitellogenic oocytes in the

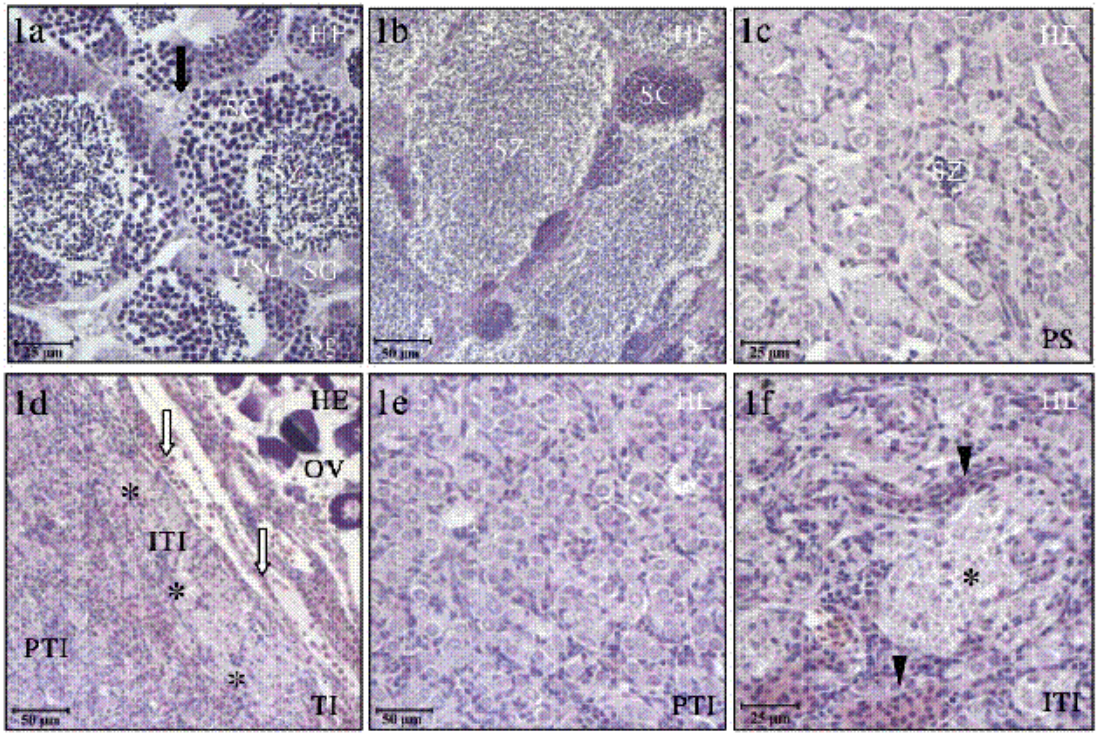

Figure 1

Testicular area of the gonad. The testicular area of the gonad at different stages of the second RC stained with hematoxylin-eosin. At spermatogenesis (**a**), spermatogonia stem cells and all germ cell type cysts formed the tubules of the testis. At spawning (**b**), the tubules are larger and full of free spermatozoa. At post-spawning (**c**), the main cell types in the tubules are spermatogonia stem cells and primary spermatogonia cysts. Some remaining spermatozoa can also be seen. At testicular involution (**d-f**), the testis is formed by spermatogonia stem cells and primary spermatogonia cysts that compose a dense tissue with no lumina in the tubules. Two morphological areas can be distinguished: the peripheral testicular area (**d,e**) and the internal testicular (**d,f**) area which is close to the efferent duct and the ovarian area and presents large necrotic areas surrounded by eosinophilic granulated cells. Scale bar = 25 μm (**a,c,f,**) and 50μm (**b,d,e**). Sg, Spermatogenesis; S, spawning; PS, post-spawning; TI, testicular involution; SG, spermatogonia cysts; PSG, primary spermatogonia cysts; SC, spermatocytes cysts; SZ, spermatozoa; PTI, peripheral testicular area in the involution stage; ITI, internal testicular area in the involution stage; OV, ovarian area; (arrow), spermatogonia stem cell; (arrow heads), eosinophilic cells; (white arrows), efferent duct; (asterisk), necrotic areas.

yolk vesicle stage (also called cortical alveoli stage) were observed. Thus, numerous vitellogenic oocytes in the yolk vesicle stage were observed in April coinciding with the testicular involution stage (April-July). In order to define the germinal cell populations, the morphology and the nuclear and cell diameters of the cells were taken into account (see Table 2 and Fig. 3c, 3d, 3e). In the ovarian area, non-apoptotic cells were observed, while scarce oogonia (Fig. 3f) and some somatic cells (Fig. 3f inset) proliferated, coinciding with the testicular involution stage of the testicular area.

Gonadal development at the end of the second/beginning of the third reproductive cycles

From September to October, the fish have a gonad with both testicular and ovarian areas, which do not undergo further development compared with the same areas described during testicular involution. However, due to the degenerative process that the testicular area underwent during testicular involution, the ovarian area represented 98% of the total gonad. From November onwards, the gametogenic activity restarted and the third RC began, allowing the distinction between both sexes, depending

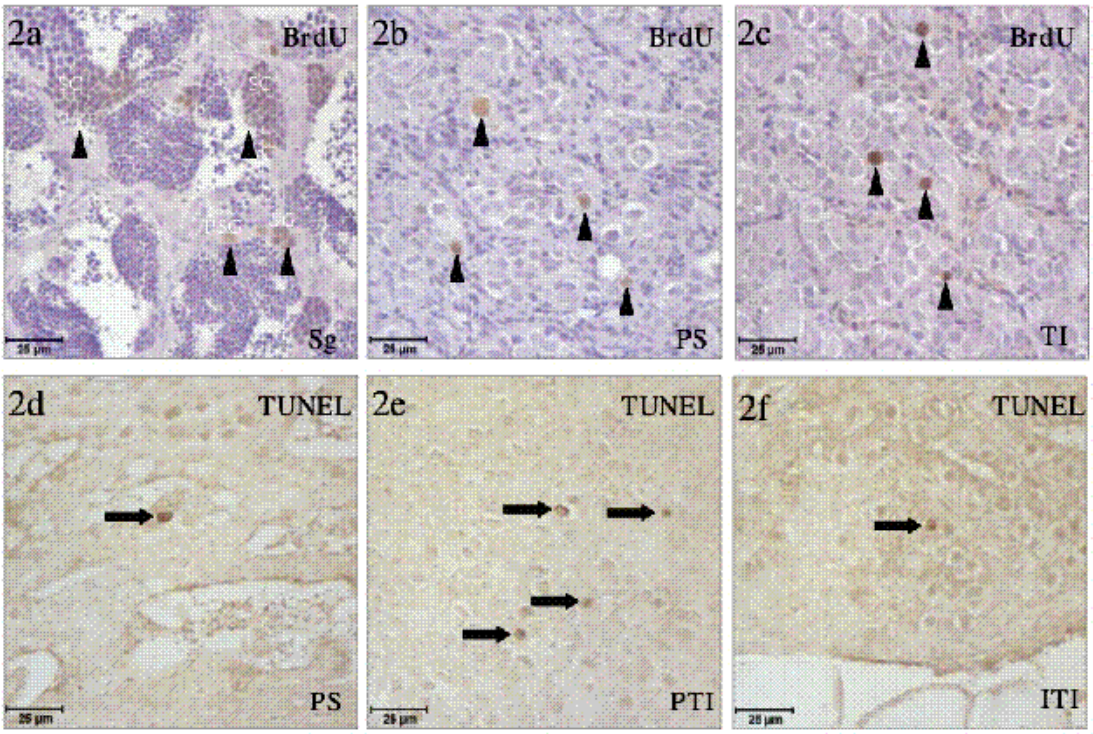

Figure 2
Cell proliferation and apoptosis in the testicular area of the gonad. Testicular area of the gonad at different stages of the second RC immunostained with the anti-BrdU mAb (**a-c**) or labeled with TUNEL (**d-f**). At spermatogenesis (**a**), spermatogonia stem cells and primary spermatogonia and spermatocytes cysts proliferate. At post-spawning (**b**) and testicular involution (**c**) spermatogonia stem cells and primary spermatogonia cysts were inmunostained. Only at post-spawning (**d**) and testicular involution (**e,f**) were apoptotic cells observed. Notice that in the testicular peripheral area (**e**) the number of TUNEL positive cells is higher than in the testicular internal area (**f**). Scale bar = 25 μm (**a-f**). (arrowheads), proliferative cells; (arrows), TUNEL positive cells; Sg, Spermatogenesis; PS, post-spawning; TI, testicular involution; PTI, peripheral testicular area in the involution stage; ITI, internal testicular area in the involution stage.

on which area progressed throughout the gametogenesis process. At this time 40% of the population was female and 60% males despite the homogeneous involution of the testicular area observed in the population at the end of the second RC described above. Females showed a more developed ovarian area with vitellogenic oocytes in the secondary yolk vesicle and tertiary yolk vesicle stages (see Table 3 and Fig. 3g,h).

Interestingly, the ovarian area of the fish developing as males contained numerous atretic follicles, while the most developed oocytes were vitellogenic oocytes at the yolk vesicle stage. The atretic follicles were formed by a degenerated oocyte surrounded by a flattened cell monolayer (see Table 3 and Fig. 3i).

Parameters related with the development of the gonad

As an index of the functional reproductive stage we measured the GSI, variations in which correlated very well with the development of the testicular area in gilthead seabream males (see Table 4 and Fig. 4a). Thus, the GSI increased during spermatogenesis, while in the spawning stage, the shedding of spermatozoa resulted in a sharp decrease in the index, which continued to decrease until the end of the post-spawning stage. In the testicular involution stage the GSI showed little variations. The gonad

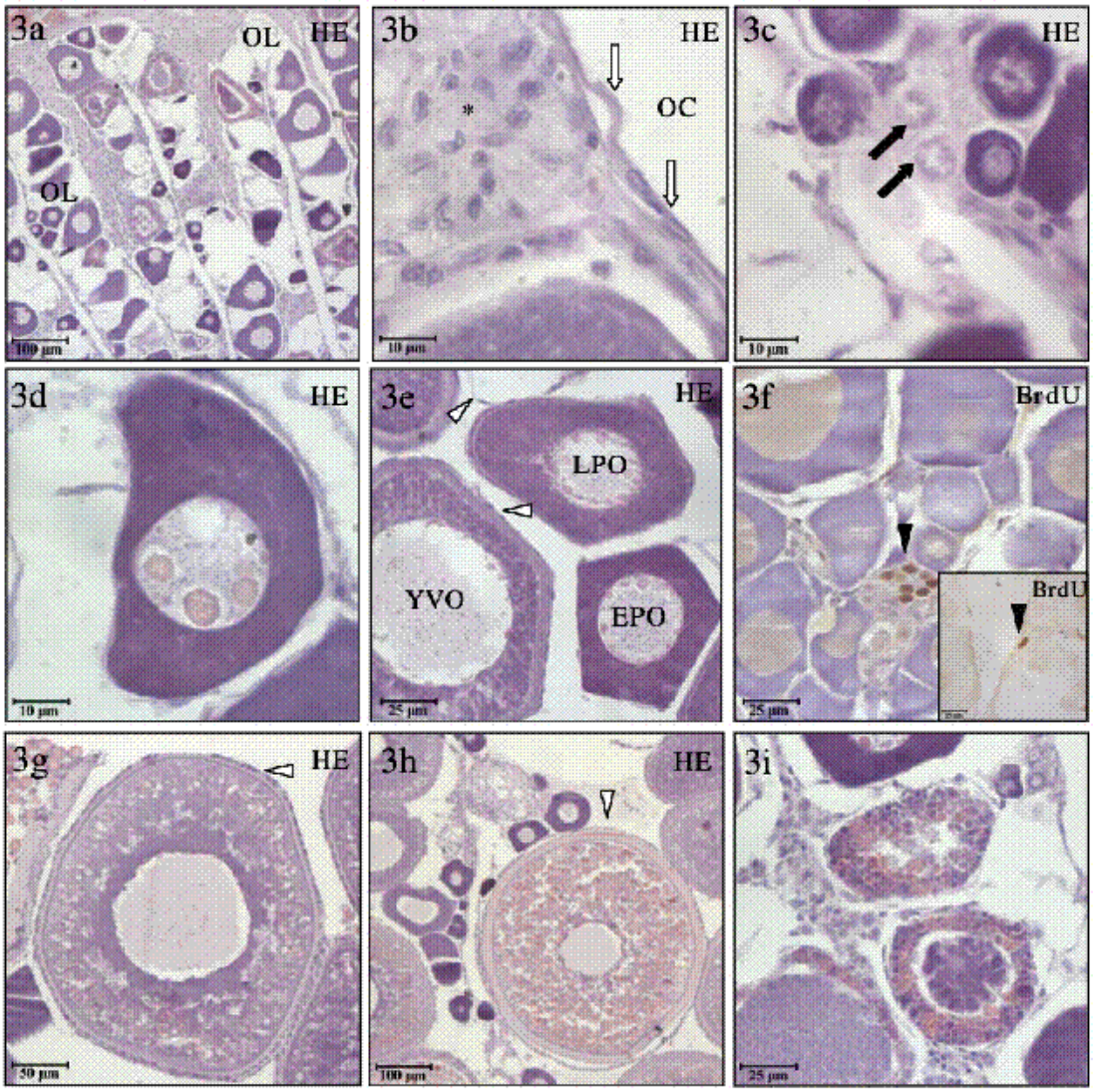

Figure 3

The ovarian area of the gonad. Ovarian area of the gonad at different stages of the second RC stained with hematoxylin-eosin (**a-e,g-i**) and immunostained with BrdU mAb (**f, inset f**). The ovarian epithelium forms longitudinal lamellae that extend into the central ovarian cavity (**a**). A squamous epithelium lines these lamellae at the luminal surface, below which a group of undifferentiated somatic cells were observed (**b**). The ovarian lamellae are formed by nests of oogonia (**c**), pre-perinucleolar oocytes (**d**), early and late perinucleolar oocytes (**e**), yolk vesicle oocytes (**e**) and secondary (**g**) and tertiary (**h**) yolk vesicle oocytes. Oogonia (**f**) and somatic cells (**inset f**) only proliferated during testicular involution. Some atretic follicles were observed in the ovarian of males at the beginning of the third RC (**i**). Scale bar = 100 µm (**a,h**), 50 µm (**g**), 25 µm (**e,f,i, inset f**) and 10 µm (**b-d**). OL, ovarian lamellae; OC, ovarian cavity; EPO, early perinucleolar oocytes; LPO, late perinucleolar oocytes; YVO, yolk vesicles oocytes; (arrow heads), proliferative cells; (asterisk), ovarian somatic cells; (white arrows), epithelium lines the ovarian lamellae; (white arrows heads), follicular epithelial cell layer.

Table 2: Feature of the ovarian area of the gonad of gilthead seabream during the second reproductive cycle.

Months	Types of cells present in the ovary	Cell diameter (μm)	Nuclear diameter (μm)	Cytoplasm	Nucleus	Follicle related structures
October-March	Oogonia	16.2 ± 0.3	9.3 ± 0.2	Slightly basophilic	A nucleolus	
	Pre-perinucleolar oocytes	35.0 ± 0.8	18.0 ± 0.4	Highly basophilic	Two or three centrally located nucleoli	
April-July	Early perinucleolar oocytes	57.6 ± 1.0	29.6 ± 0.6	Highly basophilic	Numerous nucleoli	
	Late perinucleolar oocytes	84.1 ± 3.3	46.9 ± 2.0	Highly basophilic	Numerous nucleoli close to the nuclear envelope	Granulosa
	Vitellogenic oocytes at yolk vesicle stage (cortical alveoli stage) *	96.2 ± 2.6	51.0 ± 2.1	Weak basophilic Granules randomly distributed	Numerous nucleoli close to the nuclear envelope	Granulosa Zone radiate

* In April-July also appeared the germ cell types present in the ovary in October-March

growth resumed at the beginning of the third RC (November).

The ovarian ratio (Fig. 4b) and the means of the cell and nuclear diameters of the oocytes (Fig. 4c) were calculated as an index of ovarian development. The ovarian ratio showed great variations throughout the second RC (see Table 4). From October to January the ratio decreased sharply, coinciding with the progression of spermatogenesis in the testicular area of the gonad. However, during post-spawning and testicular involution, the ovarian ratio increased gradually and then stabilized and reached its maximum value at the end of the second/beginning of the third RC (see Table 5 and Fig. 4b). As a result of the development of oocyte populations, mean cell and nuclear sizes increased gradually during the second RC. At the beginning of the third RC both cell and nuclear diameters experienced a great increase (see Tables 4, 5 and Fig. 4c).

DMRT1 gene expression in the gonad

In order to determine when the sex change process started, the mRNA level of DMRT1, a gene known to be related with the maintenance of testicular tissue, was measured by real-time RT-PCR. The DMRT1 mRNA levels increased as spermatogenesis proceeded and reached their highest level at the end of the spermatogenesis stage. The level remained steady during spawning and sharply decreased during post-spawning. The DMRT1 mRNA levels were very low during testicular involution (Fig. 5).

Leukocytes present in the gonad

Throughout the second RC, the acidophilic granulocytes (G7 positive cells) were present in the interstitial tissue of testicular (Fig. 6a–d) and ovarian areas (Fig. 6e) and in the connective tissue that limited both areas, surrounding the efferent duct and forming the tunica albuginea. The acidophilic granulocytes infiltrated the gonad in variable num-

Table 3: Feature of the ovarian area of the gonad of gilthead seabream at the end of the second reproductive cycle/beginning of the third reproductive cycle.

Months	Types of cells present in the ovary	Cell diameter (μm)	Nuclear diameter (μm)	Cytoplasm	Nucleus	Follicle related structures
October	No further development					
	Vitellogenic oocytes at secondary yolk vesicle stage *	192.8 ± 9.5	89.0 ± 4.5	Acidophilic globules distributed at the periphery Lipid dropped close to the nucleus		Granulosa Zone radiate Theca cell layer
November	Vitellogenic oocytes at tertiary yolk vesicle stage *	310.6 ± 24.9	114 ± 3.5	Numerous eosinophilic globules		Granulosa Zone radiate Theca cell layer
	Atretic follicles **			Irregular in shape Numerous acidophilic and some basophilic granules	Highly condensed and basophilic	Flattened cell monolayer

*In November also appeared the germ cell types present in the ovary in October-July. These specimens are females.
**In November, the most developed vitellogenic oocytes undergoes an atretic process. These specimens are males.

Table 4: Parameters related with the development of the gonad of gilthead seabream during the second reproductive cycle.

Months	Testicular stages	Ovarian stages	GSI (%)	Ovarian ratio (%)	Ovarian cell diameter means (μm)	Ovarian nuclear diameter means (μm)	Acidophilic granulocytes (%)
October- January	Spermatogenesis	Immature	From 1.4 ± 0.2 to 3.7 ± 1.0 and then to 1.8 ± 0.8	From 44.55 ± 15.06 to 9.52 ± 5.11	32.2 ± 1.7	16.62 ± 0.90	No detected
February	Spawning	Immature	1.3 ± 0.4	46.45 ± 3.26	37.3 ± 1.8	19.54 ± 0.91	4.79 ± 0.96
March	Post-spawning	Immature	0.52 ± 0.04	66.26 ± 3.71	39.6 ± 1.8	21.93 ± 0.99	1.12 ± 0.24
April-July	Testicular involution	Growth: vitellogenic oocytes in yolk vesicles stage	0.41 ± 0.05	From 74.43 ± 10.78 to 88.50 ± 4.20	51.5 ± 1.8	26.82 ± 0.98	From 8.76 ± 1.24 to 3.69 ± 0.67 reaching the maximum value in may (9.25 ± 1.23)

bers (Fig. 6g) and were located in different compartments of the gonad depending on the stage of the RC. The localization of acidophilic granulocytes during spermatogenesis, spawning and post-spawning during the second RC (Fig. 6a,b) coincided with that observed during the first [16]. During testicular involution (Fig. 6c,d) the acidophilic granulocytes were observed in higher numbers in the interstitial tissue of the testicular peripheral area (Fig. 6c) and around the necrotic areas in the testicular internal area (Fig. 6d). Moreover, the granules of the acidophilic granulocytes that surrounded the necrotic areas were heterogeneous in size and electrondensity. Some granules located close to the plasma membrane were beginning to fuse with each other (Fig. 6f).

During spermatogenesis the amount of acidophilic granulocytes was below the limit of detection and increased during spawning. Although the percentage of acidophilic granulocytes rapidly decreased at the end of post-spawning, they increased again during testicular involution to reach maximum numbers in the gonad. This percentage decreased at the end of the testicular involution stage and remained steady until the beginning of the next RC (see Tables 4, 5 and Fig. 6g).

Due to the lack of specific antibodies for macrophages and lymphocytes in the gilthead seabream, we analyzed the presence of these cell types by electron microscopy (Fig. 6h, i) and from the expression of M-CSFR, TCR-β and IgM-H genes in the gonad (Fig. 6j) which were specific markers for macrophages and T and B lymphocytes, respectively. The results showed that macrophage- and lymphocyte-like cells were located in the interstitial tissue of the testis during spermatogenesis. Macrophage-like cells were characterized as irregular cells with polymorphous nuclei and an electron-dense cytoplasm with numerous mitochondria and appeared in close contact with Leydig cell clusters (Fig. 6h). Lymphocyte-like cells appeared as round cells with a large and heterochromatinic nucleus (Fig. 6i). These morphological observations were confirmed by RT-PCR, since the mRNA levels of M-CSFR, TCR-β and IgM-H were found in all stages of the second RC (Fig. 6j).

Discussion

Our data showed that gilthead seabream, in the western Mediterranean area, developed as males during the first two RCs, while from the third RC onwards the population divided into males and females. This behavior has also been described in studies performed in other Mediterranean regions and indoors with simulated natural photoperiod and temperatures ranging from 15°C to 23°C [9,21]. However, our data are innovative since this is the first time that the cell renewal (proliferation, apoptosis and necrosis) process involved in testicular and ovarian development has been correlated with the leukocyte types present in the gonad. Moreover, the proliferative and apoptotic processes involved in the second RC of the gilthead seabream show interesting differences compared from the first RC [16]. In both cycles spermatogenesis, spawning and post-spawning stages show similar features. However, the last stages of each cycle (resting and testicular involution, respectively) were seen to differ completely. Thus, compared with what happened in post-spawning, the resting stage was characterized by an increase in the number of proliferative cells and no apoptotic cells [16], while during the testicular involution stage, the number of proliferative cells was similar and the number of apoptotic cells increased as did the size of the necrotic areas. In contrast, in the second RC, the degenerative process initiated at post-spawning, was enhanced in the testicular involution stage, resulting in a progressive increase in the ovarian index, which reached 98% of the total gonad at the end of the second RC. Unlike in the first RC, as the testicular area degenerates, the immature oocytes develop and the first vitellogenic oocytes appear. However, the number of proliferative oogonia and ovarian somatic cells in the second RC do not differ from the normal proliferative activity described during each resting stage of the male phase in several sparid species, including

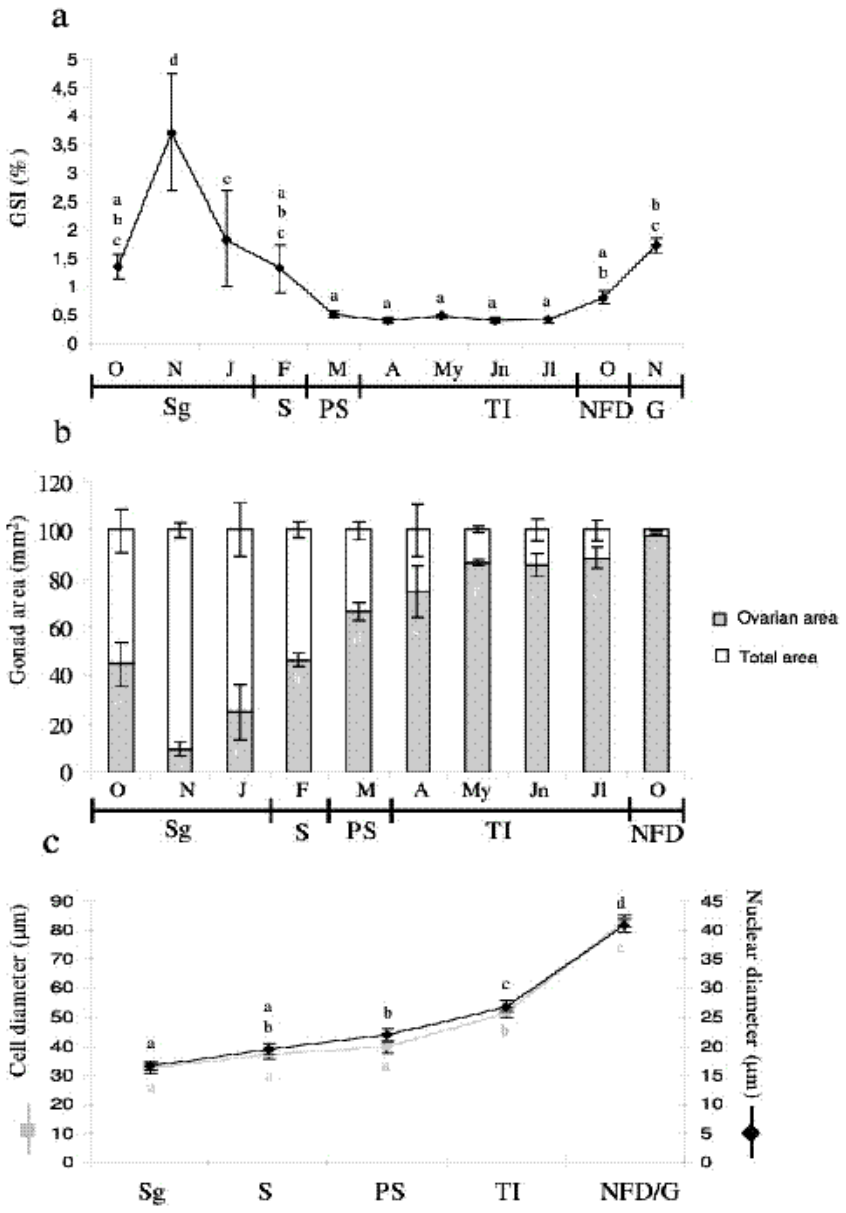

Figure 4

Parameters related with the development of the gonad. GSI (**a**), the ratio between the ovarian area (gray part of the bars) and the total area of the gonad (full bars) (**b**) and the cell and nuclear diameters of the oocytes throughout the second RC and at the beginning of the third (**c**). Data represent means ± SEM n = 10–18 fish/month (**a**), n = 3 fish/month (**b**) and n = 111–269 cell/stage (**c**). Different letters denote statistically significant differences between the groups according to a Waller-Duncan test. Sg, Spermatogenesis; S, spawning; PS, post-spawning; TI, testicular involution; NFD, no further development; G, gametogenesis.

Table 5: Parameters related with the development of the gonad of gilthead seabream at end of the second reproductive cycle/ beginning of the third reproductive cycle.

Months	Testicular stages	Ovarian stages	GSI (%)	Ovarian ratio (%)	Ovarian cell diameter means (μm)	Ovarian nuclear diameter means (μm)	Acidophilic granulocytes (%)
October	No further development		0.82 ± 0.10	97.9 ± 0.4	58.6 ± 2.0	28.2 ± 1.1	3.1 ± 0.7
November	No further development	Growth: vitellogenic oocytes at secondary yolk vesicle and tertiary yolk vesicle stages	1.72 ± 0.13		101.2 ± 5.7	51.2 ± 2.4	
	Spermatogenesis	Atretic follicles Oogonia Pre-perinuclear oocytes Early and late perinuclear oocytes Vitellogenic oocytes at yolk vesicle stage					

the gilthead seabream [22,16]. Despite what has been said before [9], our data demonstrated that during the last stage of each cycle the gonad does not remain latent since cell proliferation and apoptosis allow tissue to be renewed and the beginning of sex change in the first and second RC, respectively.

In seasonal breeding mammals, apoptosis occurs throughout the RC and is related with the amount of spermatogonia and spermatocytes present in the testis rather than being related with seasonal testicular involution [23,24]. However, in the gilthead seabream, apoptosis occurs during post-spawning in the first RC [16] and during post-spawning and testicular involution stages in the second, but not during spermatogenesis as occurs in others species [25,26]. Thus, our data and the data obtained in several fish species demonstrate that germ cell apoptosis and necrotic areas are involved in testicular involution [15,16,27-30].

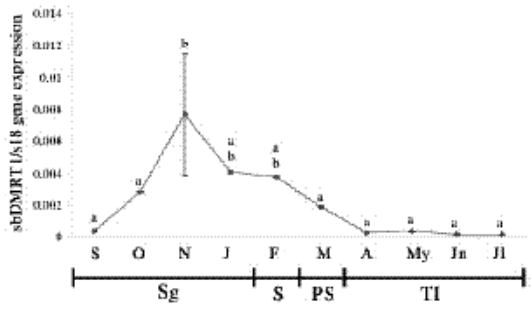

Figure 5
DMRT1 gene expression in the gonad. The mRNA levels of DMRT1 were studied by real-time RT-PCR in the gonad at the indicated month. Data represent means ± SEM of triplicate samples. Total mRNA were obtained after mixing the same amount of mRNA from 4–5 fish/month. Different letters denote statistically significant differences between the groups according to a Student-Newman-Keuls test. Sg, spermatogenesis; S, spawning; PS, post-spawning; TI, testicular involution.

One important observation of the study is that at the end of the second RC the whole seabream population undergoes a testicular regression process probably triggered by a down-regulation of the expression of genes involved in testicular maintenance. Different genes from a family of genes encoding proteins that contain a DNA-binding motif, called a DM domain, have recently been cloned from a wide range of vertebrates including fish, and these genes have been found to be expressed in the developing gonads and in the adult ovary and/or the testis [8,31-33]. In fact, one DM domain-containing gene, DMRT1 (DM-related transcription factor 1) appears to be involved in a sex-determining cascade and also in testis maintenance [8]. Our data show that the DMRT1 is related with testis development in adults since DMRT1 mRNA levels increase as spermatogenesis proceeds, slightly decreases at the end of the stage and keeps steady during spawning. Interestingly, when testicular involution starts at post-spawning, the mean levels of DMRT1 decrease and reach their minimum values when this process is enhanced during the testicular involution stage. Moreover, DMRT1 expression in trout is high during mid spermatogenesis and also occurs in the pre-vitellogenic ovary and decreases when it starts to develop [8]. This could explain why, in the gilthead seabream, the vitellogenic oocytes do not appear until down-regulation of this gene is really effective. All this supports the idea that in fish the DMRT1 is related not only with sex determination, but also with testicular functions and immature ovary maintenance. Moreover, the very low DMRT1 mRNA levels at the end of the testicular involution stage would explain the remains of a small testicular area (2% of the total gonad) which would allow 60% of the fish population to block the sex change process at the beginning of the third reproductive cycle. In this case, the testis develops again and the maturing

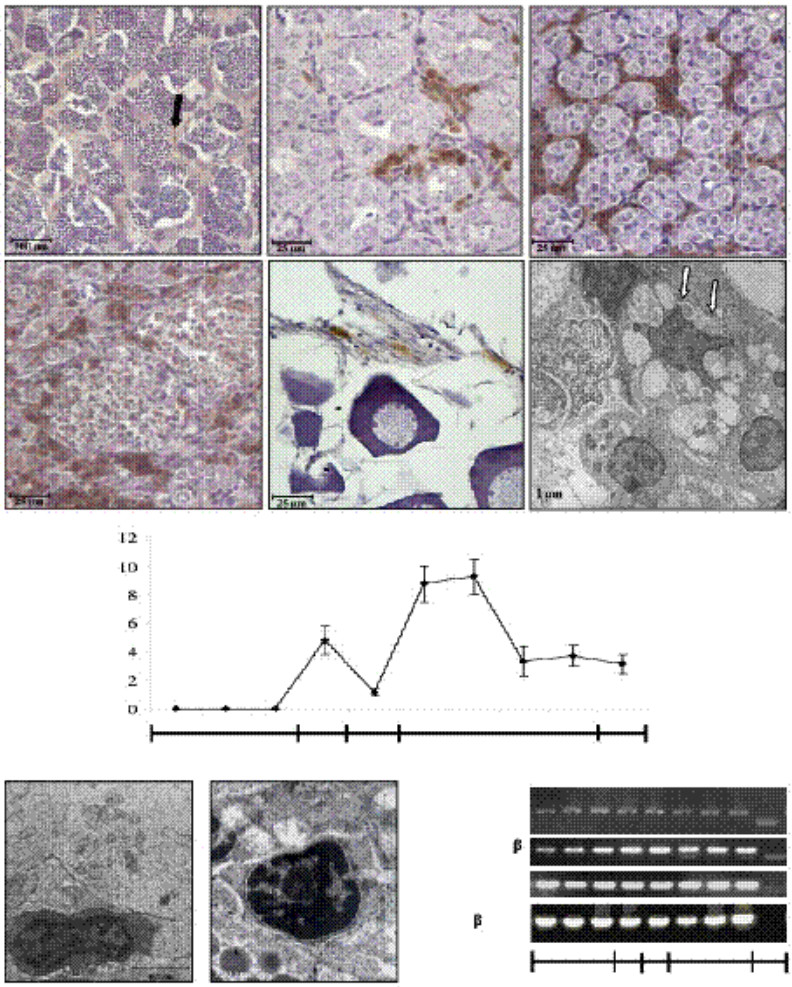

Figure 6

Leukocytes present in the gonad. The testicular and ovarian areas of the gonad at different stages of the second RC immunostained with G7 (**a-e**), the ultrastructure of testicular acidophilic granulocytes (**f**), the percentage of gonad acidophilic granulocytes (**g**), the ultrastructure of testicular macrophage-like cells (**h**) and lymphocyte-like cells (**i**) and RT-PCR analysis of M-CSFR, TCR-β and IgM-H genes, as appropriate markers of macrophages and T and B lymphocytes, respectively (**j**). The acidophilic granulocytes appeared in the interstitial tissue of the testis at spermatogenesis (**a**), post-spawning (**b**) and testicular involution (**c,d**). Note that they also appeared between the germ cells at post-spawning (**b**) and around the necrotic areas at testicular involution (**d**). Scattered acidophilic granulocytes were also observed in the interstitial tissue of the ovarian area (**e**). Heterogeneous granules fused to each other were observed in the acidophilic granulocytes closed to the testicular necrotic areas (**f**). Testicular cell suspensions (n = 3–5 fish/month) were immunostained with the G7 and then analyzed by flow cytometry (**g**). The macrophage-like cells in the interstitial tissue in the vicinity of Leydig cells at spermatogenesis stage (**h**). Lymphocyte-like cells in the interstitial tissue (**i**). Total mRNA was obtained to mix the same amount of each mRNA from 4–5 fish/sample (**j**). Scale bar = 100 μm (**a**), 25 μm (**b-e**), 1 μm (**f**), 0.5 μm (**h**), 0.3 μm(**i**). Different letters denote statistically significant differences between the groups according to a Waller-Duncan test. (arrow), G7 positive cells; (white arrows), granules fused to each other; MLC, macrophage-like cell; LLC, lymphocyte-like cell; L, Leydig cell; Sg, Spermatogenesis; S, spawning; PS, post-spawning; TI, testicular involution; NFD, no further development; PTI, peripheral testicular area in the involution stage; ITI, internal testicular area in the involution stage; O, ovary; G, gametogenesis and C-, negative control.

oocytes degenerate, becoming atretic follicles as described previously [13,21]. The lack of discernible sex-determining genes such as Sry gene [6], and the existence of genes whose up- or down-regulation determine the development of one sex or the other, would explain the characteristic of the gonad (ovo-testis) in hermaphroditic sparids and the sexual plasticity of teleosts. However, further studies are needed in order to fully understand the gene regulation of the variable pattern of sex determination in fish.

Several studies have dealt with the gilthead seabream sex change and the corresponding female physiology [13,14], but few studies have followed the male physiology throughout the RC and none have dealt with immune and reproductive system interactions. However, as in mammals, the immune and the reproductive systems interact in a complex manner in the gilthead seabream testis, as our previous data on testicular acidophilic granulocytes suggests [15,17].

As regards the presence of leukocytes in the fish gonad, little is known about their role in the seasonal changes observed in this organ. Our previous data from the first RC showed that acidophilic granulocytes infiltrate the gonad following physiological stimuli produced by testicular cells and display impaired immune functions, although they are the only testicular cells that are able to produce reactive oxygen intermediate (ROIs) and intracellularly accumulate IL-1β [15-18]. Interestingly, their location in the gonad during the first RC is similar to that observed during the second one. However, unlike in the first RC, the number of testicular acidophilic granulocytes peaks twice: (i) at the end of spawning/beginning of post-spawning, and (ii) at the beginning of the testicular involution stage when they reach their highest numbers. This finding supports the idea that testicular acidophilic granulocytes are somehow involved in the degenerative process that occurs during these stages. The morphology of testicular acidophilic granulocytes observed in the testicular involution stage also supports this hypothesis. This is the first time that acidophilic granulocytes have been shown to have a different ultrastructure from that observed in testicular and non-activated acidophilic granulocytes [15,20]. Fusion of the granules was observed close to the plasma membrane of the cell, suggesting that these cells might be actively involved in tissue remodeling during testicular involution.

In fish, only a few morphological studies have described macrophages and lymphocytes in the testis [22,34,35] but no experimental studies on the possible roles of these cells in this organ exist due to the lack of specific markers. In rainbow trout, a few macrophages have been observed during spermatogenesis while, after spawning, they were more numerous and appeared near the Sertoli cells, phagocytosing the non-emitted spermatozoa [28,29]. In mammals, macrophages are considered as essential accessory cells for normal reproductive functioning as they are found abundantly in the reproductive tract of males but are somewhat immunosuppressed compared with other resident macrophage populations [1,2,4]. Moreover, Leydig cells and testicular macrophages are functionally related and ROIs and IL-1β produced by testicular macrophages significantly affect Leydig cell physiology [36]. Lymphocytes are also present in the mammalian testis, and approximately 15% of immune cells in the normal adult testis were shown to be lymphocytes [1,2]. Most of these lymphocytes expressed T cell markers with a predominance of CD8+ T cells, whereas B cells were not detectable [1]. In spite of the relatively small number of lymphocytes, the testicular immune-privilege may be a localized phenomenon affecting T cell activation and maturation events [1].

We used electron microscopy analysis of the gonads and studied the expression of specific gene markers to demonstrate that macrophages and both T and B lymphocytes are present in the gonad of the gilthead seabream throughout the second RC, as has been described in mammals [1,37]. Our data show that both macrophage-like cells and lymphocyte-like cells are present in the interstitial tissue of the testicular area of the gonads. Interestingly, in contrast to acidophilic granulocytes, macrophages appear mostly during spermatogenesis in close relation with Leydig cell clusters. Taking all this into account, we hypothesise that macrophages are involved in spermatogenesis, while acidophilic granulocytes are involved in the testicular involution process. However, further studies are necessary to understand whether these cell types are involved in the development and physiology of the gonad as they are thought to be in mammalian vertebrates [1].

Conclusions

The gilthead seabream specimens from the western Mediterranean area developed as males during the first two RCs. The whole population underwent a testicular degenerative process at the end of the second RC, which was initiated at post-spawning and enhanced at the testicular involution stage, coinciding with maturation of the ovary. However, only 40% of specimens were females in the third RC. DMRT1 might be related with testicular functions and immature ovary maintenance since its expression sharply decreased during the last two stages of the second RC. Interestingly, innate and adaptive immune cells were present in the gonads of gilthead seabream, strongly suggesting a role in spermatogenesis and/or the testicular degenerative process that occur prior to sex change. In fact, two massive infiltrations of acidophilic granulocytes were observed at post-spawning and testicular involution stages.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

SL carried out the sampling, gonad processing, developed the methods and wrote the manuscript. ECP participated in the planning of the experiments and carried out the sampling and gene expression analysis, supervised the reproductive stage, took part in the discussion of the results, and helped write the manuscript. AlGa reared the specimens. VM provided the gene sequences, supervised the gene expression analysis and participated in discussing the results. JM participated in the discussion of the results. AGA devised the study, made the statistical analysis, participated in the discussion of the results, and helped write the manuscript.

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2. Natural and synthetic estrogens modulate the inflammatory response in the gilthead seabream (*Sparus aurata* L.) through the activation of endothelial cells

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Natural and synthetic estrogens modulate the inflammatory response in the gilthead seabream (*Sparus aurata* L.) through the activation of endothelial cells

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ABSTRACT

Sex steroids are known to deeply alter processes other than fish reproduction, including fish growth, int
mediary metabolism, osmoregulation and immunity. We have previously reported that 17β-estrad
(E_2), the main fish estrogen, promotes the mobilization of acidophilic granulocytes from the head kidn
the bone marrow equivalent in fish, to the gonad in the bony fish gilthead seabream (*Sparus aurata*
The aim of this study was to investigate the effects of E_2 and 17α-ethinylestradiol (EE_2), an endocr
disruptor with strong estrogenic effects commonly found in the aquatic environment, on the ability
gilthead seabream endothelial cells (ECs) to promote leukocyte infiltration. E_2 and EE_2 were seen to af
ECs in different ways. Thus, E_2 was able to increase the production of nitric oxide (NO) and up-regul
the expression of the key activation markers, interleukin-1β, CC chemokine ligand 4, interleukin-8
selectin and matrix metalloproteinase 9, when used alone or combined with bacterial DNA. In contr
EE_2 failed to affect NO release and reduced the up-regulation of the above genes promoted by bacte
DNA. Moreover, we found that leukocyte adhesion to ECs was enhanced by E_2 treatment. Collectiv
these results suggest that estrogens modulate fish leukocyte trafficking during an inflammatory proc
by activating ECs.

1. Introduction

Leukocyte recruitment is an early and pivotal event in any inflammatory response, in which the endothelium plays a crucial role due to its unique position at the interface between the blood stream and the inflamed area. The ability of estrogens to modulate the activity of endothelial cells (ECs) has been widely studied due to the vasculoprotective and anti-inflammatory properties that show in cardiovascular disease in humans. In mammalian ECs, exposure to the main estrogen 17β-estradiol (E_2) is recognized to promote vasodilatation, enhance vascular permeability and, more importantly, alter platelet and leukocyte adhesion through the increased production of nitric oxide (NO) and modulation of the expression of several cytokines, chemokines and adhesion molecules. These effects, among others, are triggered by the activation of estrogen receptor (ER) α and/or ERβ (Nilsson, 2007; Arnal et al., 2010). On the other hand, environmental factors which may disrupt the endocrine system have been implicated in the development of reproductive and immune disturbances. 17α-Ethinylestradiol (EE_2) is a pharmacological compound with strong estrogenic activity widely used as oral contraceptive and in hormone replacement therapy commonly found in waste water-treatment plants eff
ents (Ternes et al., 1999; Johnson et al., 2005; Hinteman et
2006; García-Reyero et al., 2011). The effect of EE_2 exposure
been studied in several fish species from a reproductive point
view, which is considered the most sensitive approach for evalu
ing endocrine disruptive chemicals (Hashimoto et al., 2009; Ho
et al., 2010; Kaptaner and Unal, 2010; Lai et al., 2002; Lange et
2008; Marlatt et al., 2010; Peters et al., 2007; Xu et al., 2008; Cal
et al., submitted for publication). However, there is a compl
lack of knowledge about how estrogens modulate EC activities
whether E_2 and EE_2 exert similar or distinct effects on EC functio

The gilthead seabream (*Sparus aurata* L.) is a seasonally bre
ing, protandrous hermaphrodite teleost with a bisexual gon
which, during the male phase, has a functional testicular area an
non-functional ovarian area. Moreover, the testis undergoes abn
morphological changes, especially after spawning (Chaves-P
et al., 2005a). These changes, include the intensive remodel
and massive infiltration of the head kidney (bone marrow equ
alent in fish) acidophilic granulocytes (AGs) (Chaves-Pozo et
2005b; Liarte et al., 2007), the main phagocyte cell type of
gilthead seabream, coinciding with endogenous increases of
levels (Chaves-Pozo et al., 2008), phenomena that can be mi
icked by the exogenous administration of E_2 (Chaves-Pozo et
2007). Moreover, *in vitro*, E_2 modulates the activation of leu
cytes by all three ERs (ERα, ERβ1 and ERβ2), which are descrit

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

Gene	Accession number	Name	Sequence (5′–3′)	Used
era	AF136979	F1	GCTTGCCGTCTTAGGAAGTG	PCR/real
		R1	TGCTGCTGATGTGTTTCCTC	time
erb1	AF136980	F1	CAGCTCCAGAAGGTGGACTC	PCR
		R1	CGATTGGCATAGCTGAAAT	
erb2	AJ580050	F3	TGATGATGTCACTCACCAACC	PCR
		R3	TTCAGCTCACGAAACCGA	
actb	X89920	F	ATCGTGGGGCGCCCCAGGCACC	PCR
		R	CTCCTTAATGTCACGCACGATTTC	
il1b	AJ277166	F2	GGGCTGAACAACAGCACTCTC	Real time PCR
		R3	TTAACACTCTCCACCCTCCA	
Il6	AM749958	F1	AGGCAGGAGTTTGAAGCTGA	Real time PCR
		R2	ATGCTGAAGTTGGTGGAAGG	
tnfa	AJ413189	FE1	TCGTTCAGAGTCTCCTGCAG	Real time PCR
		RE1	CATGGACTCTGAGTAGCGCGA	
tgfb1	AF424703	F	AGAGACGGGCAGTAAAGAA	Real time PCR
		R	GCCTGAGGAGACTCTGTTGG	
ccl4	AM765840	F1	GCTGTGTTTGTGCTGATGCT	Real time PCR
		R1	GCTGGCTGGTCTTTTGGTAG	
il8	AM765841	F2	GCCACTCTGAAGAGGACAGG	Real time PCR
		R2	TTTGGTTGTCTTTGGTCGAA	
sele	AM749963	F1	GACAGTGAGCAGGCGTACAA	Real time PCR
		R1	ATCGCTTCATGATCCACACA	
mmp9	AM905938	F1	GGGGTACCCTCTGTCCATTT	Real time PCR
		R1	CCTCCCCAGCAATATTCAGA	
mmp2	FN649419	F1	ACTATGACCGCGACAAGTCC	Real time PCR
		R1	GTAACCTTGGTCGGGACAGA	
tlr5mb	AM296028	F	CCTGTCTGCAACTGTCAGGA	Real time PCR
		R	TGTGGATCTGGTTCAAGCTG	
tlr9	AY751798	F	GGAGGAGAGGGACTGGAT	Real time PCR
		R	GATCACACCGTCACTGTCTC	
tlr22	AM920660	F	GCGAATCTGATTTCCCTCAA	Real time PCR
		R	GGGTGCCACTGATTTGATTT	
rps18	AM490061	F	AGGGTGTTGGCAGACGTTAC	Real time PCR
		R	CTTCTGCCTGTTGAGGAACC	

in gilthead seabream (Pinto et al., 2006) and expressed in both naïve and stimulated leukocytes (Liarte et al., 2011a). This evidence contrasts with observations made in mammals, in which E_2 treatment primarily promotes immunosuppressive and anti-inflammatory effects in leukocytes and ECs (reviewed in Härkönen and Väänänen, 2006; Nilsson, 2007). However, estrogens can also show pro-inflammatory activities, depending on the concentration and exposure time, indicating their dual role in the immune system (Straub, 2007).

In this study, molecular and functional assays were performed to investigate the effects of E_2 and EE_2 on gilthead seabream EC physiology, in order to determine whether estrogens modulate the ability of ECs to recruit leukocytes under physiological or pollutant-induced conditions.

2. Materials and methods

2.1. Animals

Healthy specimens (650 g mean body weight) of gilthead seabream (*S. aurata* L., Actinopterygii, Perciformes, Sparidae) were bred and kept at the Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (IEO) (Mazarrón, Murcia) in 14 m^3 running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/h) with natural temperature (20.2 ± 4.5 °C) and photoperiod, and fed twice a day with a commercial pellet diet (Skretting, Spain) at a feeding rate of 1.5% fish biomass. Fish were fasted for 24 h before sampling. Specimens were anesthetized, decapitated and hearts and head kidneys were collected. 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 IEO for the use of laboratory animals.

2.2. EC isolation, culture and stimulation

Seabream ECs were isolated as previously described (Roca et al., 2008). In brief, the atria from 15 to 20 hearts were collected, pooled and incubated in the following solutions for the indicated times: (1) phosphate-buffered saline (PBS, pH 7.2–7.4) for 30 min; (2) PBS with 0.5 mg/ml trypsin and 0.1 mg/ml EDTA (Sigma–Aldrich) for 7 min; and (3) 0.5 mg/ml collagenase in sRPMI [RPMI-160 culture medium (Gibco) adjusted with 0.35% NaCl to gilthead seabream serum osmolarity, 353.33 mosmol] supplemented with 0.7 mg/ml $CaCl_2$ for 30 min. The cell pellet was resuspended in sRPMI supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 100 i.u./ml penicillin, 100 μg/ml streptomycin (P/S) and 250 ng/ml fungizone and seeded on 25 cm^2 plastic tissue culture flasks or 96-well culture plates previously pre-coated with PBS containing 0.1% gelatin for 30 min at room temperature. The cells were incubated at 23 °C for 36 h to allow ECs to adhere, after which time, debris and non-adherent cells were removed by vigorous shaking and washing with medium. Finally, the medium was replaced and the cultures were left undisturbed until treatment.

EC monolayers were incubated for 16 h in sRPMI containing different pathogen-associated molecular patterns (PAMPs), including flagellin (10 μg/ml, Invitrogen), *Vibrio anguillarum* genomic DNA (VaDNA) (50 μg/ml) and lipopolysaccharide (LPS) (10 μg/ml, Sigma) as previously described (Roca et al., 2008), or with sRPMI supplemented with 10% charcoal/dextran-treated hormone-free fetal bovine serum (HFS) (Hyclone), containing increasing doses of E_2 (0, 5, 50 ng/ml) (Sigma) or EE_2 (0, 5, 50 ng/ml) (Fluka) in the presence or absence of 50 μg/ml VaDNA for 3 h. In order to

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avoid the interference from estrogens naturally present in FBS, FBS-supplemented media was replaced with HFS-supplemented 24 h before estrogen exposure.

2.3. Leukocyte isolation and purification

Seabream head kidney leukocytes were obtained as described elsewhere (Chaves-Pozo et al., 2003, 2005b). AGs were isolated by magnetic-activated cell sorting (MACS) using the G7 mAb ($G7^+$ cells). The remaining cell fraction ($G7^-$ cells) was mainly constituted by hematopoietic precursor cells, macrophages (Mø) and lymphocytes (Ly), as previously described (Sepulcre et al., 2002; Roca et al., 2006).

2.4. Analysis of gene expression

Total RNA was extracted from EC monolayers with TRIzol Reagent (Invitrogen) following the manufacturer's instructions and treated with amplification grade DNase I (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 °C for 60 min.

The expression of the ER genes *era*, *erb1* and *erb2* was analyzed by RT-PCR performed with an Eppendorf Mastercycle Gradient Instrument (Eppendorf). Reaction mixtures were incubated for 2 min at 95 °C, followed by 35 cycles of 45 s at 95 °C, 45 s at the specific annealing temperature for each gene (see Table 1), 1 min at 72 °C, and finally 10 min at 72 °C. As an RT-PCR control, the expression of the housekeeping gene β-actin (*actb*) was used.

Real-time PCR performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems) was used to analyse the genes coding for (i) the pro-inflammatory molecules interleukin-1β (IL-1β), IL-6 and tumor necrosis factor α (TNFα); (ii) the anti-inflammatory molecule transforming growth factor β1 (TGF-β1); (iii) the molecules related to leukocyte infiltration CC chemokine ligand 4 (CCL-4), the CXC chemokine IL-8 and the adhesion molecule E-selectin (SELE); (iv) the matrix metalloproteinases 9 and 2 (MMP9 and MMP2); and (v) the toll like receptors 5 membrane bound (mb), 9 and 22 (TLR5mb, TLR9 and TLR22). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min 60 °C and 15 s at 95 °C. For each mRNA, gene expression was corrected by the ribosomal protein S18 gene (*rps18*) content in each sample (average Ct records 19.6 ± 0.25). The gilthead seabream specific primers used are shown in Table 1. In all cases, each PCR was performed with triplicate samples and repeated at least twice. Less than 2% variation in the *rps18* gene expression was observed between samples.

2.5. Nitric oxide determination

Nitric oxide (NO) production was analyzed by the Griess assay, which measures nitrite, the stable spontaneous oxidation product of NO (Maalouf et al., 2010), in the supernatants obtained from ECs incubated for 24 h in sRPMI supplemented with 10% HFS alone or in the presence of 5 or 50 ng/ml of E_2 and EE_2. Samples were assayed in triplicate and data are represented as the average concentration of NO_2^- of three independent experiments ± SEM (μM ± SEM).

2.6. Leukocyte migration assay

For the analysis of leukocyte migration, total head-kidney leukocytes, AGs and $G7^-$ cell fractions from independent fish were exposed to conditioned media from control or E_2-treated ECs in the presence or absence of VaDNA (E_2-ECM and E_2+VaDNA-ECM) for 24 h. The migration assay was performed using a 10-well transmigration chamber (Neuroprobe) as previously described (Roca et al., 2008). In brief, fresh medium (sRPMI supplemented with 10% HFS), 10% autologous seabream serum (as positive control) or conditioned media from ECs were placed in the lower wells, while the upper wells were filled with 2×10^6 isolated head kidney

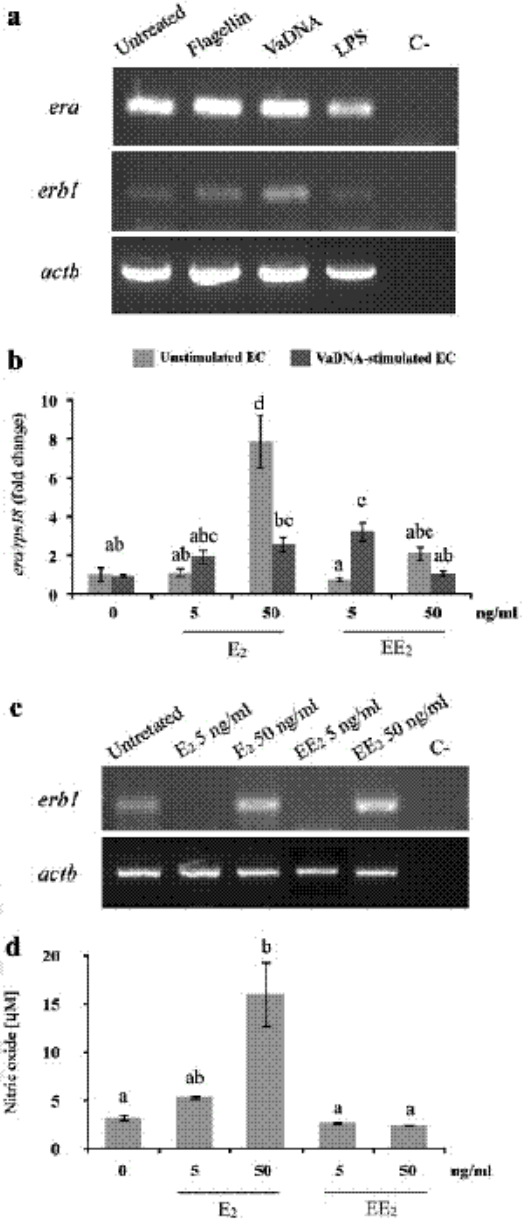

Fig. 1. RT-PCR analysis of *era*, *erb1* and *actb* genes in EC cultures untreated or treated with different PAMPs (10 μg/ml flagellin, 50 μg/ml VaDNA or 10 μg/ml LPS) for 16 h (a). mRNA levels of *era* in EC cultures incubated with 0 (control), 5 or 50 ng/ml of E_2 or EE_2 alone (unstimulated EC, light grey bars) or in the presence of 50 μg/ml VaDNA (VaDNA-stimulated EC, dark grey bars) for 3 h. Data represent means ± SEM of triplicate samples (b). RT-PCR analysis of *erb1* in EC cultures incubated with 0 (untreated), 5 or 50 ng/ml of E_2 or EE_2 for 3 h (c). Release of nitric oxide from EC cultures treated with 0, 5 or 50 ng/ml E_2 or EE_2 for 24 h. Data represent means ± SEM of triplicate samples from three experiments (d). Different letters denote statistically significant differences among the groups according to a Waller–Duncan test. C−: negative control.

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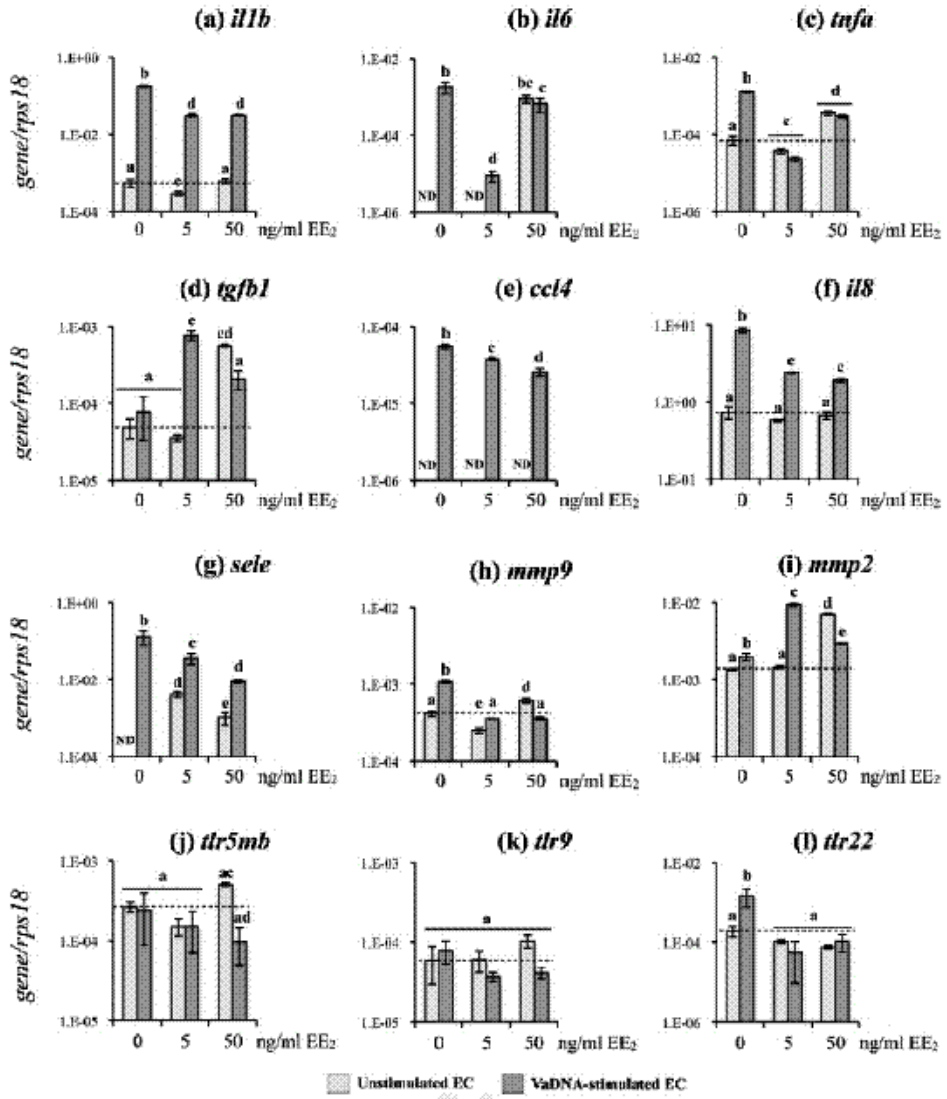

Fig. 3. The mRNA levels of *il1b* (a), *il6* (b), *tnfa* (c), *tgfb1* (d), *ccl4* (e), *il8* (f), *sele* (g), *mmp9* (h), *mmp2* (i), *tlr5mb* (j), *tlr9* (k) and *tlr22* (l) were studied by real-time RT-PCR in EC cultures incubated with 0, 5 or 50 ng/ml of EE_2 alone (unstimulated EC, light grey bars) or in the presence of 50 μg/ml VaDNA (VaDNA-stimulated EC, dark grey bars) for 3 h. Data represent means ± SEM of triplicate samples and are representative of three independent experiments. Slashed line denotes un-stimulated control expression level. Different letters denote statistically significant differences among the groups according to a Waller–Duncan test: "a" un-stimulated control grouping; "b" VaDNA stimulated control grouping when different from "a".

2.8. Statistical analysis

All data were analyzed by analysis of variance (ANOVA) and a Waller–Duncan multiple range test to determine differences between groups ($P \leq 0.05$).

3. Results

3.1. Gilthead seabream endothelial cells express constitutively *era* and *erb1* genes

The mRNA of *era* and *erb1* was constitutively expressed by gilthead seabream ECs. PAMP treatments (flagellin, VaDNA and LPS) did not alter *era* expression. However, flagellin and VaDNA apparently increased *erb1* expression (Fig. 1a). In contrast, *erb2* gene mRNA was not detected in any of the conditions studied (data not shown).

3.2. E_2 and EE_2 regulate the expression of ER genes in endothelial cells

We used one physiological (5 ng/ml) and one supraphysiological (50 ng/ml) dose of E_2 (Chaves-Pozo et al., 2008) and EE_2 in all the experiments. The *era* gene expression was notably induced in non-activated ECs at the highest concentration of E_2 used, while the effect of EE_2 was negligible (Fig. 1b). However, only EE_2 induced the expression of *era* gene in VaDNA-activated ECs (Fig. 1b). In contrast, *erb1* gene exhibited a dual modulation pattern in response to both E_2 and EE_2 treatments: low doses reduced *erb1* expression while high doses had no significant impact (Fig. 1c). Neither E_2 nor EE_2 in the absence or presence of VaDNA was able to affect *erb2* expres-

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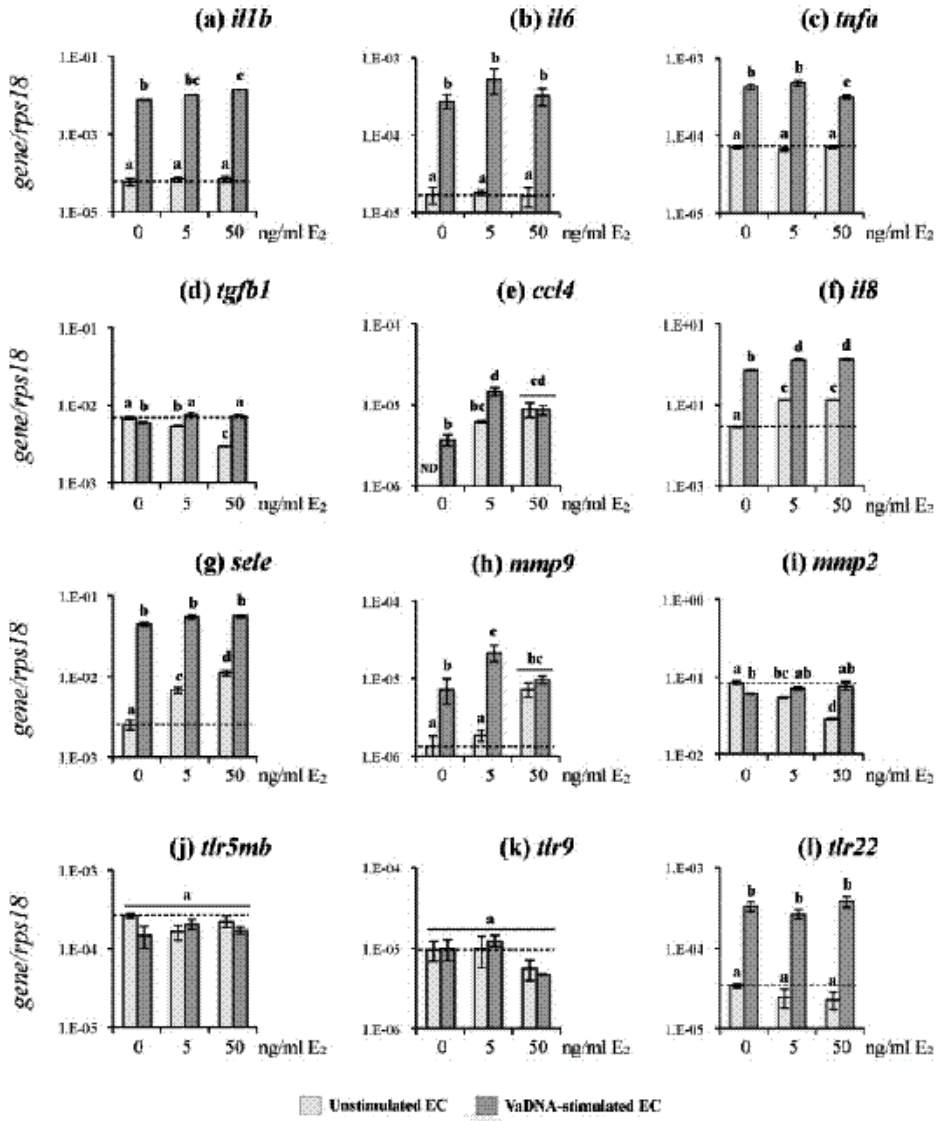

Fig. 2. The mRNA levels of *il1b* (a), *il6* (b), *tnfa* (c), *tgfb1* (d), *ccl4* (e), *il8* (f), *sele* (g), *mmp9* (h), *mmp2* (i), *tlr5mb* (j), *tlr9* (k) and *tlr22* (l) were studied by real-time RT-PCR in EC cultures incubated with 0, 5 or 50 ng/ml of E_2 alone (unstimulated EC, light grey bars) or in the presence of 50 μg/ml VaDNA (VaDNA-stimulated EC, dark grey bars) for 3 h. Data represent means ± SEM of triplicate samples and are representative of three independent experiments. Slashed line denotes un-stimulated control expression level. Different letters denote statistically significant differences among the groups according to a Waller–Duncan test: "a" un-stimulated control grouping; "b" VaDNA stimulated control grouping when different from "a".

leukocytes in supplemented sRPMI. Filled transmigration chambers were incubated at 23 °C with 5% CO_2 for 3 h. Transmigrated cells were harvested from the lower wells and counted with a Neubauer chamber. The percentage of different leukocyte populations that migrated in response to the different treatments was analyzed by flow cytometry (Sepulcre et al., 2002). Treatments did not affect leukocyte viability as no statistically significant differences between groups were observed.

2.7. Leukocyte adhesion assay

The adhesion of leukocytes to ECs was determined as previously described (Roca et al., 2008). Briefly, seabream head kidney leukocytes from independent fish were stimulated for 3 h with 10 μg/ml flagellin in supplemented sRPMI. Head kidney leukocytes (activated or not) and leukocyte fractions ($G7^+$ and $G7^-$) were then labelled with the fluorescent dye 2′,7′-bis(2-carboxyethyl) 5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) (Sigma–Aldrich) and added, in sRPMI medium supplemented with 10% HFS, to EC monolayers pre-incubated with 0, 5, 50, 500 ng/ml E_2 in the presence or in the absence of VaDNA for 24 h. BCECF-AM labelled and EC monolayer cells were incubated at 23 °C for 20 min, after which unbound cells were removed by washing twice with PBS and adhered cells were quantified using a Fluostar fluorescence analyzer (BGM, LabTechnologies). The values reported are the average of 3 readings from 6 different samples, expressed as means ± SEM of fluorescence arbitrary units.

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sion in gilthead seabream ECs (data not shown). Unfortunately, we were unable to confirm these results by real time PCR, since no primer pairs that fulfill the real time PCR requirements could be obtained due to the high degree of similarity conserved between the *erb* genes.

3.3. E_2 alters the production of nitric oxide by endothelial cells

To examine the influence of estrogen exposure on NO production, gilthead seabream ECs were exposed to various concentrations of E_2 or EE_2 and the culture supernatants were collected 24 h later to measure the NO concentration. As shown in Fig. 1d, E_2 exposure increased the NO production at the supraphysiological dose of 50 ng/ml. In contrast, EE_2 exposure did not alter NO levels in the supernatants from ECs.

3.4. E_2 and EE_2 alter the gene expression profile of endothelial cells

As far as the immune-relevant genes are concerned, E_2 (Fig. 2) modulated the expression of most of the genes directly related with the anti-inflammatory effect (*tgfb1*), the leukocyte chemoattraction (*ccl4*, *il8*), leukocyte adhesion (*sele*) and extravasation (*mmp9*, *mmp2*) in cultured ECs. Depending on the individual gene expression patterns of these genes, three groups can be distinguished: (i) those genes whose expression was stimulated by E_2 in a dose-dependent manner (*ccl4*, *sele* and *mmp9*); (ii) those whose induction was similar at both E_2 concentrations used (*il8*); and (iii) those whose expression was repressed by E_2 in a dose-dependent manner (*tgfb1* and *mmp2*). In contrast, E_2 alone barely altered the mRNA levels of the pro-inflammatory (*il1b*, *il6*, *tnfa*) and innate immunity receptor (*tlr5mb*, *tlr9*, *tlr22*) genes analyzed. When the cells were stimulated with VaDNA, E_2 treatment further up-regulated the expression of *il1b*, *ccl4*, *il8* and *mmp9* at one E_2 concentration at least, successfully recovered the E_2-induced repression of *tgfb1* and *mmp2* genes, and decreased the VaDNA-induced expression of *tnfa* but had no effect over *il6*, *sele* and *tlr* genes (Fig. 2). Surprisingly, EE_2 modulation of EC gene expression (Fig. 3) substantially differed from the results obtained with E_2. Thus, four groups of genes could be differentiated according to their expression pattern after EE_2 treatment: (i) genes whose expression was up-regulated in the presence of EE_2 at one dose at least (*il6*, *tgfb1* and *mmp2*), (ii) genes whose expression was down-regulated by at least at one EE_2 dose (*il1b*), (iii) genes whose expression was down or up-regulated, depending on the EE_2 dose used (*tnfa*, *sele* and *mmp9*) and (iv) those genes whose expression was unaltered by EE_2 treatment (*ccl4*, *il8*, *tlr5mb*, *tlr9* and *tlr 22*). Interestingly, when the cells were activated by VaDNA, EE_2 treatment further up-regulated the expression of *tgfb1* and *mmp2*, reduced the VaDNA induction of *il1b*, *tnfa*, *ccl4*, *il8*, *sele*, *mmp9* and *tlr22*, and had no effect on the expression of *il6*, *tlr5mb* and *tlr9* (Fig. 3).

3.5. Conditioned media from E_2-treated endothelial cells affects leukocyte migration

The specific induction of the genes encoding the chemokines Il8 and Ccl4 by E_2 in ECs but not by EE_2 prompted us to analyse the functional relevance of this observation. Unexpectedly, no significant differences were observed in the numbers of total head kidney leukocytes migrating to E_2-ECM or E_2+VaDNA-ECM in any of the experimental conditions tested (Fig. 4a). Similarly, flow cytometry analysis showed that the numbers of transmigrated AGs to E_2-ECMs or E2+VaDNA-ECM was unaltered (Fig. 4b). Moreover, E_2+VaDNA-ECM showed no significant alteration numbers of transmigrated Mø and Ly ($G7^-$ cells) (Fig. 4c).

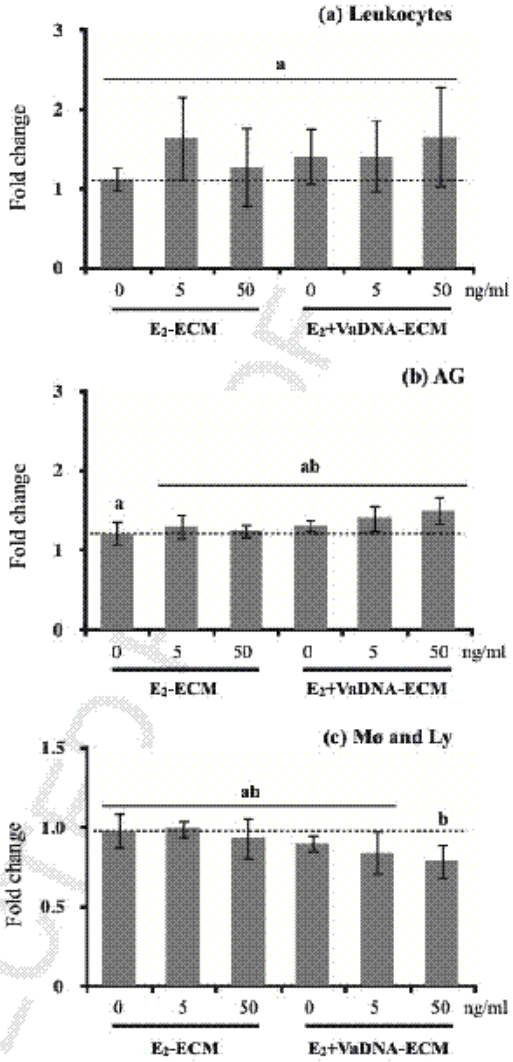

Fig. 4. Migration of freshly isolated head-kidney leukocytes towards 10% of 0, 5, 50 ng/ml E_2-ECM or E_2+VaDNA-ECM (a) and flow cytometry analysis of transmigrated acidophilic granulocytes (AG) (b) and macrophage (Mø) and lymphocyte (Ly) (c) fractions. The results are presented as mean ± SE of triplicate samples from six experiments. Slashed line denotes un-stimulated control expression level. Different letters denote statistically significant differences among the groups according to a Waller–Duncan test.

3.6. E_2 promotes leukocyte recruitment through the activation of endothelial cells

The stimulation of ECs with E_2 resulted in a dose-dependent enhanced adhesion of unstimulated head-kidney leukocytes (Fig. 5a). VaDNA stimulation of ECs resulted in increased leukocyte adhesion but E_2 treatments did not significantly alter this effect (Fig. 5a). In contrast, neither E_2 alone nor co-stimulation with E_2 and VaDNA together was able to modify the adhesion pattern of leukocytes pre-activated with flagellin (Fig. 5b). Surprisingly, the adhesion of purified AG ($G7^+$ cells) to E_2-stimulated ECs barely increased while Mø and Ly ($G7^-$ cells) showed clearly enhanced adhesion (Fig. 5c). In contrast, stimulation of ECs with E_2 and VaDNA simultaneously was able to reduce the adhesion of both leukocyte fractions compared with EC stimulated with VaDNA alone (Fig. 5c).

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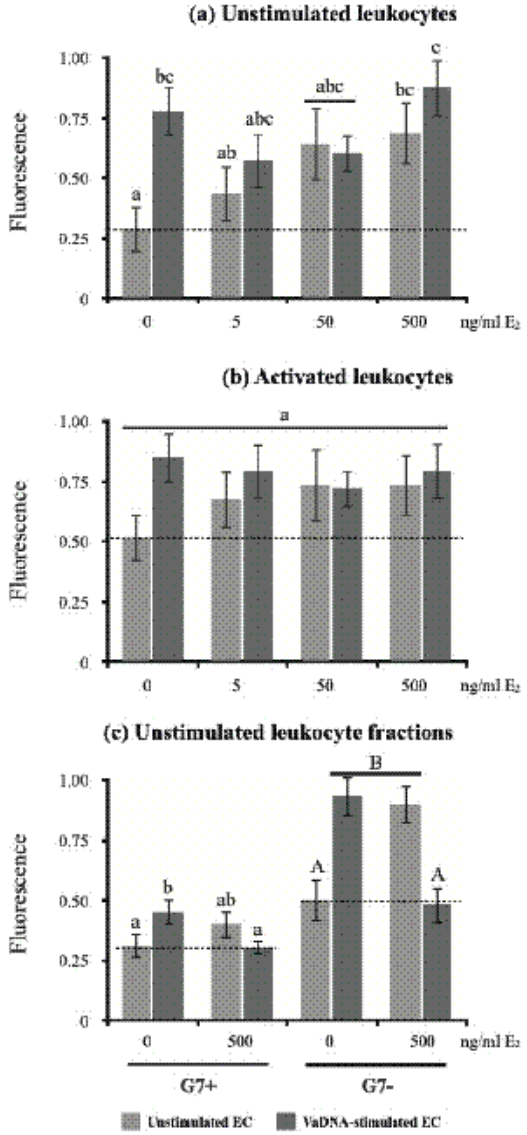

Fig. 5. Adhesion of freshly isolated head kidney leukocytes (unstimulated) (a), previously stimulated (activated) (10 μg/ml flagellin for 3 h) head kidney leukocytes (b) and unstimulated leukocyte purified fractions (acidophilic granulocytes [G7$^+$] or macrophage and lymphocyte [G7$^-$]) (c) to EC monolayers previously exposed to 0, 5, 50 or 500 ng/ml of E_2 alone (light grey bars) or in the presence of 50 μg/ml VaDNA (dark grey bars) for 24 h. Data represent means ± SEM of fluorescence arbitrary units and are representative of six independent experiments. Slashed line denotes unstimulated control expression level. Different letters denote statistically significant differences among the groups according to Waller–Duncan test.

4. Discussion

Leukocyte recruitment is an early and pivotal event in any inflammatory response. The endothelium, which is critically positioned at the interface between the inflammation site and the blood stream, is recognized as playing a crucial role in the recruitment process through the expression of adhesion molecules which facilitate the extravasation of leukocytes. Accumulated evidence on cardiovascular disease points to the prominent role of estrogens in the ability of ECs to signal inflammation and participate in the leukocyte infiltration process (Nilsson, 2007; Straub, 2007). In this trend, several pieces of evidence suggest a pro-inflammatory role for E_2 in the gilthead seabream, since it is able to stimulate leukocyte migration and, *in vitro*, up-regulate the genes encoding cytokines, chemokines and molecules related to leukocyte infiltration (MMPs and cellular adhesion molecules [CAMs]) in these cells (Liarte et al., 2011a). This evidence contrasts with observations made in mammals, in which E_2 treatments primarily exhibit immunosuppressive and anti-inflammatory activities in leukocytes and ECs (reviewed in Härkönen and Väänänen, 2006; Nilsson, 2007). However, mammals lack the physiological inflammatory process partially triggered by E_2 which occurs during and after the spawning stage of the gilthead seabream gonad (Chaves-Pozo et al., 2003, 2005a, 2007; Liarte et al., 2007).

ER isoforms show specific cell and tissue distribution patterns of expression in mammals. Thus, the high expression of *era* is generally restricted to classical estrogen-responsive tissues such as reproductive organs, while *erb1* is more widely distributed (Kuiper et al., 1996, 1997). Mammalian ECs are reported to express both *era* and *erb1* (Green et al., 1986; Mosselman et al., 1996; Couse and Korach, 1999; Andersson et al., 2001) the expression of which is modulated by estrogen exposure: *era* and *erb1* expression is up- and down-regulated, respectively, upon physiological exposure to E_2 (Nardulli and Katzenellenbogen, 1986; Read et al., 1989; Mäkelä et al., 1999; Ihionkhan et al., 2002). Similarly to mammals, gilthead seabream ER isoforms show specific cell and tissue distribution expression patterns (Pinto et al., 2006). In our study, it was found that ECs constitutively express both *era* and *erb1* genes, but *erb2* was not detected. Interestingly, while *erb1* expression showed a similar behavior to that described for mammalian species after both E_2 and EE_2 treatment, *era* expression was sharply up-regulated at a supra-physiological E_2 concentration (50 ng/ml) but it was not significantly induced by a physiological E_2 concentration (5 ng/ml), even when the cells were co-stimulated with VaDNA. Taking into consideration the fact that E_2 can bind different ER isoforms with a similar affinity (Kuiper et al., 1998), the differential ER expression profile modulated by E_2 in gilthead seabream ECs may represent a powerful system for local regulation of the endothelial function and leukocyte recruitment. Moreover, as also occurs in human endometrial cells, gilthead seabream ECs display differential behavior when exposed to E_2 or to EE_2, a recognized ERα selective agonist (Barkhem et al., 1998). However, while human endometrial cells lack the ERα (Bredhult et al., 2007), gilthead seabream ECs express both *era* and *erb1* genes. Interestingly, neither E_2 nor EE_2 in the absence or presence of VaDNA was able to induce *erb2* expression in gilthead seabream ECs, as occurs in gilthead seabream macrophages (Liarte et al., 2011a).

One of the hallmarks of the effects of estrogens on mammalian ECs is the ability of E_2 to induce NO synthesis through the induction of nitric oxide synthase (Nilsson, 2007). Surprisingly, exposure to EE_2 did not alter NO production in human ECs (Arnal et al., 1996). As in mammals, our data in gilthead seabream ECs show that E_2, but not EE_2, significantly enhances NO production, indicating that estrogens regulate NO production by ECs from fish to mammals.

In mammals, accumulated evidence on cardiovascular disease points to the prominent role of estrogens in the ability of ECs to signal and propagate inflammation (Nilsson, 2007; Straub, 2007). As in mammals, gilthead seabream ECs have been demonstrated to express several members of the TLR family of innate immunity receptors and to respond to the TLR ligands, VaDNA and flagellin (Roca et al., 2008). Taking all the above into account, we studied the expression profile of several immune-relevant genes in gilthead seabream ECs following estrogen (E_2 or EE_2) treatment and/or VaDNA co-stimulation. Our data show that E_2 induces the expression genes encoding chemokines, adhesion molecules and MMPs in gilthead seabream ECs, which agrees with previous studies that demonstrated that E_2 promotes AG infiltration

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into the testis and regulates the expression of these genes in gilthead seabream macrophages (Chaves-Pozo et al., 2007; Liarte et al., 2011a). These effects contrast with what occurs in mammals, where E_2 inhibits *in vivo* the migration of leukocytes into inflamed areas and exerts tissue-protective activities through the down-regulation of adhesion molecules and the proforms of MMPs (Straub, 2007). Nevertheless, it is important to mention that E_2 did not affect the expression in ECs of the genes encoding major pro-inflammatory cytokines, such as IL-1β, IL-6 and TNFα, which may prevent the detrimental effects of E_2-induced inflammation through leukocyte recruitment. Surprisingly, the gene expression profile displayed by EE_2-exposed ECs in the gilthead seabream varied substantially from E_2-exposed cells. Thus, our data show that EE_2 dramatically reduces the expression of most of the immune markers studied in VaDNA-activated ECs *in vitro*, while showing a variety of different modulatory effects on the expression profile of inactivated cells. The differential expression profile of most of the genes analyzed in stimulated EE_2-treated ECs, compared with E_2-treated ECs, indicates that this compound would be able to impair the recruitment and activation of fish leukocytes. Interestingly, in mammals the estrogenic transcriptional repression of chemokines, adhesion molecules and MMPs is performed via the suppression of NF-κB (Nilsson, 2007), while the stimulation of those genes observed in gilthead seabream ECs might also be orchestrated by NF-κB, as we have previously demonstrated in the case of the E_2 stimulation of several pro-inflammatory genes in Mø (Liarte et al., 2011a,b).

Taking into account that E_2 up-regulates several genes involved in the recruitment of leukocytes, including chemokines, we next analyzed whether conditioned media from E_2-treated ECs were able to promote the chemotactic mobilization of head kidney leukocytes. It was found that E_2 not only failed to promote the chemotactic mobilization of total head kidney leukocytes and AG but also inhibited the migration of activated Mø and Ly cell fractions, which could suggests that some other factors or the release of mediators that inhibit this activity, besides the chemokines IL-8 and CCL4, are needed. Although, these data may suggest that E_2-induced chemokine expression in ECs would not be sufficient to promote an effective leukocyte migration into inflamed areas, ECs might be critically involved in the regulation of leukocyte trafficking by E_2, since we observed that E_2 increases the ability of leukocytes to adhere to ECs.

In conclusion, this manuscript demonstrates that ECs express two of the three ERs known in gilthead seabream and are able to respond to estrogens through the induction of NO release, the up-regulation of the genes encoding chemokines, adhesion molecules and MMPs, and by their ability to adhere to leukocytes. These results suggest a role for ECs in the immune-modulatory role played by E_2 in this species, but they also show that the environmental exposure of fish to endocrine disruptors, such as EE_2, may compromise the immune response. Further research aimed at shedding light into the genetic regulation carried out by estrogens in this model will provide useful information for aquaculture and the sustainability of this species.

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3. 17β-Estradiol regulates gilthead seabream professional phagocyte responsesthrough macrophage activation

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17β-Estradiol regulates gilthead seabream professional phagocyte responses through macrophage activation

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ABSTRACT

In mammals, estrogens regulate the immune system, either directly or indirectly via several leukocyte types through autocrine/paracrine mechanisms. In the gilthead seabream (*Sparus aurata* L.) gonad, an intensive remodeling process accompanied by the massive infiltration of acidophilic granulocytes (AG) is partially triggered by 17β-estradiol (E_2). Once AG infiltrated the gonad, show impaired activities. In this study we first demonstrate that neither testicular nor head-kidney AG express any of the three estrogen receptor (ER) genes (ERa, ERb1 and ERb2) described in the gilthead seabream, while head-kidney macrophages (Mc) and lymphocytes (Ly) constitutively express ERa gene. Moreover, Mc are important in the immune-modulatory role of E_2, as suggested by its ability to induce ERb2 gene expression and up-regulate the expression of genes coding for ERa, ERb1, pro-inflammatory cytokines, chemokines and tissue remodeling molecules. Furthermore, the soluble factors produced by E_2-treated Mc decreased in head-kidney phagocytes, their phagocytic ability and capacity, while no effects were observed on their reactive oxygen intermediate (ROI) production or their migratory capabilities. However, the role of Ly in the regulation of AG migration and the modulation of phagocytic and ROI production activities triggered by E_2 can not be ruled out, so that further studies are necessary to clarify these issues.

1. Introduction

In mammals, the immune-modulating role of estrogens is controversial due to the fact that the effects of estrogens differ, depending on: (i) the immune stimulus (foreign antigens or autoantigens) and subsequent antigen-specific immune responses; (ii) the cell types involved; (iii) the target organ and its specific microenvironment; (iv) the concentration of estrogens; and (v) the variability in expression of estrogen receptor (ERa and ERb) genes depending on the microenvironment and the cell type (Straub, 2007). This last issue is of outstanding importance taking into account that the preponderance of one ER subtype over the other might alters the effects that estrogens have on the immune system responses (Straub, 2007). Estrogens regulate the differentiation, maturation and function of many cell types of the monocyte–macrophage system in a context-dependent manner, either directly or indirectly via other cells by means of autocrine/paracrine mechanisms. Most of these effects are mediated by repression of the expression of cytokine genes and the modulation of other inflammatory mediators by the ER-dependent or by non-genomic pathways (Harkonen and Vaananen, 2006). There is increasing evidence that estrogens indirectly modulates other leukocyte activities, such as the migration, degradation and rearrangement of tissues and T and B cell responses (Harkonen and Vaananen, 2006; Nilsson, 2007). In fish, there are a few studies on the effects of estrogens on immune responses review in (Iwanowicz and Ottinger, 2009) and among them none has dealt with the regulation of ER gene expressions in immune cells by immune- and hormone-related stimuli or with the ability of macrophages (Mc) to response to 17β-estradiol (E_2) and modulate the immune responses of fish.

The gilthead seabream (*Sparus aurata* L.) is a seasonally breeding, protandrous hermaphrodite teleost with a bisexual gonad, which, during the male phase, has a functional testicular area and a non-functional ovarian area. The testis undergoes abrupt morphological changes especially after spawning, including an intensive remodeling of the testis and the massive infiltration of acidophilic granulocytes (AG) (Chaves-Pozo et al., 2005a; Liarte et al., 2007). These immune cells are produced in the head-kidney, the main hematopoietic organ in fish, but when they infiltrate the testis show heavily impaired reactive oxygen intermediate (ROI) production, phagocytic activity and the constitutive production of interleukin-1β (IL-1b) (Chaves-Pozo et al., 2003, 2005b, 2008a). Interestingly, it is the gonad itself which actively regulates the presence of these

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immune cells in the testis by stimulating their extravasations from the blood (Chaves-Pozo et al., 2005b). Endogenous increases of E_2 in serum is correlated with AG migration into the gonad after spawning (Chaves-Pozo et al., 2008b), while exogenous E_2 accelerates the final events of spermatogenesis and induces the infiltration of AG into the gonad (Chaves-Pozo et al., 2007). The Mc and lymphocytes (Ly) are also present in the interstitial tissue of the gilthead seabream gonad; however, the number of testicular Mc is scarce and no data related to Ly are available (Chaves-Pozo et al., 2008a). All these observations in the gilthead seabream testis prompted to us to examine the role of estrogens in the innate immune response of this species.

For this, we studied the ER gene expressions in the three leukocyte cell types of the head-kidney of gilthead seabream, namely AG, Mc and Ly, as well as in testicular AG, and determined the regulation of these genes in Mc upon E_2 and/or *Vibrio anguillarum* genomic DNA (VaDNA) stimulation. Moreover, in order to determine the role of Mc in the immune-modulatory effects of E_2, we analyzed: (i) the expression of genes coding for key immune molecules, including inflammatory and anti-inflammatory molecules, innate immune receptors, molecules related to leukocyte infiltration, matrix metalloproteinases (MMP) and the anti-viral molecule myxovirus (influenza) resistance protein (Mx) in Mc treated with E_2 with or without co-stimulation with VaDNA, and (ii) the capability of E_2-treated Mc to modulate the phagocytosis, ROI production and migratory activities of the professional phagocytic cell types of the gilthead seabream.

2. Materials and methods

2.1. Animals and cell suspensions

Healthy specimens (650 g mean weight) of gilthead seabream (*S. aurata* L., Actinopterygii, Perciformes, Sparidae), were breed and kept at the Centro Oceanográfico de Murcia (IEO, Mazarrón, Murcia) in 14 m^3 running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/h) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Skretting, Spain) at a feeding rate of 1.5% of fish biomass. Fish were fasted for 24 h before sampling. Specimens were decapitated, and the testes and head-kidneys were removed and dissociated, as described elsewhere (Chaves-Pozo et al., 2003, 2005b). The experiments described comply with the Guidelines of the European Union Council (86/609/EU), the Bioethical Committee of the University of Murcia (Spain) and the Instituto Español de Oceanografía (Spain) for the use of laboratory animals.

2.2. Acidophilic granulocyte, macrophage and lymphocyte cell enriched-fractions

The AG-enriched cell fractions were obtained from head-kidney and testis by magnetic-activated cell sorting (MACS), as previously described (Chaves-Pozo et al., 2005b; Roca et al., 2006). In brief, head-kidney and testicular cell suspensions were incubated with an 1:10 optimal dilution of a monoclonal antibody (mAb) specific to gilthead seabream AG (G7) (Sepulcre et al., 2002), washed twice with phosphate-buffered saline (PBS, pH 7.2–7.4) with 2 mM EDTA (Sigma) and 5% fetal bovine serum (Gibco), and then incubated with 100 μl per 10^7 cells of micromagnetic bead-conjugated anti-mouse immunoglobulin G antibody (Miltenyi Biotec). After washing, G7+ (enriched in AG) and G7− (depleted of AG) cell fractions were collected by MACS following the manufacturer's instructions and their purity was analyzed by flow cytometry (Roca et al., 2006). The Ly-enriched cell fractions and Mc monolayers were obtained as non-adherent and adherent cells, respectively, after incubating G7− cell fractions from head-kidney overnight in serum free sRPMI medium [RPMI-1640 culture medium (Gibco) adjusted with 0.35% NaCl to gilthead seabream serum osmolarity and supplemented with 100 i.u./ml penicillin and 100 μg/ml streptomycin (Biochrom)]. The Mc monolayers generated were then repeatedly washed with PBS (pH 7.2–7.4) to remove the non-adhering cells. The identity of the obtained fractions was confirmed by the specificity of the G7 mAb and the expression of the macrophage colony stimulating factor receptor (MCSFR), the light chain of the immunoglobulin (Ig) and the β subunit of T cell receptor (TCRb) genes, as previously described (Roca et al., 2006; Sepulcre et al., 2002).

2.3. Cell culture, treatments and conditioned media

The Mc conditioned medium were obtained after incubating Mc in sRPMI medium supplemented with 5% charcoal/dextran-treated hormone-free fetal bovine serum (hf-FBS, Hyclone) alone (MCM) or with 5, 50 or 500 ng/ml E_2 (Sigma) (E_2-MCM) for 24 h.

Aliquots of 0.5×10^6 total head-kidney cells were incubated in sRPMI medium with 5% hf-FBS and 0, 5, 50 or 500 ng/ml E_2 or 10% of MCM or 10% of 5, 50 or 500 ng/ml E_2-MCM in the presence or absence of 50 μg/ml VaDNA for 3 and 24 h. Afterwards, phagocytosis, ROI production and migration assays were performed (see below). The Mc were also incubated in sRPMI medium with 5% hf-FBS and 0, 5 or 50 ng/ml E_2 both in the presence or absence of 50 μg/ml VaDNA for 3 h and processed by gene analysis (see below).

2.4. Reactive oxygen intermediate production assay

Reactive oxygen intermediate production was measured as the luminol-dependent chemiluminescence produced by 0.5×10^6 total head-kidney cells pre-incubated with 0, 5, 50 or 500 ng/ml E_2 or 10% of MCM or 10% of 5, 50 or 500 ng/ml E_2-MCM for 3 or 24 h (Mulero et al., 2001). This was brought about by adding 100 μM luminol (Sigma) and 1 μg/ml phorbol myristate acetate (PMA, Sigma), while the chemiluminescence was recorded every 127 s for 1 h in a FLUOstart luminometer (BGM, LabTechnologies). The values reported are the average of triple readings from 6 different samples, expressed as the slope of the reaction curve from 127 to 1016 s, from which the apparatus background was subtracted.

2.5. Phagocytosis assay

Aliquots of 0.5×10^6 total head-kidney cells pre-incubated with 0, 5, 50 or 500 ng/ml E_2 or 10% of MCM or 10% of 5, 50 or 500 ng/ml E_2-MCM were challenged with FITC-labelled *V. anguillarum* (strain R-82, serotype 01) for 60 min as described (Esteban et al., 1998). The number and the intensity of green fluorescent cells were analyzed by using flow cytometry (Esteban et al., 1998).

2.6. Migration assay

A migration assay was performed using a 10-well transmigration chamber (Neuroprobe) as previously described (Gonzalez et al., 2007). 0, 5, 50 or 500 ng/ml E_2 or 10% of MCM or 10% of 5, 50 or 500 ng/ml E_2-MCM, 10% autologous seabream serum or sRPMI medium were placed in the lower wells, while the upper wells were filled with 2×10^6 freshly isolated head-kidney cells or head-kidney cells pre-incubated for 24 h with sRPMI medium alone or containing 5, 50 or 500 ng/ml E_2 or 10% of MCM or 10% of 5, 50 or 500 ng/ml E_2-MCM. Filled transmigration chambers were incubated at 23 °C with 5% CO_2 for 3 h. Transmigrated cells were harvested from the lower wells, analyzed using flow cytometry and counted with a Neubauer chamber.

2.7. Viability assay

Aliquots of 0.5×10^6 total head-kidney cells pre-incubated for 24 h with 0, 5, 50 or 500 ng/ml E_2 or 10% of MCM or 10% of 5, 50 or 500 ng/ml E_2-MCM were diluted in 200 μl PBS containing 40 μg/ml propidium Iodide. The number of red fluorescent cells (dead cells) from duplicated samples was analyzed by flow cytometry.

2.8. Analysis of gene expression

Total RNA from aliquots of freshly isolated AG, Mc and Ly from the head-kidney as well as testicular AG and the testicular cells of the AG-depleted fraction alone or pre-incubated for 3 h with 0, 5 or 50 ng/ml E_2 in the presence or absence of 50 μg/ml VaDNA were extracted with TRIzol Reagent (Invitrogen) following the manufacturer's instructions 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 °C for 60 min.

The ERa, ERb1, ERb2, MCSFR, Ig and TCRB genes were analyzed by semi-quantitative PCR performed with an Eppendorf Mastercycle Gradient Instrument (Eppendorf). Reaction mixtures were incubated for 2 min at 95 °C, followed by 35 cycles of 45 s at 95 °C, 45 s at the specific annealing temperature for each gene (see Table 1), 1 min at 72 °C, and finally 10 min at 72 °C. As a RT-PCR control, β actin (Actb) gene was used.

The following were analyzed by real-time PCR performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems): (i) the expression of the genes coding for the pro-inflammatory molecules IL-1b, IL-6, tumor necrosis factor α (TNFa) and cyclooxygenase 2 (Cox-2); (ii) the anti-inflammatory molecules transforming growth factor β1 (TGFb1) and the type II interleukin-1 receptor (IL-1R2); (iii) the innate immune receptors toll like receptor 9 (TLR9), soluble and membrane-anchored TLR5 (TLR5s and TLR5mb), TLR22, mannose receptors, C type 1 (MRC1a and MRC1b); (iv) the molecules related to leukocyte infiltration the CC chemokine ligand 4 (CCL4) and the CXC chemokine IL-8; (v) the MMP9 and MMP13; and (vi) the anti-viral molecule myxovirus (influenza) resistance protein (Mx). Reaction mixtures were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min 60 °C and 15 s at 95 °C. For each mRNA, gene expression was corrected by the ribosomal protein S18 (RPS18) gene content in each sample. The gilthead seabream specific primers used are shown in Table 1. In all cases, each PCR was performed with triplicate samples and repeated at least twice. Less than 2% variation in the RPS18 gene expression was observed between samples.

2.9. Statistical analysis

All data are representative of 3–6 independent samples or experiments and were analyzed by analysis of variance (ANOVA) and a Waller–Duncan multiple range test to determine differences between groups ($P \leq 0.05$).

3. Results

3.1. Gilthead seabream acidophilic granulocytes do not express ER genes, while macrophages constitutively express the ERa gene

Neither testicular nor head-kidney AG expresses any of the ER genes, while head-kidney Mc and Ly expresses the ERa gene. However, the mRNA of ERb1 gene was detected in the testicular AG-depleted cell fraction, while ERb2 gene was not constitutively expressed in any of these fractions (Fig. 1a and b). Those results were confirmed by sequencing of the amplicons.

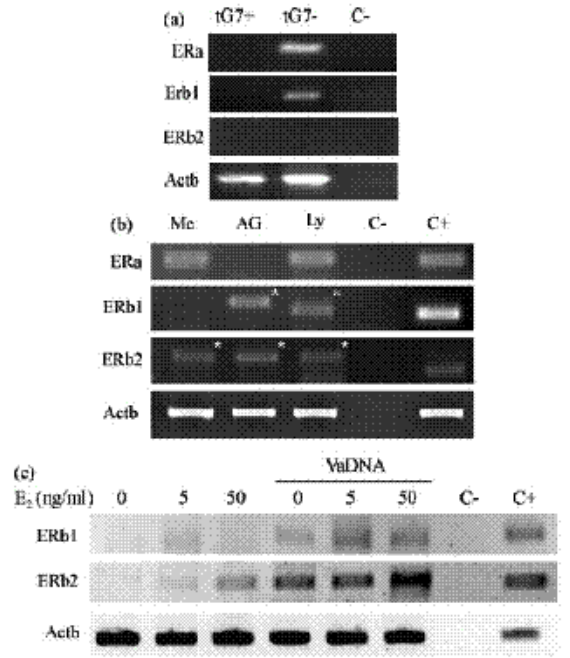

Fig. 1. RT-PCR analysis of ERa, ERb1, ERb2 and Actb genes in testicular cell fractions (a), head-kidney cell fractions (b) and Mc monolayer incubated with 0, 5 or 50 ng/ml of E_2 alone or in the presence of 50 μg/ml VaDNA for 3 h (c). tG7+: testicular acidophilic granulocyte-enriched fraction; tG7−: testicular acidophilic granulocyte-depleted fraction. Mc: macrophage cell fraction; AG: acidophilic granulocyte cell fraction; Ly: lymphocyte cell fraction; C−: negative control; C+: positive control; *: non-specific PCR product. Data are representative of four independent experiments.

3.2. Estradiol alters the gene expression profile of macrophages

As regards the regulation of ER genes expression in Mc, it is interesting to note that ERb1 gene is only induced by VaDNA, while E_2 up-regulated the VaDNA-stimulated ERb1 gene expression (Fig. 1c). In contrast, both E_2 and VaDNA induced the expression of ERb2 gene and co-incubation with E_2 and VaDNA triggered higher ERb2 mRNA levels than they did separately (Fig. 1c). Those results were confirmed by sequencing analysis. Unfortunately, we were unable to confirm these results by real-time PCR, since no primer pairs that fulfill the real-time PCR requirements were obtained due to the high similarity conserved between the three ER genes.

In contrast, although the ERa mRNA levels could be stimulated by either stimulus, they did not reach higher levels upon co-stimulation with both stimuli (Fig. 2a). Notably, VaDNA stimulation failed to induce the expression of ERa, ERb1 and ERb2 in AG (data not shown), suggesting that the effects of E_2 on these cells (Chaves-Pozo et al., 2007) were indirectly mediated by other immune cells. Among them, Mc are good candidates since they constitutively expressed ERa together with ERb1 and ERb2 upon activation with E_2 and pathogens-associated molecular patterns (PAMPs).

In so far as the immune-relevant genes are concerned (Fig. 2b–r), E_2 up-regulated the expression of all the genes analyzed, except IL-1R2 and MRC1b, whose mRNA levels were not modified by E_2. Depending on individual gene expression patterns we can dis-

Table 1
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Ig	EF555070	F R	TTACTACTGTCAGAGTTTTC ACTCCAGTGTGAGGGGAAGC	PCR
TCRb	AM490435	F1 R1	GCTTCTTCAATGGGACAGGA CCGTACACACAGCCCTTGAT	PCR
ERa	AF136979	F1 R1	GCTTGCCGTCTTAGGAAGTG TGCTGCTGATGTGTTTCCTC	PCR/real-time PCR
ERb1	AF136980	F1 R1	CAGCTCCAGAAGGTGGACTC CGATTGGCATAGCTGAAAT	PCR
ERb2	AJ580050	F3 R3	TGATGATGTCACTCACCAACC TTCAGCTCACGAAACCGA	PCR
Actb	X89920	F R	ATCGTGGGGCGCCCCAGGCACC CTCCTTAATGTCACGCACGATTTC	PCR
IL-1b	AJ277166	F2 R3	GGGCTGAACAACAGCACTCTC TTAACACTCTCCACCCTCCA	Real-time PCR
TNFa	AJ413189	FE1 RE2	TCGTTCAGAGTCTCCTGCAG CATGCACTCTCAGTAGCCCCA	Real-time PCR
IL-6	AM749958	F1 R1	AGGCAGGAGTTTGAAGCTGA ATGCTGAAGTTGGTGGAAGG	Real-time PCR
Cox-2	AM296029	F1 R1	GAGTACTGGAAGCCGAGCAC GATATCACTGCCGCCTGAGT	Real-time PCR
TGFb1	AF424703	F R	AGAGACGGGCAGTAAAGAA GCCTGAGGAGACTCTGTTGG	Real-time PCR
IL-8	AM765841	F2 R2	GCCACTCTGAAGAGGACAGG TTTGGTTGTCTTTGGTCGAA	Real-time PCR
CCL4	AM765840	F1 R1	GCTGTGTTTGTGCTGATGCT GCTGGCTGGTCTTTTGCTAC	Real-time PCR
Mx	NM.001007284	F R	GAGGCTTCACTTGGCAACTC TTGTTCCAATAAGGCCAAGC	Real-time PCR
MMP9	AM905938	F1 R1	GGGGTACCCTCTGTCGATTT CCTCCCCAGCAATATTCAGA	Real-time PCR
MMP13	AM905935	F R	CGGTGATTCCTACCCATTTG TGAGCGGAAAGTGAAGGTCT	Real-time PCR
TLR5mb	AM296028	F R	CCTGTCTGCAACTGTCAGGA TGTGGATCTGGTTCAAGCTG	Real-time PCR
TLR5s	AM953332	F R	CTTCCTGAGCCAAGTTGAGG TCAGCTTGTGGAGGTCCTTT	Real-time PCR
TLR9	AY751798	F R	GGAGGACAGGCACTGCAT GATCACACCCTCACTGTCTC	Real-time PCR
TLR22	AM920660	F R	GCGAATCTGATTTCCCTCAA GGGTGCCACTGATTTGATTT	Real-time PCR
MRC1a	FG264028	F R	GTTTTGACAGTTGGGCTGGT TCAACAATGAGCGATTCAGG	Real-time PCR
MRC1b	AM968568	F R	ATAAAACTCATAAAGGGACATGG GCTCAGAATCAAAAGCTGGG	Real-time PCR
IL-1r2	AM296027	F R	AAGGACTCCAGCTCCACTGA ACGCCTTCTACATGGACCAC	Real-time PCR
RPS18	AM490061	F R	ACGGTGTTGGCAGACGTTAC CTTCTGCCTGTTGAGGAACC	Real-time PCR

criminate two groups of genes. Those genes whose expression was stimulated by E_2 in a dose dependent manner (IL-1b, TNFa, CCL4, Mx, MMP9, TLR5s, TLR9, TLR22) and those which expression was similar at both E_2 concentrations used (IL-6, Cox-2, TGFb1, IL-8, MMP13, TLR5mb and MCR1a). Furthermore, when the cells were co-stimulated with VaDNA and E_2 most of the genes analyzed (IL-1b, TNFa, IL-6, IL-8, CCL4, MMP9, MMP13, TLR5mb, TLR5s, TLR22 and MRC1a) were up-regulated in a synergistic way at one E_2 concentration at least. In contrast, E_2 did not modify the VaDNA-induced mRNA levels of Cox-2 gene, while VaDNA did not modify the E_2-induced mRNA levels of TGFb1 gene and decreased those of Mx and TLR9. Interestingly, VaDNA alone decreased the mRNA levels of TLR9 and MRC1b compared with control cells.

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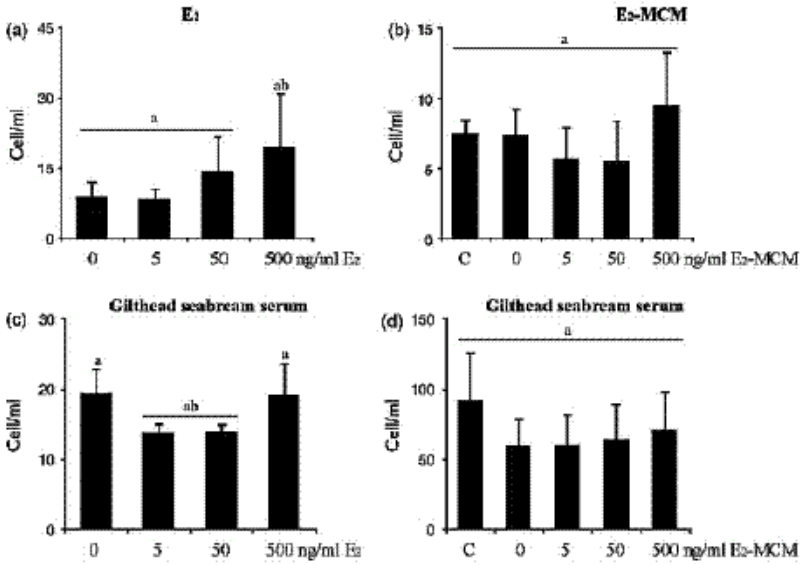


Fig. 3. Migration of head-kidney leukocytes: freshly isolated (a and b) towards 0, 5, 50 or 500 ng/ml E_2 (a) or 10% of 0, 5, 50, 500 ng/ml E_2-MCM (b); or pre-incubated for 24 h (c and d) with 0, 5, 50 or 500 ng/ml E_2 (c) or 10% of 0, 5, 50, 500 ng/ml E_2-MCM (d) towards 10% of autologous gilthead seabream serum was assayed by cell counting and flow cytometry. C: cells pre-incubated with medium alone. The results are presented as mean ± SE in triplicate of 6 independent samples. Different letters denote statistically significant differences between the groups according to a Waller–Duncan test ($P \leq 0.05$).

E_2 (Fig. 3c) and E_2-MCM (Fig. 3d), their migratory capability to gilthead seabream serum was not significantly modified. The percentage of different head-kidney cell populations that migrated in response to the different treatments was analyzed by flow cytometry as described by (Sepulcre et al., 2002) and no differences were observed (data not shown).

3.4. Estradiol reduces the phagocytic activity of leukocytes

The percentage of head-kidney phagocytes remained constant after E_2 treatments (Fig. 4a), while their phagocytic capacity decreased when the cells were treated with 50 or 500 ng/ml E_2 (Fig. 4b). We then analyzed whether the soluble molecules produced by Mc upon E_2 treatment modulate head-kidney phagocyte (mainly AG) activity. Our data demonstrated that E_2-MCM decreased the phagocytic ability and capacity of head-kidney cells when 500 ng/ml E_2 were used to stimulate Mc (Fig. 4c and d).

3.5. Estradiol modulates the VaDNA-induced ROI production of head-kidney leukocytes

The ability of E_2 to modulate *in vitro* the ROI production of head-kidney leukocytes, both in the presence or absence of VaDNA, was analyzed. Independently of the concentration used, E_2 alone was unable to alter the ROI production of head-kidney cells after 3 h (Fig. 5a) and 24 h (Fig. 5b) of incubation. Surprisingly, E_2 was able to partially decrease the ROI production induced by VaDNA after 3 h (Fig. 5a), while it showed the opposite effect after 24 h (Fig. 5b). Similar results were obtained with flagellin (data not shown).

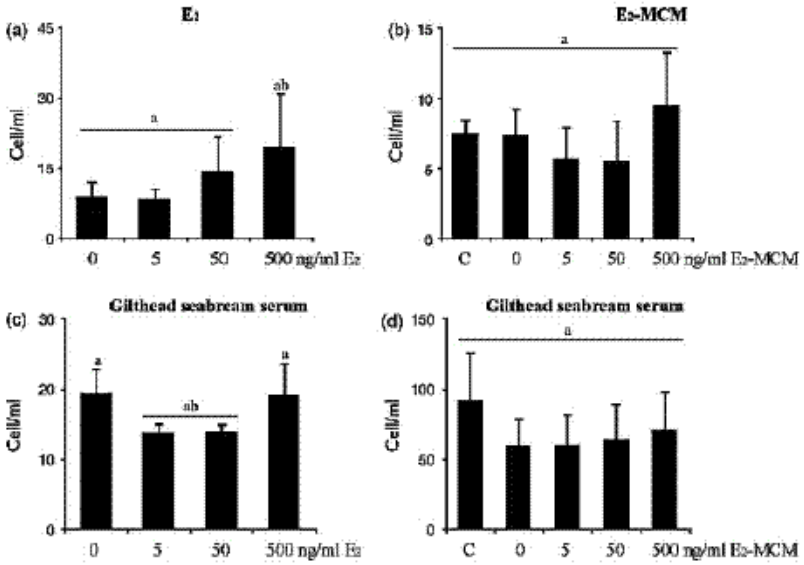
In order to determine whether Mc are involved in the E_2 effect on ROI production, which is mainly mediated by AG, we incubated head-kidney leukocytes with E_2-MCM and observed that the ROI production of head-kidney leukocytes was not modified at any of the E_2 concentrations or incubation times assayed (Fig. 5c and d). However, MCM inhibited VaDNA-stimulated ROI production in the same manner as E_2-MCM did after 3 h of incubation (Fig. 5c). Interestingly, MCM slightly increased the ROI production activity of head-kidney leukocytes after 24 h, while E_2-MCM did not significantly modify this effect (Fig. 5d).

3.6. Neither E_2 nor MCM affect leukocyte viability

Treatment with MCM, E_2, or E_2-MCM did not affect gilthead seabream head-kidney leukocyte viability as no statistically significant differences between groups were observed (data not shown).

4. Discussion

In the gilthead seabream, the gonad itself actively regulates the presence of AG by stimulating their extravasations from the blood (Chaves-Pozo et al., 2005b). This infiltration resembles an inflammatory process triggered by physiological stimuli, in which the main activities of AG are strongly inhibited by the testicular microenvironment in order to preserve reproductive functions (Chaves-Pozo et al., 2005b). Previous data showed that E_2 is related with the mobilization of AG from the head-kidney to the gonad and probably with the magnitude of this infiltration (Chaves-Pozo et al., 2007, 2008a). However, our present data show that neither testicular nor head-kidney AG expresses any of the known ER, namely ERa, ERb1 or ERb2, while head-kidney Mc and Ly constitutively express the ERa gene. In mammals, however, Mc and B Ly express both ERa and ERb genes, while there is a debate on whether or not T cells contain classical nuclear ER (Benten et al., 1998; Harkonen and Vaananen, 2006). Interestingly, in different mammalian models, the preponderance of ERa gene over the ERb gene is accepted to be a mechanism controlling the effects of E_2 on the immune system (Straub, 2007). In gilthead seabream Mc, VaDNA drastically up-regulates the expression of ERa, ERb1 and ERb2 genes, suggesting that the immune system is able to increase its sensitivity to



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The ability of E_2 to modulate *in vitro* the ROI production of head-kidney leukocytes, both in the presence or absence of VaDNA, was analyzed. Independently of the concentration used, E_2 alone was unable to alter the ROI production of head-kidney cells after 3 h (Fig. 5a) and 24 h (Fig. 5b) of incubation. Surprisingly, E_2 was able to partially decrease the ROI production induced by VaDNA after 3 h (Fig. 5a), while it showed the opposite effect after 24 h (Fig. 5b). Similar results were obtained with flagellin (data not shown).

In order to determine whether Mc are involved in the E_2 effect on ROI production, which is mainly mediated by AG, we incubated head-kidney leukocytes with E_2-MCM and observed that the ROI production of head-kidney leukocytes was not modified at any of the E_2 concentrations or incubation times assayed (Fig. 5c and d). However, MCM inhibited VaDNA-stimulated ROI production in the same manner as E_2-MCM did after 3 h of incubation (Fig. 5c). Interestingly, MCM slightly increased the ROI production activity of head-kidney leukocytes after 24 h, while E_2-MCM did not significantly modify this effect (Fig. 5d).

3.6. Neither E_2 nor MCM affect leukocyte viability

Treatment with MCM, E_2, or E_2-MCM did not affect gilthead seabream head-kidney leukocyte viability as no statistically significant differences between groups were observed (data not shown).

4. Discussion

In the gilthead seabream, the gonad itself actively regulates the presence of AG by stimulating their extravasations from the blood (Chaves-Pozo et al., 2005b). This infiltration resembles an inflammatory process triggered by physiological stimuli, in which the main activities of AG are strongly inhibited by the testicular microenvironment in order to preserve reproductive functions (Chaves-Pozo et al., 2005b). Previous data showed that E_2 is related with the mobilization of AG from the head-kidney to the gonad and probably with the magnitude of this infiltration (Chaves-Pozo et al., 2007, 2008a). However, our present data show that neither testicular nor head-kidney AG expresses any of the known ER, namely ERa, ERb1 or ERb2, while head-kidney Mc and Ly constitutively express the ERa gene. In mammals, however, Mc and B Ly express both ERa and ERb genes, while there is a debate on whether or not T cells contain classical nuclear ER (Benten et al., 1998; Harkonen and Vaananen, 2006). Interestingly, in different mammalian models, the preponderance of ERa gene over the ERb gene is accepted to be a mechanism controlling the effects of E_2 on the immune system (Straub, 2007). In gilthead seabream Mc, VaDNA drastically up-regulates the expression of ERa, ERb1 and ERb2 genes, suggesting that the immune system is able to increase its sensitivity to

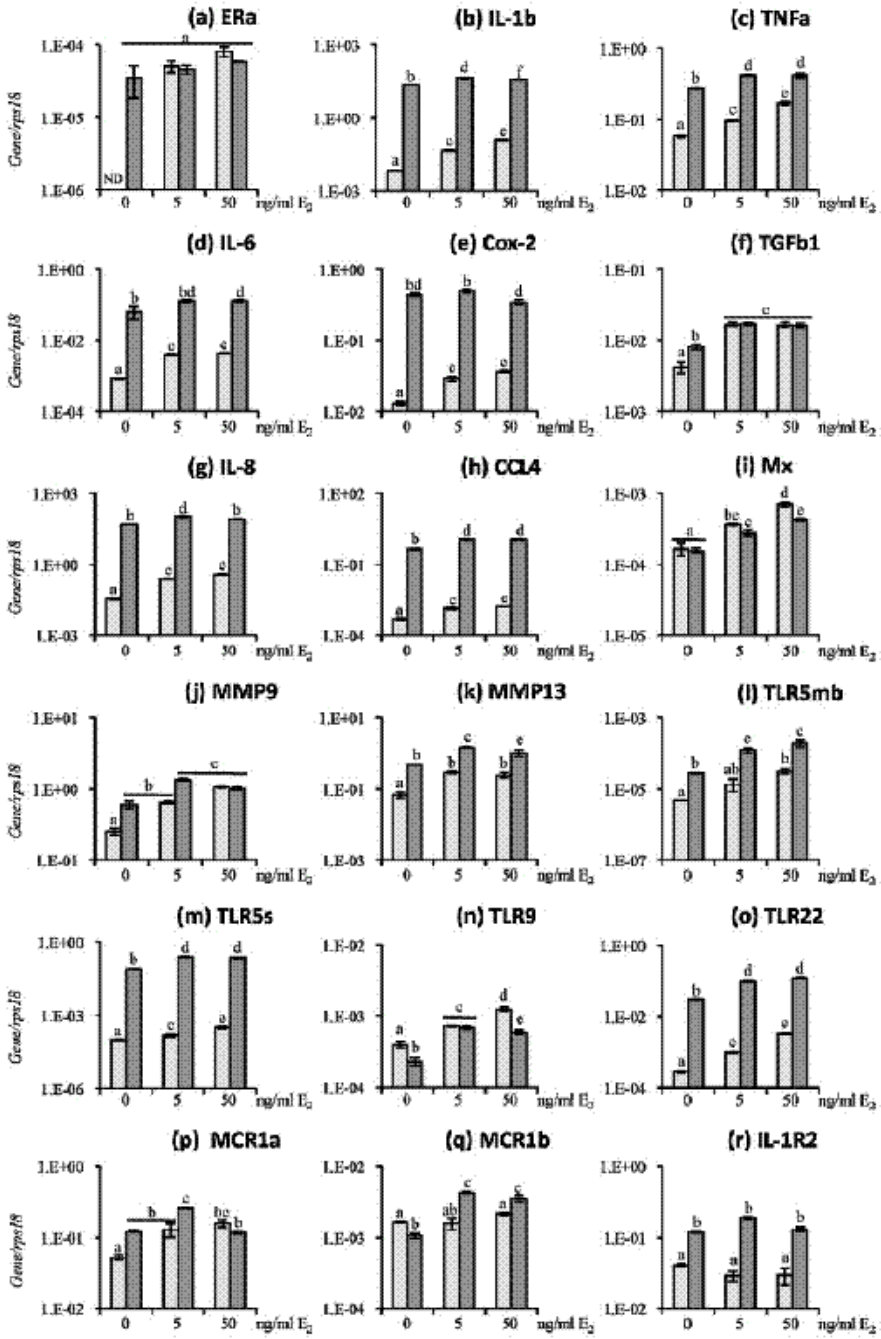

Fig. 2. The mRNA levels of ERa (a), IL-1b (b), TNFa (c), IL-6 (d), Cox-2 (e), TGFb1 (f), IL-8 (g), CCL4 (h), Mx (i), MMP9 (j), MMP13 (k), TLR5mb (l), TLR5s (m), TLR9 (n), TLR22 (o), MRC1a (p), MRC1b (q), and IL-1R2 (r) were studied by real-time RT-PCR in Mc monolayer incubated with 0, 5 or 50 ng/ml of E_2 alone (light grey bars) or in the presence of 50 μg/ml VaDNA (dark grey bars) for 3 h. Data represent means ± SEM of triplicate samples and are representative of three independent experiments. Different letters denote statistically significant differences between the groups according to a Waller–Duncan test ($P \leq 0.05$).

3.3. *Neither E_2 nor E_2-MCM affects in vitro leukocyte migration*

To test the ability of E_2 to directly promote leukocyte migration, transmigration chamber assays were performed. No significant differences were observed between the cell numbers migrating to the culture medium containing E_2 or E_2-MCM at any of the E_2 concentrations used (Fig. 3a and b). Interestingly, when the cells were pre-incubated for 24 h with different concentrations of

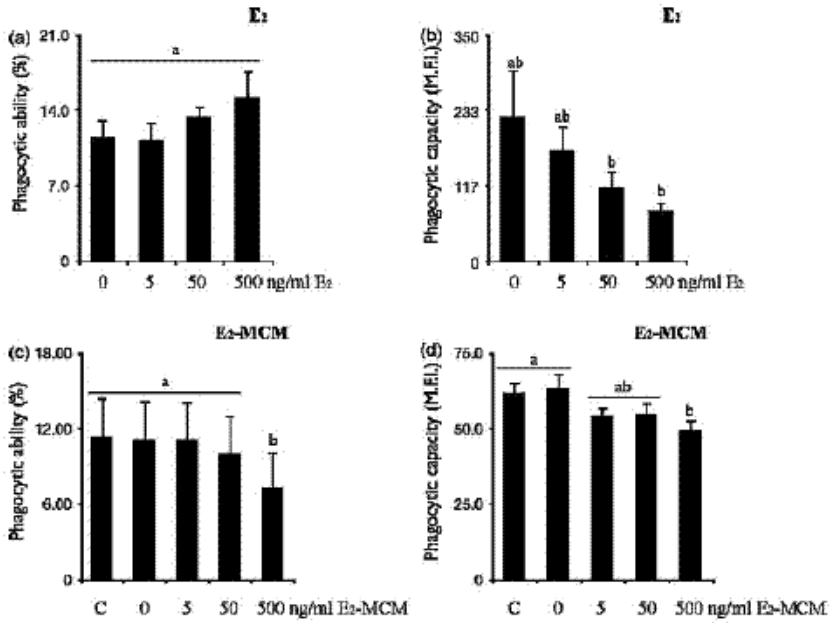

Fig. 4. Phagocytosis of head-kidney cells pre-incubated with 0, 5, 50 or 500 ng/ml E_2 (a and b) or 10% of 0, 5, 50, 500 ng/ml E_2-MCM (c and d). Whole head-kidney cell suspensions were incubated for 24 h with the indicated treatments, and then the phagocytosis of FITC-labeled *Vibrio anguillarum* was assayed by flow cytometry. C: cells pre-incubated with medium alone. The results are presented as mean ± SE in triplicate of 6 independent samples. Different letters denote statistically significant differences between the groups according to a Waller–Duncan test ($P \leq 0.05$).

E_2 during the development of the immune response. Although evidence conclusively demonstrates that fish leukocytes express ER genes, the literature in this respect does not deal with the possible differential roles of the two ERb genes (ERb1 and ERb2) present in fish. Our data demonstrate for the first time that ERb1 and ERb2 are differentially regulated in Mc. Thus, ERb1 gene expression is only induced by VaDNA and its VaDNA-induced expression is slightly increased by E_2, in contrast to ERb2 gene whose expression is induced by both stimuli, which, moreover, have a synergic effect on ERb2 gene expression.

Taking all the above into account, we explored the pattern of several immune-relevant genes of gilthead seabream Mc following E_2 and/or VaDNA stimulation. In mammals, the strongest action mechanism of estrogen in the monocyte–macrophage system is the transcriptional repression of cytokine genes (Harkonen and Vaananen, 2006). In contrast to findings in mammals, we found

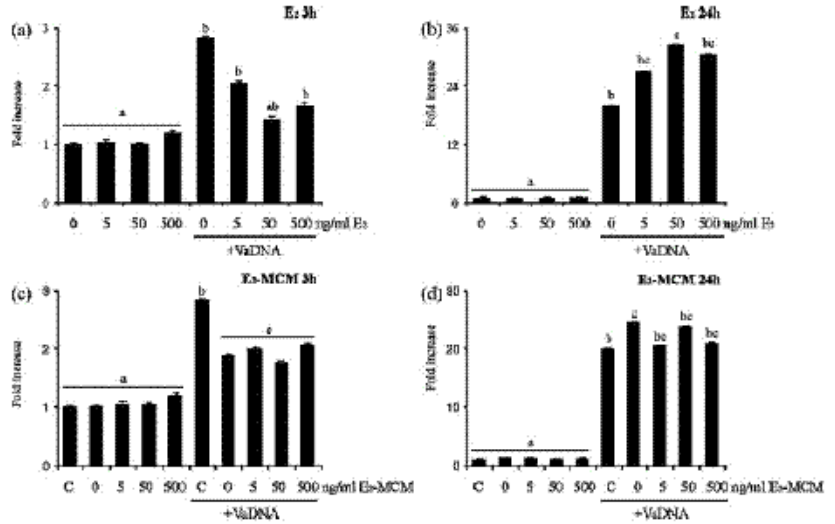

Fig. 5. ROI production of head-kidney cells incubated with 0, 5, 50 or 500 ng/ml E_2 alone or in the presence of 50 μg/ml VaDNA (a and b) or with 10% of 0, 5, 50, 500 ng/ml E_2-MCM alone or in the presence of 50 μg/ml VaDNA (c and d) for 3 h (a and c) or 24 h (b and d). The results are presented as mean ± SE in triplicates of 6 independent samples. Different letters denote statistically significant differences between the groups according to a Waller–Duncan test ($P \leq 0.05$). C: cells incubated with medium.

that E_2 is able to stimulate most of the genes analyzed, promoting a higher activation status in both naïve and VaDNA co-stimulated Mc. The up-regulation of chemokines, pro-inflammatory cytokines and MMPs might promote the mobilization of leukocytes from the head-kidney to the gonad. The Mc are present in the interstitial tissue of the gonad and we cannot exclude the possibility that they might orchestrate head-kidney AG infiltration into the testis. In particular, TNFa up-regulation might be involved in the recruitment of AG, a possibility also supported by the fact that TNFa gene expression is up-regulated in the gonad prior to the infiltration of AG during the first two reproductive cycles (RCs) when the specimens are males (Chaves-Pozo et al., 2005a, 2008a; Liarte et al., 2007) and the observation that gilthead seabream Tnfa recombinant protein promotes the mobilization of AG from the head-kidney to the injection site (García-Castillo et al., 2004) through the up-regulation of E-selectin and chemokines in endothelial cells (Roca et al., 2008). In mammals, E_2 inhibits *in vivo* the migration of leukocytes into inflamed areas and exerts tissue-protective effects by up-regulation of TIMPs and by down-regulation of the proforms of MMPs (Straub, 2007). In contrast, in fish, our data show a stimulatory effect of E_2 on MMP9 and MMP13 gene expressions, correlating with previous studies in which E_2 promotes AG infiltration into the testis and regulates MMPs, which are probably involved in this process (Chaves-Pozo et al., 2007, 2008c). In fact, the immune system of the gilthead seabream is able to respond after tissue injury or remodeling, as occurs in the gonad, through a specific signal promoted by MMP-derived collagen fragments that induces a specific set of immune-related molecules and extracellular matrix remodeling enzymes that are substantially different from those induced by PAMPs (Castillo-Briceño et al., 2009). Despite the fact that E_2 up-regulates several genes related with the mobilization of leukocytes, including those coding for MMPs and chemokines, neither E_2 nor E_2-MCM promotes chemotactic mobilization of head-kidney leukocytes towards them or towards serum. All these data suggest that head-kidney Mc are not involved in the mobilization of AG and identify Ly as the cell type that might orchestrate the mobilization of AG from the head-kidney to the gonad in response to E_2. However, Mc might be involved in the modulation of the immune system response to E_2 treatment. In fact, the up-regulation of TLR genes by E_2 observed in both naïve and VaDNA co-stimulated Mc suggest that E_2 is involved in increasing the response capacity of the immune system against future infections and explains the synergistic effect of E_2 and VaDNA, since the gene coding for VaDNA receptor (TLR9) is also up-regulated by E_2. Furthermore, gilthead seabream Mc have been demonstrated to be involved in the negative feedback that prevents the harmful effects of the inflammatory response by blocking the action of IL-1b through up-regulating the expression of IL-1R2 gene, a decoy receptor for IL-1b (López-Castejón et al., 2007). However, our data show that E_2 does not modify basal or VaDNA-stimulated IL-1R2 gene expression. Similarly, E_2 induces, rather than inhibits, the expression of MRC1 genes, which are classical markers of anti-inflammatory Mc (Pelegrín and Surprenant, 2009). All these data suggest that E_2 acts on gilthead seabream Mc, in which it promotes an acute inflammatory process. These data contrast with observations made in mammals, in which Mc exhibit immunosuppressive activities (Harkonen and Vaananen, 2006). However, in the mammalian gonad there is no physiological inflammatory process partially triggered by E_2, as occurs in the gonad of the gilthead seabream during and after the spawning stage of the first two RCs (Chaves-Pozo et al., 2003, 2005b, 2007).

In order to test whether Mc are able to modulate the main activities of AG, which does not express ER genes, we compared the effects of E_2 and E_2-MCM on the phagocytic, ROI production and migration activities of head-kidney leukocytes. Surprisingly AG infiltrates the gonad in a process regulated as if there was inflammation; however, its main functions are suppressed by the microenvironment of the gonad at the same time that several pro-inflammatory molecules are up-regulated in the gonad (Chaves-Pozo et al., 2008a). Similarly, although E_2 promotes the up-regulation of several relevant-immune genes in Mc, both E_2 and E_2-MCM impair the phagocytic activity of head-kidney leukocytes, suggesting that the soluble factors produced by testicular Mc in response to E_2 contribute to blocking the phagocytic activity of testicular AG. In goldfish Mc, E_2 inhibits the percentage of phagocytic cells (Wang and Belosevic, 1995). In contrast, in the gilthead seabream, E_2 reduces the phagocytic capacity of head-kidney phagocytic cells, while the percentage of these cells remains steady. As regards ROI production activity, Mc ROI production in goldfish and carp is not modified by E_2 treatment (Wang and Belosevic, 1995; Yamaguchi et al., 2001). However, in the gilthead seabream, PAMP-stimulated ROI production of head-kidney leukocytes decreased after 3 h of E_2 treatment and increased after 24 h. Interestingly, and in contrast to the phagocytic activity, this effect is not promoted by an E_2-triggered soluble factor produced by Mc, since E_2-MCM and MCM similarly decreased the level of VaDNA-stimulated ROI production after 3 h and showed no statistical differences between both groups after 24 h of incubation. Further studies are necessary to determine whether Ly, which also expresses the *era* gene, are involved in the modulation of phagocytic and ROI production activities triggered by E_2.

5. Conclusions

In conclusion this manuscript demonstrates that (i) only Mc and Ly constitutively express one of the three ER known in gilthead seabream (Pinto et al., 2006) and (ii) the Mc are key cells in the immune-modulatory role played by E_2, as suggested by their pattern of gene expression upon E_2 treatment. Strikingly, the soluble factors produced by E_2-treated Mc decrease the phagocytic ability and capacity of professional phagocytes, while no effects were observed on ROI production and migratory capabilities of head-kidney leukocytes.

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4. Estrogen-responsive genes in macrophages of the bony fish gilthead seabream: A transcriptomic approach

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Estrogen-responsive genes in macrophages of the bony fish gilthead seabream: A transcriptomic approach

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ABSTRACT

The role of sex steroids in the modulation of fish immune responses has received little attention. Previous studies have demonstrated that 17β-estradiol (E_2) is able to alter the response of gilthead seabream leukocytes to infectious agents. We have used suppression subtractive hybridization to identify genes upregulated by E_2 (50 ng/ml) in macrophage cultures from gilthead seabream. We isolated 393 up-regulated cDNA fragments that led to the identification of 162 candidate estrogen-responsive genes. Functional analyses revealed the presence of several enriched immune processes and molecular pathways. The E_2 up-regulation of some immune-relevant genes was further confirmed by real time RT-PCR. Bioinformatics analysis revealed the ability of E_2 to orchestrate profound alterations in the macrophage expression profile, especially immune-related processes and pathways. This is the first report on E_2-dependent modifications of fish macrophage transcriptome and lends weight to a suggested role for estrogen in the immune system, the possible significance of which is discussed.

1. Introduction

In mammals, estrogens regulate the differentiation, maturation and function of many cell types of the monocyte–macrophage system in a context-dependent manner, either directly or indirectly via other cells by means of autocrine/paracrine mechanisms. Most of these effects are mediated by modulation of the expression of cytokine genes and/or of other inflammatory mediators by the classic estrogen receptor (ER) or by non-genomic pathways (Härkönen and Väänänen, 2006). Among the actions indirectly modulated by estrogens are several leukocyte activities (Härkönen and Väänänen, 2006; Nilsson, 2007). However, this immune-modulating role of estrogens remains controversial due to the fact that these effects differ, depending on: (i) the immune stimulus (foreign antigens or auto-antigens) and subsequent antigen-specific immune responses; (ii) the cell types involved; (iii) the target organ and its specific microenvironment; (iv) the concentration of estrogens; and (v) the variability in expression of ER isoforms according to the microenvironment and the cell type (Straub, 2007).

Fish represent the earliest class of vertebrates with a completely developed immune response, a fully functional complement system, unique receptors that recognize pathogens and/or danger signals, soluble mediators that can regulate the inflammatory processes, and immune cell populations characteristic of the innate or the adaptative immune system (Plouffe et al., 2005; Randelli et al., 2008). In fish, few studies have addressed the effects of estrogens on immune responses (reviewed in Iwanowicz and Ottinger, 2009) and none has applied "omic" approaches to characterize the effect of the main estrogen 17β-estradiol (E_2) on the transcriptome of macrophages.

The gilthead seabream (*Sparus aurata* L.) is a seasonal breeding, protandrous hermaphrodite teleost with a bisexual gonad, which, during the male phase, has a functional testicular area and a non-functional ovarian area. The testis undergoes abrupt morphological changes especially after spawning (Chaves-Pozo et al., 2005a), including an extensive remodeling of the testis and a massive infiltration of acidophilic granulocytes (AG); interestingly, it is the gonad itself that actively regulates the presence of these immune cells in the testis by stimulating their extravasation from the blood (Chaves-Pozo et al., 2005b). Moreover, macrophages (MØ) and lymphocytes have also been observed in the interstitial tissue of the gonad (Chaves-Pozo et al., 2008a; Liarte et al., 2007). However, the number of testicular MØ remains steady throughout the reproductive cycle (RC) and no data related to lymphocytes are available (Chaves-Pozo et al., 2008a). All these immune cells are produced in the head-kidney, the main hematopoietic organ in fish. Endogenous increases of E_2 in serum are correlated with AG migration into the gonad after spawning (Chaves-Pozo et al., 2008b), while exogenous E_2 brings forward the final events of spermatogenesis and induces the infiltration of AG into the gonad (Chaves-Pozo

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et al., 2007). Surprisingly, MØ and lymphocytes express ERα while no ER isoforms are expressed by AG. Moreover, E_2 modulates ER expression in MØ, increases their response to an immune challenge and up-regulates the expression of several pro-inflammatory cytokines (*il1b*, *il6*, *tnfa*), chemokines (*ccl4*, *il8*) and immune receptors (*tlr5*, *tlr9*, *tlr22*) (Liarte et al., 2011). All these observations on this immune–reproductive systems interaction prompted us to examine the ability of E_2 to modulate the transcriptome of MØ in gilthead seabream males and its ability to affect the innate immune response of this species.

For this purpose, we performed a suppression subtractive hybridization (SSH) analysis to identify expressed sequence tags (ESTs) of physiological relevance, mainly from an immune point of view, in gilthead seabream MØ treated with E_2. Functional and computational genomic analyses to determine the particular enrichment of cellular processes and molecular pathways reveal the ability of E_2 to orchestrate profound alterations in the macrophage transcriptome, affecting its whole physiology, especially immune-related processes and pathways.

2. Material and methods

2.1. Animals and cell suspensions

Healthy specimens (650 g mean weight) of gilthead seabream (*S. aurata* L., Actinopterygii, Perciformes, Sparidae) were bred and kept at the Centro Oceanográfico de Murcia, Instituto Español de Oceanografía (IEO) (Mazarrón, Murcia) in 14 m^3 running seawater aquaria (dissolved oxygen 6 ppm, flow rate 20% aquarium volume/h) with natural temperature and photoperiod, and fed twice a day with a commercial pellet diet (Skretting, Spain) at a feeding rate of 1.5% of fish biomass. Fish were fasted for 24 h before sampling. Specimens were decapitated, and head-kidneys were removed and dissociated, as described elsewhere (Chaves-Pozo et al., 2003, 2005b). The experiments described comply with the Guidelines of the European Union Council (86/609/EU), the Bioethical Committee of the University of Murcia (Spain) and the IEO for the use of laboratory animals.

2.2. Isolation of macrophages and treatment

Macrophage (MØ) monolayers were obtained as adherent cells from head-kidney cellular suspensions (Chaves-Pozo et al., 2008a). In brief, head-kidney cellular suspensions from 6 individuals were seeded at 10^6 cells/ml, and incubated overnight in serum-free sRPMI medium [RPMI-1640 culture medium (Gibco) adjusted with 0.35% NaCl to gilthead seabream serum osmolarity and supplemented with 100 i.u./ml penicillin and 100 μg/ml streptomycin (Biochrom)] in 25 cm^2 culture flasks. The MØ monolayers generated were then repeatedly washed with PBS (pH 7.2–7.4) to remove the non-adhering cells. The identity of the monolayer was confirmed by the expression of the macrophage colony stimulating factor receptor (*mcsfr*) (Roca et al., 2006). Once MØ monolayers had been obtained, they were incubated in sRPMI medium supplemented with 5% charcoal/dextran-treated hormone-free fetal bovine serum (HF-FBS, Hyclone) alone (control) or containing 5 or 50 ng/ml of 17β-estradiol (E_2, Sigma) for 3 h, as previously described (Liarte et al., 2011).

2.3. RNA isolation and cDNA synthesis

Total RNA was extracted using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. To perform cDNA synthesis the Super SMART PCR cDNA Synthesis Kit (Takara Clontech, USA) was used: 500 ng of total RNA pooled from 6 MØ monolayers samples was placed into 50 μl MilliQ water, adding 7 μl of 12 μM 3′SMART CDS Primer IIA, and 7 μl of 12 μM template switching SMART IIA oligonucleotide. The mixture was heated to 65 °C for 3 min and then incubated at 42 °C for 2 min. The following reagents were then added: 20 μl of 5× first-strand buffer, 2 μl of DTT (100 mM), 10 μl of dNTP (10 mM), 4.5 μl of RNase Inhibitor (20 U/μl), and 5 μl of Powerscript Reverse Transcriptase (Takara-Clontech, USA). The cDNA synthesizing reaction was performed at 42 °C for 90 min, and stopped by adding 2 μl of EDTA (0.5 M). To purify the cDNA generated, NucleoSpin Extract II (Macherey-Nagel, USA) was used according to the manufacturer's instructions. cDNA was recovered in 35 μl MilliQ water and subsequently used for PCR amplification: 30 μl of 10× Advantage2 PCR buffer, 6 μl of dNTP (10 mM), 6 μl of 12 μM Primer IIA, and 6 μl of 50× Advantage2 Polymerase Mix (Takara-Clontech, USA) were added to the PCR solution. The reaction profile was 1 min at 95 °C; 28 cycles of 5 s at 95 °C, 5 s at 65 °C, 3 min at 68 °C.

2.4. Suppression subtractive hybridization and cloning

The PCR-Select™ cDNA Subtraction Kit (Clontech, Palo Alto) was used to generate the subtractive library between 50 ng/ml E_2-treated (tester) and control MØ monolayers (driver), following the manufacturer's instructions. In brief, both cDNA populations were digested with RsaI and specific adaptors were ligated to the tester before the tester and driver were hybridized to exclude common sequences; the resultant cDNA was subjected to two rounds of PCR for the selective amplification of differentially expressed sequences using adaptor specific primers. A fraction of the adaptor ligated tester cDNA was not hybridized to serve as unsubtracted control. Subtracted and unsubtracted secondary PCR products samples were run on 2% agarose gel to check for differential band patterns. To isolate the differentially expressed sequences, 0.5 ml of the subtracted secondary PCR product were directly cloned into pGEM-T Easy vector (Promega, Madison, USA) and transfected into DH5α bacteria.

2.5. Sequencing and clustering

Colonies were randomly picked and cloned. Inserts were amplified by PCR using the T7 primer pair in 96 well plates and after amplification check on a 2% agarose gel, PCR products were sent for purification and direct sequencing to Macrogen Inc. (Seoul, Korea). After removal of the vector and adaptor sequences, the resultant sequence tags (ESTs) were clustered using UCLUST software (http://www.drive5.com/) with default parameters (Edgar, 2010). Clustered ESTs were submitted to contiguous sequence (contig) assembly using a web interface of the CAP3 software (http://pbil.univ-lyon1.fr/cap3.php) (Huang and Madan, 1999) and redundancy within the library was calculated as the ratio between sequences belonging to a contig and the total number of sequences considered.

2.6. Homology search and annotation

All the unigenes generated in the assembly process, whether singlets or contigs, were annotated by querying against SP, TR, NR, NCBI non-human non-mouse ESTs (EST_others) and NT databases using the BLAST algorithm (BLASTX and BLASTN) (Altschul et al., 1997) and Blaststation software (TM Software Inc., USA).

For each putative transcript the ten most similar entries were recorded. The assignment of putative transcripts required an *e*-value lower than $1e^{-10}$ for protein database entries and $1e^{-40}$ for nucleotide database entries. Sequences failing to match the SP database entries were successively searched with the TR and NR databases. Sequences that failed to match entries in any of the protein databases were then searched with the EST_others

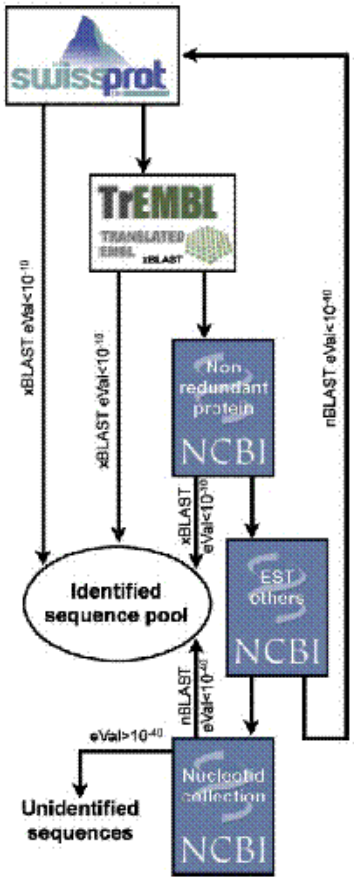

Fig. 1. Principal steps followed during the BLAST search process. Cut off e-values for protein (BLASTX) and nucleotide (BLASTN) databases were set at $1e^{-10}$ and $1e^{-40}$, respectively. Sequences failing to match the Swiss-Prot (SP) database were successively queried in the TrEMBL (TR) and NCBI non-redundant protein (NR) databases. Sequences not matching entries in any of the protein databases were queried in the NCBI non-human non-mouse ESTs (EST_others) database. Retrieved gilthead seabream ESTs were successively queried in SP, TR and NR databases. Sequences failing to match entries in the protein databases were queried to the NCBI nucleotide collection (NT) database.

database; sequences corresponding to matches of previously known gilthead seabream ESTs were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/), clustered and successively queried in the SP, TR and NR databases. Sequences failing to match entries in the protein databases were then searched with the NT database (Fig. 1).

Once the corresponding orthologs had been determined, genes were annotated as putative housekeeping genes or ERG by searching in lists of genes showing constitutive expression or responsiveness to estrogen treatments (Eisenberg and Levanon, 2003; Hsiao et al., 2001; Tang et al., 2007). Genes were classified and mapped according to GO consortium rules and KEGG (Ashburner et al., 2000; Kanehisa et al., 2006). Functional analysis in terms of GO and KEGG annotations was based on the Genecodis software using those unigenes matching known genes with existing human orthologs. Using individual reference Uniprot gene names, the Genecodis software (http://genecodis.dacya.ucm.es/) determines biological annotations and combinations of annotations that are significantly associated in a list of genes (Carmona-Saez et al., 2007; Nogales-Cadenas et al., 2009).

Table 1
Summary of the BLAST results obtained for the 367 isolated ESTs.

BLAST result	Number of hits	Percentage
Unidentified ESTs	205	56
Identified ESTs	162	44
Unamed protein/EST	4	1
Known genes	158	43
Redundant	3	0.8
Housekeeping genes	9	2.5
Estrogen responsive	1	0.25

2.7. Real-time PCR analysis

Quantitative real-time PCR experiments were carried out to test the ability of E_2 to alter the macrophage gene expression profile at low doses as well as to validate some of the genes isolated by SSH and characterized by GO. Among the whole dataset of sequences, one reported housekeeping gene and four sequences belonging to key immune relevant markers were selected: *actb*, *mcsfr*, *mmp13*, *mhc1a*, *marco* and *nlrc3*. Specific primer pairs for each of the sequences were designed (Table 3). The qRT-PCR was performed using a SuperScript III ReverseTranscriptase Kit (Invitrogen) to synthesize first strand cDNA from 1 μg of total RNA, and SYBR Green PCR Core Reagents (Applied Biosystems) with an ABI PRISM 7500 instrument (Applied Biosystems) according to the respective manuals. For each mRNA, gene expression was normalized to the ribosomal protein S18 (*rps18*) content of each sample using the comparative Cq method ($2^{-\Delta\Delta Cq}$). In all cases, each PCR was performed in triplicate. Less than 2% variation in the *rps18* gene expression was observed between samples.

2.8. Statistical analysis

qRT-PCR results from selected candidate genes were analyzed by analysis of variance (ANOVA) and a Waller–Duncan multiple range test to determine differences between groups ($p \leq 0.05$), using the SPSS software package (SPSS Inc., Chicago, IL).

3. Results

3.1. Subtractive library expressed sequence tags statistics

A SSH library was constructed to isolate and identify mRNA species up-regulated by a supraphysiological dose of E_2 (50 ng/ml) in gilthead seabream MØ cultures. A total of 393 sequences were obtained by cloning randomly selected colonies, with an average length of 450 base pairs (bp), ranging between 56 and 935 bp.

After clustering and assembling, the library was arranged into 20 contigs and 347 singletons (11.7% redundancy). The number of EST per contig ranged from 2 to 5, with a median of 2. The length of the contigs ranged from 285 to 762 bp, with an average length of 461 bp. All sequences were submitted to the expressed sequence tag database (dbEST) at the European Bioinformatics Institute (EBI) with consecutive accession numbers from FR719326 to FR719692.

3.2. Gene annotation

Out of 367 putative unigenes, 162 (44%) were identified as orthologs to known genes or ESTs, while 205 (56%) could not be identified by similarity searches in the databases (Table 1).

Each of the 367 putative unigenes was compared sequentially against Swiss-Prot (SP), TrEMBL (TR) and NCBI non-redundant protein (NR) databases. Of these, 160 (43.6%) matched at least one significantly similar sequence. The remaining 207 sequences that matched no protein sequence were then compared against the NCBI nucleotide collection (NT) database, identifying two more

Table 2
Summary of unique enriched GO terms found by computational analysis ($p < 0.05$).

GO term	Term description	N	GO term	Term description	N
Cellular component					
GO:0000502	Proteasome complex	4	GO:0005885	Arp2/3 protein complex	3
GO:0005634	Nucleus	39	GO:0015629	Actin cytoskeleton	4
GO:0005737	Cytoplasm	58	GO:0016020	Membrane	33
GO:0005739	Mitochondrion	23	GO:0016459	Myosin complex	3
GO:0005743	Mitochondrial inner membrane	6	GO:0016469	Proton-transporting two-sector ATPase complex	2
GO:0005759	Mitochondrial matrix	7	GO:0019773	Proteasome core complex, alpha-subunit complex	3
GO:0005764	Lysosome	4	GO:0030863	Cortical cytoskeleton	2
GO:0005783	Endoplasmic reticulum	13	GO:0032587	Ruffle membrane	2
GO:0005829	Cytosol	25	GO:0042470	Melanosome	5
GO:0005839	Proteasome core complex	2	GO:0044451	Nucleoplasm part	1
GO:0005840	Ribosome	5	GO:0070469	Respiratory chain	3
Molecular function					
GO:0000049	tRNA binding	2	GO:0005515	Protein binding	73
GO:0000166	Nucleotide binding	19	GO:0008233	Peptidase activity	11
GO:0000386	Second spliceosomal transesterification activity	1	GO:0008553	Hydrogen-exporting ATPase activity	2
GO:0003723	RNA binding	9	GO:0015207	Adenine transmembrane transporter activity	1
GO:0003735	Structural constituent of ribosome	4	GO:0016491	Oxidoreductase activity	8
GO:0003779	Actin binding	7	GO:0016656	Monodehydroascorbate reductase (NADH) activity	1
GO:0003924	GTPase activity	5	GO:0016805	Dipeptidase activity	2
GO:0003954	NADH dehydrogenase activity	2	GO:0016937	Short-branched-chain-acyl-CoA dehydrogenase activity	1
GO:0004163	Diphosphomevalonate decarboxylase activity	1	GO:0019002	GMP binding	1
GO:0004197	Cysteine-type endopeptidase activity	3	GO:0019899	Enzyme binding	4
GO:0004298	Threonine-type endopeptidase activity	4	GO:0030348	Syntaxin-3 binding	1
GO:0004364	Glutathione transferase activity	2	GO:0030628	Pre-mRNA 3'-splice site binding	1
GO:0004365	Glyceraldehyde-3-phosphate dehydrogenase (phosphorilating activity)	1	GO:0030791	Arsenite methyltransferase activity	1
GO:0004555	Alpha,alpha-trehalase activity	1	GO:0030792	Methylarsonite methyltransferase activity	1
GO:0004818	Glutamate–tRNA ligase activity	1	GO:0034450	Ubiquitin–ubiquitin ligase activity	2
GO:0004824	Lysine–tRNA ligase activity	1	GO:0042586	Peptide deformylase activity	1
GO:0005011	Macrophage colony stimulating factor receptor activity	1	GO:0042802	Identical protein binding	7
GO:0005200	Structural constituent of cytoskeleton	4	GO:0046961	Proton-transporting ATPase activity, rotational mechanism	3
GO:0005506	Iron ion binding	3	GO:0051082	Unfolded protein binding	4
Biological process					
GO:0005991	Trehalose metabolic process	1	GO:0019747	Regulation of isoprenoid metabolic process	1
GO:0005993	Trehalose catabolic process	1	GO:0022900	Electron transport chain	4
GO:0006364	rRNA processing	4	GO:0030574	Collagen catabolic process	3
GO:0006414	Translational elongation	4	GO:0030833	Regulation of actin filament polymerization	2
GO:0006430	Lysyl–tRNA aminoacylation	1	GO:0030845	Inhibition of phospholipase C activity involved in G-protein coupled	1
GO:0006457	Protein folding	5	GO:0031145	Anaphase-promoting complex-dependent proteasomal ubiquitin-dependent	6
GO:0006508	Proteolysis	9	GO:0032366	Intracellular sterol transport	1
GO:0006511	Ubiquitin-dependent protein catabolic process	4	GO:0032872	Regulation of stress-activated MAPK cascade	1
GO:0006608	snRNP protein import into nucleus	1	GO:0033157	Regulation of intracellular protein transport	1
GO:0006892	Post-Golgi vesicle-mediated transport	3	GO:0035166	Post-embryonic hemopoiesis	1
GO:0006928	Cellular component movement	5	GO:0042069	Regulation of catecholamine metabolic process	1
GO:0008039	Synaptic target recognition	1	GO:0051436	Negative regulation of ubiquitin–protein ligase activity during mitosis	6
GO:0009399	Nitrogen fixation	1	GO:0051437	Positive regulation of ubiquitin–protein ligase activity during mitosis	6
GO:0010920	Negative regulation of inositol phosphate biosynthetic process	1	GO:0051603	Proteolysis involved in cellular protein catabolic process	4
GO:0010925	Positive regulation of inositol-polyphosphate 5-phosphatase activity	1	GO:0055114	Oxidation reduction	9
GO:0015868	Purine ribonucleotide transport	1	GO:0060305	Regulation of cell diameter	1
GO:0016192	Vesicle-mediated transport	6	GO:0070560	Protein secretion by platelet	1
GO:0018872	Arsonoacetate metabolic process	1			

N, number of genes per term association.

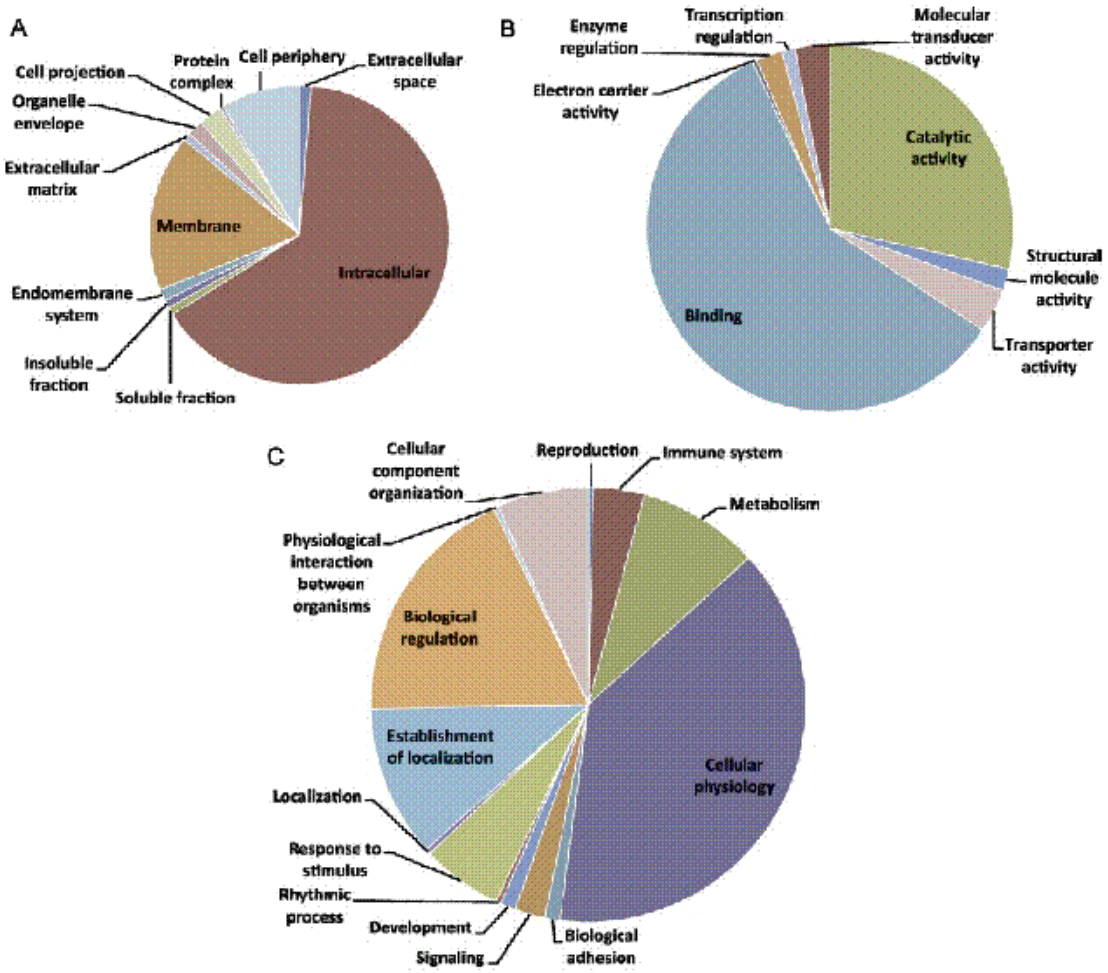

Fig. 2. Gene Ontology terms distribution in the cellular components (A), molecular functions (B), and biological processes (C) vocabularies. In (A) intracellular (70%) and membrane (19%) were the most represented cellular compartments. In (B) the most represented categories was binding (59%), followed by catalytic activities (29%). In (C) cellular physiology (39%), biological regulation (18%) and establishment of localization (11%) were the most represented processes. GO subcategories immune system process (4%), response to stimulus (6%) and physiological interaction between organisms (0.5%) are probably involved in the interaction of immune cells with pathogens or danger signals. It should be emphasised that the second most represented category, biological regulation, could contain genes whose functions may alter the cellular physiology, thus affecting the ability of gilthead seabream macrophages to respond to an immunological challenge.

additional sequences (0.5%) that significantly matched at least one similar EST (Fig. 1).

Of the 162 unigenes identified, 158 (43%) matched known gene products, while 4 (1%) matched proteins or ESTs for which no annotation is yet available. Surprisingly, only 3 unigenes corresponding to non-overlapping regions of three different genes were found. Detailed annotations and descriptions are available in Additional file 1.

Around 57.5% of the BLAST highest homology hits in the library were homologs with coding sequences present in placental mammal genome (17% human, 16% mouse, 9% rat), while the second homologous group was teleost fish with approximately 31.5% of hits (13% zebrafish). Other metazoan groups were also represented: birds (5%), amphibians (4%), monotrema (0.5%), elasmobranches (0.5%) and arthropods (1%) (Additional file 2). Searches in reference databases identified 9 sequences of the housekeeping genes β-actin (*actb*), ADP-ribosylation factor 1 (*arf1*), actin-related protein 2/3 complex subunits 3 and 4 (*arpc3*, *arpc4*), dolichyl-diphosphooligosaccharide-protein glycosyltransferase subunit DAD1 (*dad1*), isocitrate dehydrogenase subunit beta (*idh3b*), peroxiredoxin-1 gene (*prdx1*), 26S proteasome regulatory subunit 8 (*psmd8*) and WD repeat-containing protein 1 gene (*wdr1*). One sequence, previously known as estrogen responsive gene (ERG), was identified as the matrix metalloproteinase 13 (*mmp13*).

3.3. Functional annotation analysis

EST unigenes matching known gene products were classified according to biological process, molecular function and cellular component, according to Gene Ontology (GO) consortium rules. A total of 368 different GO terms were assigned. Fig. 2 shows a more detailed assignment of sequences corresponding to the third (biological process [BP] and molecular function [MF]) and fourth (cellular component [CC]) hierarchical levels of the associated GO functional categories. According to CC terms, most of the annotated genes were internal to cell (70%) or related to the membrane (19%). MF terms were mainly represented by binding and catalytic activity with 59% and 29% of hits, respectively. Of the BP terms, a significant proportion of the genes were involved in cell physiology (39%), regulation (18%) or the establishment of localization (11%).

Table 3
Gene accession numbers and primer sequences used for gene expression analysis.

Gene	Accession number	Name	Sequence (5′–3′)
actb	FR719326	F	ATCGTGGGGCGCCCCAGGCACC
		R	CTCCTTAATGTCACGCACGATTTC
mcsfr2	FR719584	F3	CTGCCCTACAATGACAAG
		R4	TCAGACATCAGAGCTTCC
mmp13	FR719337	F	CGGTGATTCCTACCCATTTG
		R	TGAGCGGAAAGTGAAGGTCT
hmr1	FR719646	F	AGCTGTTCAGTGTCCCATCC
		R	GACCCACAGTCAGACCCAGT
marco	FR719667	F	CTGGGCTACACCAGAGCTTC
		R	TTCCAGAATTAGGGCAGTCG
nlrc3	FR719637	F	GGCAGCTCTCCTCTCCTTCT
		R	CAGCAGAATCATCTCCCACA

Computational analysis allowed the detection of significantly enriched GO terms within the whole category set. MF related terms were seen to be mainly enriched for protein binding, with 73 genes involved in that category. Enrichment analysis for CC established a more detailed relationship of genes mainly related to the cytoplasm (58 genes), the nucleus (39 genes) and the plasma membrane (33). It should be noted the presence of up to three enriched categories related with the proteasome, involving 5 different genes. For BP enriched terms, the most represented categories were oxidation–reduction and proteolysis with 9 genes, and the regulation of ubiquitin–protein ligase activity with 12 genes divided into positive/negative regulation categories (Table 2).

Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology is a pathway-based classification of orthologous gene groups. Computational enrichment analysis also provided by Genecodis showed that while several KEGG pathways involving different aspects of the cellular physiology were enriched, a high number of pathways can be directly related to the immune processes, with 5 out of 18 enriched pathways (Fig. 3). Moreover, while the remaining cannot be qualified as immune pathways, many of them are probably involved in the immune response.

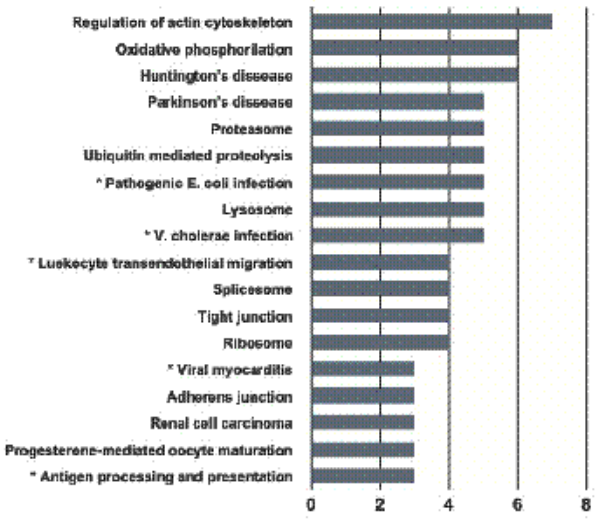

Fig. 3. Classification of enriched pathways ($p < 0.05$) based on the Kyoto Encyclopedia of Genes and Genomics according to the genes reported in the subtractive hybridization library belonging to each pathway. Note the high proportion of enriched pathways on known immune processes (5 out of 18), while the remaining pathways, although not directly related to the immune system are probably to occur during the immune response. (*) Known immune pathways.

3.4. RT-PCR analysis of selected candidate genes

Quantitative real-time PCR (qRT-PCR) experiments were performed to validate the SSH results. For that purpose, one gene identified as a housekeeping gene and five immunologically relevant genes were amplified using specific primers. The expression patterns of β-actin (*actb*), metalloproteinase 13 (*mmp13*), macrophage colony-stimulating factor receptor 2 (*mcsfr2*), MHC class I alpha antigen (*mhc1a*), macrophage receptor with collagenous structure (*marco*) and NOD-like receptor *c*3 (*nlrc3*) were clearly induced upon 50 ng/ml E_2 treatment (Fig. 4), validating the results obtained by SSH. Moreover, a physiological dose of E_2 (5 ng/ml, Chaves-Pozo et al., 2008b) was also found to be able to modulate the expression of all the selected genes (Fig. 4), confirming the experimental approach used in this study and the biological relevance of the genes identified by SSH.

4. Discussion

A functional genomics approach was followed to examine the response of gilthead seabream MØ to E_2 treatment *in vitro*, in order to identify genes involved in the modulation of MØ functions. This showed the ability of this estrogen to orchestrate profound alterations in the MØ transcriptome, alterations that may affect its whole physiology, especially immune-related processes and pathways.

Previous functional studies suggest a pro-inflammatory role for E_2 in gilthead seabream MØ. Thus, E_2 is able to increase the expression of cytokine, chemokine and toll like receptor (TLR) genes *in vitro* in both naïve and VaDNA-stimulated MØ (Liarte et al., 2011). These results contrast with observations made in mammals, where E_2 exhibits immunosuppressive activities (Härkönen and Väänänen, 2006). However, it is not surprising that some differences may exist between mammals and the gilthead seabream. For example, this teleost species shows a physiological inflammatory process in the testis partially triggered by E_2 (Chaves-Pozo et al., 2007) not present in mammals.

SSH is a powerful method for large-scale gene transcription analysis, and has been used to investigate various aspects of fish biology (reviewed in Larkin et al., 2003) as well to identify genes involved in the response of several fish species to pathogens, toxicants, hormones and endocrine disruptors (Bayne et al., 2001; Brown et al., 2004; Chapman et al., 2004; Moens et al., 2007; Pinto et al., 2006; Prieto-Alamo et al., 2009; Tsoi et al., 2004; Wang and Wu, 2007; Xia and Yue, 2010). After analysis of the library, 363 unique transcripts were obtained, of which 162 were similar to 155 known genes and 4 unannotated ESTs available in public databases. The remaining 205 unique transcripts may represent novel sequences or UTRs of known genes. Some of these genes might play important roles in reproductive–immune interactions or in the innate immune response of fish. Future studies focusing on their functional characterization would improve our understanding of the effects of estrogenic factors on immune cell physiology and perhaps provide suitable biomarkers to assess the exposure to endocrine disruptors affecting the immune system. It is not surprising that most of the differentially expressed genes identified have orthologs in mammalian species, while only 2 matched previously know gilthead seabream sequences, demonstrating the little knowledge accumulated on fish immune transcriptomics compared with human or mammalian models.

Expression analysis of selected E_2-enriched candidate genes by qRT-PCR further confirmed the up-regulation of all the selected genes and validated the SSH results presented here. Searches in reference databases retrieved 9 hits as housekeeping genes (*actb*, *arf1*, *arpc3*, *arpc4*, *dad1*, *idh3b*, *prdx1*, *psmd8*, *wdr1*) and one hit as

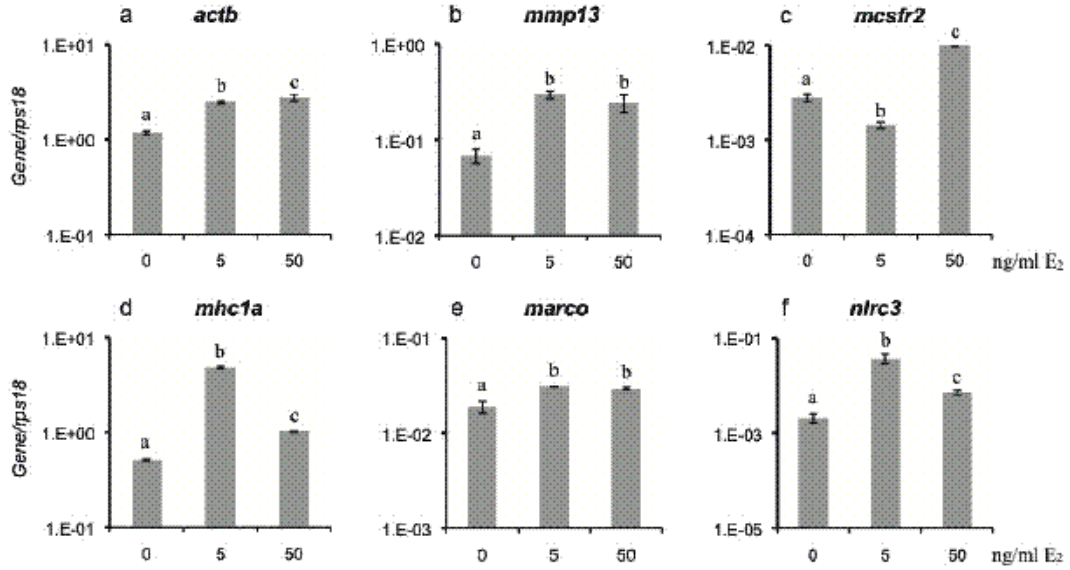

Fig. 4. Quantitative real-time PCR analyses of selected candidate genes identified in the subtractive hybridization library: (a) β-actin (*actb*), (b) metalloproteinase 13 (*mmp13*), (c) macrophage colony-stimulating factor receptor 2 (*mcsfr2*), (d) MHC class I alpha antigen (*mhc1a*), (e) macrophage receptor with collagenous structure (*marco*), and (f) NOD-like receptor c3 (*nlrc3*). Data represent means ± SEM of triplicate samples. Different letters denote statistically significant differences between the groups according to a Waller–Duncan test.

ERG (*mmp13*). The discovery in MØ of housekeeping genes that are responsive to estrogens make those markers unsuitable to be used as reference genes in future functional genomics assays (e.g. microarrays for E_2-treated samples). Furthermore, results regarding ERG are quite surprising since previous SSH analysis of gilthead seabream males exposed to E_2 revealed the presence of multiple differentially expressed ERG (Pinto et al., 2006), however, these differences might be explained by the different nature of the samples used; that is, testis as opposed to MØ.

Available bioinformatic databases such as GO and KEGG provide useful information for analyzing the functional profile of annotated genes. GO annotation retrieved 368 terms, which, following computational analysis, allowed the establishment of 95 enriched terms (22 CC, 38 MF, 35 BP). Enrichment analysis for CC and MF showed that the main enriched terms for these categories were "cytoplasm" and "nucleus" together with "protein binding", which is in accordance with the existence of a very complex regulation of intracellular networks to control the pro-inflammatory processes triggered by E_2. BP annotation and enrichment showed the dysregulation of a variety of processes, affecting several fields of the cell physiology. Genes in the GO subcategories "immune system process", "response to stimulus" and "physiological interaction between organisms" are likely to be involved in the interaction of immune cells with immune stimulus. The number of identified genes within these categories was relatively low (Xia and Yue, 2010). However, other well-represented subcategories such as "biological regulation" could contain genes whose functions may influence the behavior of MØ and thus affecting their ability to respond to an immunological challenge upon estrogens exposure. KEGG functional pathway analysis also revealed the enrichment of a variety of processes of MØ physiology represented mostly by immune pathways. However, as was the case with GO terms, some of the remaining enriched pathways very likely take place during the events following immune challenge or in the naïve immune cell physiology. This further supports a role for E_2 in the regulation of the gilthead seabream MØ physiology and in the orchestration of an immune response.

As previously mentioned, E_2 treatments have been shown to have a pro-inflammatory effect both in naïve and VaDNA-stimulated gilthead seabream MØ, through modulation of the expression of cytokines, chemokines and pattern recognition receptors (PRR) (Liarte et al., 2011). The nucleotide-binding domain leucine-rich repeat-containing (NLR) family of intracellular sensors is a critical component of the innate immune system. Among PRR, TLR and NLR are capable of recognizing highly conserved markers that are specific to microbes (PAMP) as well as danger-associated molecular patterns (DAMP) released in conditions of cellular damage or stress. Considered a basal member of the NLR family, *nlrc3* has been detected in both myeloid and lymphoid mammalian cell lines as well as in zebrafish and channel catfish leukocytes. Moreover, upon challenge, *nlrc3* has been shown to promote the down-regulation of NF-κB activation by inhibiting the degradation of the NF-κB inhibitor IκBα (Chen et al., 2009; Conti et al., 2005; Sha et al., 2009). Upon PAMP or DAMP recognition, conformational changes and/or PRR translocation activate the downstream signaling, that leads to the formation of the inflammasome and the release of pro-inflammatory cytokines (Pedra et al., 2009; Trinchieri and Sher, 2007). Recently, the chaperone heat-shock protein 90 (*hsp90b*) has been shown to play a role in NLR function and inflammasome formation by mediating conformational changes (Pedra et al., 2009). Interestingly, *hsp90b* seems to be necessary for the proper assembly and functioning of steroid receptor complexes (Taherian et al., 2008). The pro-inflammatory role of E_2 treatments on gilthead seabream MØ is further supported by the identification of both *nlrc3* and *hsp90b* genes in our library, since they would probably participate in the establishment of the inflammasome and the development of an inflammatory state.

The macrophage receptor with collagenous structure (*marco*) represents another important element, since expression of this scavenger receptor, which is able to exert PRR activities, is induced upon lipopolysaccharide (LPS) challenge or exposure to the macrophage colony-stimulating factor (MCSF) of myeloid-derived cell lines and mammalian MØ, thereby participating in the profound cell rearrangement that takes place upon cellular activation

(Chen et al., 2010; Granucci et al., 2003). MCSF regulates the survival, proliferation and differentiation of mononuclear phagocytic cells via MCSFR, which have been shown to be specifically expressed by purified MØ in the gilthead seabream (Roca et al., 2006). The up-regulation of *mcsfr2* by E_2 treatment would thus contribute to the consolidation of an inflammatory state in the gilthead seabream MØ.

Expansion of the immune cellular response leads to expression of the acute-phase proteins, among which, together with cytokines and chemokines, antimicrobial peptides (AMPs) and ion homeostasis proteins (IHPs) are the most representative. The ability of E_2 treatments to promote cytokine and chemokine expression has already been demonstrated (Liarte et al., 2011), while here we show that E_2 treatment also induces the expression of AMPs and IHPs. Genes that encode for AMPs (*bpi*, *perf*) and proteins that are involved in ion homeostasis (*frinl*) are commonly responsive in fish following stimulation with stressors or bacterial challenge (Chang et al., 2005; Feng et al., 2009; Goetz et al., 2004; Maier et al., 2008; Prieto-Alamo et al., 2009). While AMPs lyse bacterial cells by disrupting their cellular walls, IHP reduce the availability of ions that are essential for microbe development. The resulting accumulation of free intracellular iron is toxic, as it reacts with oxygen and creates H_2O_2 as a byproduct (Theil, 2003). Although in such situations ferritin captures free iron, thus limiting cell damage, the up-regulation of other anti-oxidative pathways is a common response to the oxidative stress generated. We also found that E_2 up-regulates guanine nucleotide-binding proteins (*gbg10*), gluthatione transferases (*gsta*, *gsto1*) and NADH-ubiquinone oxidoreductases (*ndua9*, *ndub8*), all of them endogenous antioxidants that protect intracellular membranes from lipid peroxidation and are up-regulated following environmental stress or immunological challenge (Colombo et al., 2006; Li and Brouwer, 2009; Prieto-Alamo et al., 2009). Despite the anti-oxidative system described, the peroxides generated can cause DNA damage and ultimately lead to cell death (Bertram and Hass, 2008). B-cell lymphoma family members (*bcl7b*) provide anti-apoptotic regulation as well as tumor suppressing activity by protecting DNA from oxidative damage (Kratz et al., 2006; Morton et al., 2009; Potter et al., 2008). Moreover, 14-3-3 protein family members (*143b1*) positively regulate proteins with survival promoting functions, such as telomerase, and negatively regulate the proteins, including p53 and p27, that induce cell apoptosis and senescence (Tzivion et al., 2006). Also related to the immune response, 14-3-3 proteins have been shown to collaborate in the events following activation of the tumor necrosis factor α (TNF-α)/NF-κB signal transduction pathway (Bouwmeester et al., 2004), even though recent work by Medina and Ghahary (2010) also demonstrated that exosomes containing 14-3-3 *beta* are released by transdifferentiating monocytes, triggering the expression of matrix metalloproteinases by the surrounding cells. The accumulated evidence in fish regarding 14-3-3 protein also demonstrates its important role during stress responses: its expression is up-regulated following handling stress in the rainbow trout (Koskinen et al., 2004; Fiol and Kültz, 2007; Kültz et al., 2001). The identification in our SSH library of elements related with all the processes described further demonstrates the ability of E_2 to promote an inflammatory state and thus an oxidative stress in gilthead seabream MØ. Moreover, although the presence of 14-3-3 members also suggests a pro-survival effect of E_2 on MØ, the involvement of MØ expressed factors like *143b1* in the infiltration of leukocytes and the remodeling processes can not be discarded if we bear in mind the physiological inflammatory process that takes place in the gilthead seabream gonad during and after the spawning stages. In addition, the presence in our library of two matrix metalloproteinase genes (*mmp8*, *mmp13*), which have previously been reported to be involved in these seasonal processes and to be up-regulated by DAMP signals (collagen proteolytic fragments) in the gilthead seabream (Castillo-Briceño et al., 2009; Chaves-Pozo et al., 2008c), also confirms the pro-inflammatory activity of E_2.

The expression of ubiquitin (*ubiq*), ubiquitin transferases (*ube4b*, *rn121*) and 26S proteasomal subunits (*psmd8*) is up-regulated upon viral infection or viral challenge both in humans and fish (Dios et al., 2007; Feng et al., 2009; Huang et al., 2006; Rise et al., 2008) by the triggering of interferon (IFN) signaling pathways, which results in improved processing of major histocompatibility complex class I antigens (*mhc1a*, *b2m*) as a result of the adaptation of the ubiquitin/26S proteasome system proteolytic properties to the requirements of the immune system (Krüger et al., 2003; Kloetzel, 2004; Kloetzel and Ossendorp, 2004). The conjugation of small ubiquitin-related modifiers (*sumo2*) has also been found to play a role in the signaling events that take place after viral challenge. In contrast to ubiquitination, SUMOylation, which depends on ubiquitin-conjugating enzyme 9 (*ubc9*), is a reversible post-translational modification that regulates the function of labeled proteins rather than targeting them to the proteasome, seemingly to be crucial for IFN-I signaling (Chang et al., 2009; Fu et al., 2011; Kubota et al., 2008). Moreover, the ability of the *ubc9*/*sumo2* system to regulate IκBα and NF-κB interactions, thus affecting the humoral response, is well established (Anckar and Sistonen, 2007; Chiu et al., 2005; Saltzman et al., 1998; Tashiro et al., 1997). The ubiquitin/26S proteasome system and SUMOylation have also been found to play important roles in ER physiology since the activity of the 26S proteasome is essential for the turnover of ERα and its cofactors (Lonard et al., 2000). Moreover, SUMOylation of the co-repressors that interact with ERs increases their activity, while direct SUMOylation of ERα has also been reported (Gill, 2005; Rytinki and Palvimo, 2008; Sentis et al., 2005). Interestingly, ER expression has been detected in human monocytes and B lymphocytes in a similar fashion to gilthead seabream, in which ERs are expressed by MØ and lymphocytes (Liarte et al., 2011; Nalbandian and Kovats, 2005). Surprisingly, the expression of ERα by dendritic cells (DC) seems to be crucial for their differentiation, while E_2 treatment on activated DC is able to increase IFN production (Douin-Echinard et al., 2008; Siracusa et al., 2008), suggesting the existence of shared estrogenic regulation mechanisms between gilthead seabream MØ and mammalian DC.

Immune cells are known to develop complex mechanisms to assess the magnitude of their responses, and even slight alterations in signal strengths result in different cellular outcomes. Taking into account all the above and the fact that gilthead seabream MØ have been show to drastically up-regulate the expression of ERs following immune challenge, increasing their sensitivity to E_2 during the development of the immune response (Liarte et al., 2011), the idea that E_2 may play a critical role in the regulation of important immune processes like the onset of the inflammasome or the presentation of antigens through the direct regulation of NF-κB activity is quite tempting. Further research into this possibility will elucidate the importance of this regulation.

In conclusion, our study shows that E_2 is able to regulate the expression of many genes associated with both cellular and immune physiology in gilthead seabream MØ. Although qRT-PCR confirmation analysis was limited to a small subset of genes, the great consistency achieved in these analyses lead us to conclude that most of the identified genes should behave similarly. We have also identified NF-κB as a common element up-stream in the molecular pathways, as revealed by the presence of the identified genes, GO and KEGG enrichment. This conclusion, which agrees with existing evidence, and further substantiates the involvement of E_2 in the innate immunity of the gilthead seabream, in a model that is of great interest for studying the modulation of immunity by hormones. For this, the exact mechanism of the interaction between E_2 and NF-κB should be addressed. Finally, further work with the genes seen to be up-regulated by E_2 may identify suit-

able markers for assessing innate immune alterations following exposure to estrogenic endocrine disruptors, which are widely distributed in the environment.

Competing interests

The authors have declared that no competing interests exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2011.03.015.

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CAPÍTULO II: RESUMEN EN CASTELLANO

1 INTRODUCCIÓN

En un mundo poblado por, aproximadamente, 7000 millones de personas, la demanda de alimentos de origen marino, en concreto pescado, ha elevado la presión pesquera sobre los caladeros hasta el punto de poner en peligro su capacidad de renovación. La pesca extractiva difícilmente cubre el 70% del volumen pesquero total anual [FAO 2006], situación ante la cuál la acuicultura se perfila como la única posibilidad de cubrir la demanda en un futuro próximo. En España, situada en el puesto 14 del ranking mundial de productores acuícolas y en el puesto 7 del ranking mundial de exportadores de pescado, la acuicultura representa el 3% de la producción mundial y el 25% de la europea, perfilándose como un área de actividad económica de gran relevancia estratégica.

La práctica de la acuicultura supone el confinamiento de un gran número de ejemplares en espacios limitados y su sometimiento a una manipulación más o menos periódica, desencadenándose situaciones de estrés en los animales que afectan tanto a los ejemplares adultos coma a larvas y juveniles. Estas condiciones favorecen el desarrollo de enfermedades infecciosas que son responsables de cuantiosas pérdidas económicas. Por ello, el conocimiento del sistema inmunitario de los peces, en general, y de las especies objeto de cultivo, en particular, se ha convertido en uno de los objetivos primarios en la investigación acuícola.

Por otra parte, los peces representan 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. El predominio de la respuesta inmunitaria innata en peces frente al predominio de la respuesta adaptativa en vertebrados superiores es la más importante de estas diferencias [Anderson 1992].

El grupo de investigación "Sistema Inmunitario Innato de Peces Teleósteos" en el que se ha desarrollado la presente Tesis Doctoral, tiene una larga trayectoria en el estudio del sistema inmunitario de especies mediterráneas de interés acuícola, tales como la dorada (*Sparus aurata* L.) y la lubina (*Dicentrarchus labrax* L.),

especies que son de gran interés comercial para la economía de la Comunidad Autónoma de la Región de Murcia así como para la economía nacional.

Ubicada en el marco de referencia expuesto, la presente Tesis Doctoral se centra en el estudio del sistema inmunitario de dorada, con especial interés en la comprensión de las relaciones existentes entre los sistemas inmunitario y reproductor y en como las hormonas sexuales producidas por éste último pueden afectar el desarrollo de los procesos inmunitarios.

1.1 EL SISTEMA INMUNITARIO

La respuesta inmunitaria constituye un mecanismo de defensa frente a agentes (p.e. virus, bacterias, hongos, protozoos y parásitos multicelulares) o sustancias (p.e. ácidos nucleicos, proteínas y polisacáridos) que resultan ajenas al organismo, sin implicar la consecuencia patológica de tal reacción [Abbas y col. 2001]. El sistema inmunitario de mamíferos está formado por células y moléculas capaces de generar una gran variedad de respuestas inmunitarias acordes con la diversidad de agentes infecciosos y, en general, la respuesta inmunitaria se puede dividir en dos ramas: innata (natural o inespecífica) y adaptativa (adquirida o específica) [Male y Roitt 1996].

La respuesta innata incluye barreras físicas, células fagocíticas, células citotóxicas naturales y varias moléculas de la sangre (complemento y proteínas de la fase aguda, etc.) que actúan como una primera defensa ante infecciones o daños [Male y Roitt 1996; Abbas y col. 2001; Mollen y col. 2006]. La respuesta adaptativa incluye a linfocitos y los anticuerpos que secretan y aparece exclusivamente en los vertebrados [Abbas y col. 2001]. Los linfocitos son capaces de reconocer patógenos individuales, para lo que existen dos categorías principales de células: los linfocitos B (células B) y los linfocitos T (células T). Las células B se encargan de reconocer y combatir patógenos extracelulares y sus productos mediante anticuerpos que se unen específicamente a una molécula diana o antígeno mientras que las células T realizan actividades complementarias como la regulación de la producción de anticuerpos o la destrucción de patógenos fagocitados.

1.1.1 El sistema inmunitario en peces teleósteos

En peces teleósteos, el sistema inmunitario muestra características similares al de aves y mamíferos, con respuestas celulares y humorales que poseen las características de especificidad y memoria [Van Muiswinkel 1995]. Su respuesta innata consta de barreras físicas (epitelios y mucosas), efectores celulares (células fagocíticas y citotóxicas no específicas) y factores humorales (complemento y otras proteínas de la fase aguda) y su respuesta adaptativa engloba un componente celular (linfocitos) y otro humoral (anticuerpos). Sin embargo, pese a sus semejanzas con el sistema inmunitario de otros vertebrados, existen diferencias claras como por ejemplo que los peces dependen en mayor grado de los mecanismos de defensa innatos, principalmente, en condiciones ambientales de baja temperatura (los peces son animales poiquilotermos) ya que la respuesta inmunitaria adaptativa es dependiente de la temperatura [Cuchens y Clem 1977; Avtalion 1981; Abruzzini y col. 1982; Clem y col. 1984, 1991; Miller y Clem 1984].

Los órganos y tejidos del sistema inmunitario en teleósteos se han clasificado, como en mamíferos, en órganos primarios y secundarios [Zapata y col. 1996]. Entre los órganos primarios, el riñón constituye su órgano hematopoyético por excelencia y consta de dos partes: la anterior o cefálica, con función fundamentalmente hematopoyética, y la posterior, con función básicamente excretora. El bazo es el órgano linfoide secundario más importante aunque presenta pocos linfocitos que pueden incrementar en número mediante la administración de un antígeno [Roca 2009].

Después de las escamas, la piel y el mucus actúan como la primera barrera defensiva. La respuesta celular innata de los peces incluye una gran variedad de leucocitos, entre ellos se incluyen los fagocitos (monocitos/macrófagos y granulocitos) y las células citotóxicas no específicas [Secombes 1996]. Los fagocitos tienen una mayor importancia en la inmunidad innata por su capacidad de eliminar virus, bacterias y parásitos [Rowley y col. 1988; Secombes y Fletcher 1992; Sepulcre y col. 2002] y, además, pueden ser los iniciadores de la activación y regulación de la respuesta inmunitaria específica [Clem y col. 1985, 1991; Vallejo y col. 1992]. El proceso de fagocitosis en peces presenta las mismas etapas descritas para los leucocitos de mamíferos, finalizando con dos mecanismos responsables de la muerte de los microorganismos fagocitados: la producción de intermediarios

reactivos de oxígeno (ROIs) y la producción de óxido nítrico (ON) y otros intermediarios reactivos de nitrógeno (RNIs). Además se conoce que los ROIs producidos por los fagocitos de peces tienen actividad bactericida [Sharp y Secombes 1993; Skarmeta y col. 1995].

Junto a los efectores celulares existe una gran variedad de sustancias que actúan en la defensa innata de los peces [Alexander e Ingram 1992]. Éstas se pueden clasificar funcionalmente en: (1) inhibidores del crecimiento bacteriano como la transferrina, antiproteasas y ceruloplasmina, (2) inhibidores de la replicación viral como el interferón, (3) inhibidores de toxinas bacterianas, (4) lisinas como la lisozima o la quitinasa, (5) aglutininas y precipitinas como las lectinas y la proteína C reactiva, y por último (6) los componentes del complemento que desempeñan una gran cantidad de funciones, entre las que destaca la actividad quimiotáctica para leucocitos [Lamas y Ellis 1994], la opsonización [Sakai 1984a, b], la inactivación de determinadas toxinas [von Eschen y Rudbach 1974; Ellis 1980; Sakai, 1984b], la actividad bactericida [Sakai 1983], la citotoxicidad y la inactivación viral [Sakai 1992].

El sistema inmunitario innato utiliza un amplio espectro de receptores que reconocen patrones moleculares asociados a patógenos (PAMPs) y que han sido denominados receptores de reconocimiento de patrones (PRRs). Estos receptores pueden estar presentes en la superficie celular, en compartimentos intracelulares, en el citosol o pueden estar solubles en la sangre o en los fluidos tisulares [Medzhitov y Janeway 2000]. Las principales funciones de estos receptores incluyen la opsonización, la activación de las cascadas del complemento y la coagulación, la fagocitosis, la activación de vías de señales pro-inflamatorias y la inducción de la apoptosis [Medzhitov y Janeway 2000].

Asimismo, en la regulación de la respuesta inmunitaria de teleósteos participan las citoquinas [Vilcek 1997], moléculas que median gran parte de las fases efectoras de la inmunidad innata y la adaptativa [Abbas y col. 2001]. Las citoquinas ejercen múltiples funciones entre las que se incluyen la regulación de la proliferación y la diferenciación de poblaciones linfocitarias así como la activación y la regulación de las células inflamatorias (fagocitos mononucleares, neutrófilos y eosinófilos). La secreción de citoquinas es breve y autolimitada y su síntesis se inicia por una nueva transcripción génica. En general, las citoquinas inician su acción uniéndose a receptores de superficie presentes en la célula diana, los cuales

tienen una elevada afinidad y su expresión es regulada por señales específicas (otra citoquina o incluso la misma citoquina) [Roca 2009].

Finalmente, el sistema inmunitario adaptativo de teleósteos, al igual que el de mamíferos, presenta memoria [Van Muiswinkel 1995]. Tras un primer contacto con el antígeno (respuesta primaria) se produce un menor título de anticuerpos específicos en el suero, que el producido en posteriores contactos (respuesta secundaria), siendo esta respuesta dependiente de la temperatura. La iniciación de la respuesta inmunitaria adaptativa está controlada por los macrófagos que funcionan como células presentadoras de antígenos para los linfocitos [Clem y col. 1985; Vallejo y col. 1992]. Los linfocitos, a su vez, producen citoquinas que activan a los macrófagos tras ser estimulados con un antígeno [Graham y Secombes 1988]. De esta manera se mantiene una coordinación y regulación mutuas entre la respuesta inmunitaria innata y la adaptativa.

En distintos trabajos sobre inmunidad innata en dorada, realizados en el seno de nuestro grupo de investigación, se ha descrito un tipo de leucocito, el granulocito acidófilo, que constituye el tipo de fagocito más activo y abundante y que desarrolla funciones similares tanto a las de los neutrófilos como a las de los macrófagos humanos [Abbas y col. 2001; Sepulcre y col. 2002; Chaves-Pozo y col. 2004, 2005a, b].

1.2 EL SISTEMA REPRODUCTOR

En vertebrados existe una gran diversidad de estrategias de reproducción que hacen que la actividad gametogénica sea constante a lo largo del año o cíclica, en el caso de vertebrados de puesta estacional [Johnson y Nguyen 1986; Sinha-Hikim y col. 1988]. En este último caso, la gónada sufre importantes fluctuaciones en la actividad gametogénica así como procesos de remodelación tisular. La regulación de estos procesos depende, en gran medida, de las gonadotropinas (GTHs), sintetizadas y secretadas por la hipófisis, las cuales inducen la secreción de esteroides y/o factores de crecimiento en distintos tipos celulares de la gónada. Entre los esteroides destacan las hormonas sexuales, con especial relevancia del andrógeno testosterona (T) y del estrógeno 17ß-estradiol (E_2) [Johnson y Nguyen 1986; Sinha-Hikim y col. 1988].

1.2.1 El sistema reproductor en peces teleósteos

Los peces constituyen el grupo de vertebrados con mayor diversidad de estrategias reproductivas. Así existen especies que desarrollan un único sexo funcional a lo largo de su vida (gonocoristas) y especies que presentan ambos sexos funcionales a lo largo de su vida (hermafroditas). Las especies gonocoristas pueden ser: i) primarios o diferenciados si únicamente desarrollan un tipo gonadal, ii) indiferenciados si todos los individuos inicialmente desarrollan un ovario indiferenciado, el cual degenera en la mitad de la población para desarrollar un testículo funcional o iii) secundarios si desarrollan una gónada hermafrodita que posteriormente evoluciona hacia un sexo único. Las especies hermafroditas pueden ser secuenciales, hablando entonces de especies protogínicas o protándricas en función del sexo que desarrollen antes, el femenino o el masculino, respectivamente, o pueden ser especies ambisexuales si desarrollan ambas gónadas simultáneamente [Devlin y Nagahama 2002].

De forma similar a lo que ocurre con las estrategias reproductivas, los ciclos reproductivos presentan una gran diversidad dependiendo de abundantes factores ambientales tales como fotoperiodo, temperatura, osmolaridad, disponibilidad de alimento, etc. y se pueden clasificar en función de la longitud del periodo de puesta. Así, hay i) especies como los salmónidos, características de clima frío, que ponen durante varias semanas al año, ii) especies como la dorada y la carpa común, características de clima templado, que ponen durante varios meses al año y iii) especies características de clima tropical, que ponen durante todo el año. Este comportamiento se ve reflejado en el ciclo gonadal ya que en las especies de puesta estacional este ciclo se puede dividir en cuatro etapas: gametogénesis, puesta, post-puesta y quiescencia, mientras que las especies que ponen durante todo el año carecen de las fases de post-puesta y quiescencia [Scott 1987; Miura 1999]. Sin embargo, a pesar de esta gran diversidad de estrategias y ciclos reproductivos, las características morfológicas y funcionales de la gónada de teleósteos están muy conservadas incluso en las especies hermafroditas [Le Gac y Loir 1999].

La dorada es una especie hermafrodita protándrica de puesta estacional que presenta una gónada bisexual compuesta por un área ovárica, mediodorsal, y un área testicular, lateroventral. Los ejemplares de dorada se desarrollan,

funcionalmente, como machos durante los dos primeros ciclos reproductivos, pudiendo posteriormente pasar a hembras [D'Ancona 1941; Pascuali 1941; Zohar y col. 1978]. Como se ha descrito en esta especie, el primer ciclo reproductivo consta de las cuatro etapas mencionadas: gametogénesis, puesta, post-puesta y quiescencia. Durante las etapas de post-puesta y quiescencia, la gónada de la dorada sufre importantes cambios morfológicos, fruto de una remodelación tisular, conducente a la eliminación de los espermatozoides remanentes del ciclo anterior y a la reorganización del tejido germinal [Chaves-Pozo y col. 2005c]. Estos procesos se correlacionan con un marcado aumento en los niveles séricos de E_2 y de la disminución de los andrógenos, T y 11-cetotestosterona (11-KT), principal andrógeno en esta especie [Chaves-Pozo y col. 2008a].

1.3 INTERACCIÓN INMUNO-REPRODUCTORA

En mamíferos existen diferentes poblaciones de leucocitos en la gónada (linfocitos, granulocitos, células cebadas y macrófagos) cuya presencia y funcionalidad así como su capacidad de activación y la intensidad de la misma están fuertemente reguladas por factores solubles. Estos factores solubles (citoquinas y hormonas) son producidos por las células gonadales a fin de impedir el desarrollo de reacciones inflamatorias crónicas que den lugar a reacciones autoinmunes y esterilidad funcional. A su vez, factores solubles (citoquinas, quimioquinas, etc.) liberados por las poblaciones de leucocitos gonadales, participan en la regulación de funciones testiculares y ováricas tales como la esteroidogénesis, la espermatogénesis y la folículogenesis [Hedger 1997; Hunt y Johnson 1999; Bukulmez y Arici 2000].

Fuera de la gónada, las hormonas sexuales esteroideas son capaces de regular las funciones de los leucocitos circulantes en sangre o presentes en otros tejidos. Diversos estudios han demostrado que diferentes poblaciones de leucocitos presentan receptores intracelulares y de membrana tanto para E_2 como para T, siendo estas hormonas capaces de modular sus actividades [Benten y col. 1998, 2001; Zhang y col. 2001; Guo y col. 2002]. Sin embargo, los efectos más estudiados en mamíferos son los relacionados con las variaciones fisiológicas de los niveles de E_2 y su influencia en la respuesta inmunitaria [Straub 2007]. Así, las variaciones en los niveles de E_2 se han correlacionado con alteraciones del comportamiento migratorio y de la expresión génica de citoquinas y otros mediadores de la

inflamación en linfocitos y en macrófagos [Yoshida y col. 1996; Härkönen y Väänänen 2006]. Además, la evidencia acumulada en diferentes modelos de mamíferos y en humanos demuestra la capacidad de los estrógenos y, en concreto, del E_2 para alterar las actividades de las células endoteliales promoviendo (i) vasodilatación, (ii) incremento de la permeabilidad capilar, (iii) alteración de la expresión génica de citoquinas y otros mediadores de la inflamación y (iv) alteración de la adhesión de plaquetas y leucocitos, representando estos efectos un gran impacto en el desarrollo de las enfermedades cardiovasculares mediante la reducción de los procesos de inflamación y extravasación de leucocitos de las zonas estenosadas [Nilsson 2007; Straub 2007].

1.3.1 Interacción inmuno-reproductora en peces teleósteos

En la gónada de teleósteos hay leucocitos cuyo tipo (granulocitos, macrófagos y linfocitos), abundancia y localización varían en función de la época del ciclo reproductor y de la especie [Billard y Takashima 1983; Scott y Sumpter 1989; Besseau y Faliex 1994; Bruslé-Sicard y Fourcault 1997; Lo Nostro y col. 2004]. Estos leucocitos gonadales de peces, al contrario de lo que ocurre en mamíferos, no eran considerados como una población propia de la gónada, sino que infiltraban el órgano, en mayor o menor medida, dependiendo de la época del ciclo reproductor. Sin embargo, trabajos realizados en nuestro laboratorio han demostrado que en la dorada, al menos un tipo de leucocito, el granulocito acidófilo, es reclutado mediante factores solubles secretados por la gónada y, aparentemente, forma parte activa de los procesos de reorganización tisular que ocurren tras la puesta [Chaves-Pozo y col. 2005a]. Además, estudios *in vitro* han demostrado que una vez infiltrados, éstos se especializan funcionalmente y constituyen una subpoblación específica del testículo de forma similar a lo que ocurre en mamíferos con los macrófagos testiculares [Hedger 2002; Chaves-Pozo y col. 2005a]. Así, estos granulocitos acidófilos muestran una alteración de sus principales funciones: producción de ROIs, capacidad fagocítica y producción de interleuquina-1ß (il1b) [Chaves-Pozo y col. 2003, 2005a]. Además, esta infiltración de granulocitos acidófilos se correlaciona con aumentos fisiológicos de los niveles séricos de E_2 [Chaves-Pozo y col. 2008a], mientras que la administración exógena de E_2 es capaz de inducir su movilización desde el riñón cefálico hasta la gónada [Chaves-Pozo y col. 2007].

Al igual que ocurre en mamíferos, en teleósteos, las hormonas sexuales

regulan las funciones de los leucocitos. Así, inyecciones intraperitoneales de E_2 o 11-KT inhiben de forma dosis dependiente la fagocitosis, la explosión respiratoria y la producción de ON en macrófagos de riñón cefálico de carpa [Yamaguchi y col. 2001; Watanuki y col. 2002]. Además se ha comprobado que la T, al igual que el cortisol, inhibe la activación de células B en salmónidos [Slater y Schreck 1993], el E_2 deprime el sistema inmunitario y aumenta su susceptibilidad a infecciones por tripanosomas en el pez dorado [Wang y Belosevic 1994] y disminuye la síntesis de péptidos antimicrobianos como las hepcidinas en la perca americana [Robertson y col. 2009]. En la dorada, un estudio reciente [Cuesta y col. 2007] demuestra que inyecciones intraperitoneales de T y E_2 alteran los valores de algunos parámetros inmunológicos. Así, la T incrementa las actividades del complemento y peroxidasa mientras que el E_2 reduce la actividad del complemento y los valores plasmáticos de inmunoglobulina M.

Todas estas observaciones nos han conducido, en primer lugar, a examinar el papel de los granulocitos acidófilos de dorada en el correcto funcionamiento de la gónada durante el segundo ciclo reproductor de los machos, previo al cambio de sexo y, en segundo lugar, a estudiar los efectos de los estrógenos sobre la respuesta inflamatoria en esta especie. En conjunto, esta investigación representa un esfuerzo por intentar comprender como las actividades del sistema reproductor pueden comprometer las actividades del sistema inmunitario. Sin duda se trata de investigación básica, de gran interés, que resulta de mayor relevancia si tenemos en cuenta la creciente presencia en el ambiente acuático de contaminantes, producidos por el hombre, con capacidad para actuar como disruptores endocrinos, la mayoría de ellos con capacidad estrogénica. Entre ellos destaca el 17α-etinilestradiol (EE_2), compuesto farmacológico, que forma parte de la casi totalidad de las píldoras o anticonceptivos orales mas modernos, con una actividad estrogénica de 10-50 veces la actividad del estrógeno natural E_2. El EE_2 se caracteriza por su vida media amplia en el medio y su capacidad de bioacumularse, alcanzando aumentos de 650 veces en el total del organismo con respecto a la concentración de exposición [Lai y col. 2002; Gibson y col. 2005; Fenlon y col. 2010]. El EE_2 está presente en aguas superficiales y efluentes europeas en concentraciones de 0.5 a 62 ng/l [Kuch y Ballschmiter 2000; Johnson y col. 2005; Hinteman y col. 2006], concentraciones capaces de alterar la fisiología de los peces [Ternes y col. 1999; Johnson y col. 2005; Hinteman y col. 2006; García-Reyero y col. 2011].

2 OBJETIVOS

2.1. Caracterizar la gónada durante el segundo ciclo reproductor de dorada mostrando una especial atención a los leucocitos presentes en ella.

2.2. Estudiar el efecto de los estrógenos, naturales y sintéticos, sobre las células endoteliales y como éstos pueden alterar el reclutamiento y extravasación de los leucocitos de dorada.

2.3. Estudiar el efecto del 17ß-estradiol sobre las principales actividades de los leucocitos de riñón cefálico de dorada.

2.4. Analizar el papel de los macrófagos como mediadores de los efectos del 17β-estradiol en los leucocitos de dorada.

2.5. Estudiar mediante técnicas transcriptómicas la modulación de la expresión génica de los macrófagos de dorada expuestos a 17ß-estradiol.

3 PRINCIPALES RESULTADOS Y DISCUSIÓN

3.1 Los leucocitos de dorada infiltran la gónada y participan en el proceso de regeneración tisular que precede al cambio de sexo

Aunque la presencia de leucocitos en la gónada de mamíferos, donde realizan tareas de vigilancia inmunológica y participan en la regulación de diferentes aspectos reproductivos, está establecida [Hedger 1997; Hunt y Johnson 1999; Bukulmez y Arici 2000], el estudio de leucocitos en la gónada de peces ha recibido, hasta ahora, poca atención, y la mayoría de los estudios tienen un enfoque, principalmente, morfológico debido a la carencia de marcadores específicos [Billard y Takashima 1983; Scott y Stumper 1989; Besseau y Faliex 1994; Bruslé-Sicard y Fourcault 1997; Lo Nostro y col. 2004]. Así, aunque varios trabajos habían abordado el cambio de sexo de la dorada desde un punto de vista eminentemente reproductor [D'Ancona 1941; Pascuali 1941; Zohar y col. 1978], ninguno lo había hecho considerando el papel de los leucocitos en este proceso. La gónada de la dorada, al tratarse de una especie de puesta estacional y hermafrodita protándrica, presenta un interés especial debido a los procesos de involución y remodelación tisular que ocurren en ella tras la puesta y en el periodo de tiempo previo al cambio de sexo. En el primer ciclo reproductor de los machos de dorada destaca, entre otros procesos, la infiltración de numerosos granulocitos acidófilos en el área testicular de la gónada en la época de post-puesta. Los granulocitos acidófilos muestran características funcionales específicas, una vez que infiltran el testículo [Chaves-Pozo y col. 2005a, b, c].

En este trabajo [Liarte y col. 2007] estudiamos la morfología de la gónada durante el segundo ciclo reproductor de la dorada, previo al posible cambio de sexo, prestando especial atención a los procesos degenerativos que ocurren en el testículo en las etapas que tienen lugar tras la puesta, así como a la presencia de leucocitos. Tras las fases de *espermatogénesis* y *puesta*, en el área testicular de la gónada se observa un proceso degenerativo caracterizado por la aparición de amplias áreas necróticas y la presencia de espermatogonias primarias en apoptosis, procesos que son mas intensos y están mas extendidos que en el primer ciclo reproductor [Chaves-Pozo y col. 2005c]. Paralelamente a estas observaciones se detectó una caída en los niveles de expresión del marcador de actividad testicular

dmrt1, acompañado de una leve actividad proliferativa en la porción ovárica y el inicio de la maduración de los oocitos inmaduros presentes en ella, alcanzando los primeros estadios vitelogénicos al final del segundo ciclo reproductor. Todos estos fenómenos que comienzan durante la etapa de ***post-puesta*** y aumentan en intensidad durante una etapa posterior no descrita en el primer ciclo reproductor, que sustituye a la etapa de ***quiescencia*** y que denominamos etapa de ***involución testicular***, parecen preparar a la gónada para el cambio de sexo. Sin embargo, los cambios observados no son restrictivos ni irreversibles, como indica la detección de tan solo un 40% de hembras en el siguiente ciclo reproductor.

La presencia de leucocitos en la gónada a lo largo del segundo ciclo reproductor quedó confirmada mediante la detección inmunocitoquímica de los granulocitos acidófilos y de la expresión de los siguientes genes marcadores: i) el receptor del factor estimulante de colonias de macrófagos (*mcsfr*) (macrófagos), ii) la cadena β del receptor de células T (*tcrb*) (células T) y iii) la cadena pesada de la inmunoglobulina M (*igmh*) (células B). Si bien la participación de macrófagos y linfocitos en los procesos de remodelación descritos no pudo ser confirmada, el patrón de infiltración que muestran los granulocitos acidófilos, con un claro aumento de su presencia al inicio de la etapa de post-puesta y un posterior repunte durante la de involución testicular, confirmaría la hipótesis de que estas células participan en dichos procesos. Esta suposición estaría además apoyada por la detección, alrededor de las áreas necróticas, de grupos grandes de granulocitos acidófilos que presentan una ultraestructura alterada y caracterizada por la presencia de gránulos de contenido heterogéneo fusionados unos a otros y con la membrana plasmática. Posteriormente, estas observaciones fueron corroboradas al comprobarse que los granulocitos acidófilos presentes en el testículo muestran actividad metaloproteasa, la cual está involucrada en los procesos de infiltración leucocitaria y reorganización de la matriz extracelular [Chaves-Pozo y col. 2008b].

Datos obtenidos por nuestro grupo de investigación en dorada relativos a (i) los niveles plasmáticos de E_2 a lo largo de los dos primeros ciclos reproductores, (ii) la infiltración de granulocitos acidófilos en la gónada tras la inyección intraperitoneal de E_2, (iii) la capacidad de la gónada de alterar la actividad metaloproteasa de los granulocitos acidófilos procedentes del riñón cefálico, afectando por tanto su capacidad de movilización y (iv) la variación de los niveles de expresión de diversos genes de relevancia inmunológica [***selectina-E*** (***sele), quimioquina CC ligando 4***

(*ccl4*), *factor de necrosis tumoral a* (*tnfa*), *factor de crecimiento transformante b* (*tgfb*), *receptor del factor de crecimiento transformante b (tgfbr)*] [Chaves-Pozo y col. 2007, 2008a, b, c] nos llevaron a plantearnos el conocer los mecanismos mediante los cuales las hormonas esteroideas y, en concreto, los estrógenos podrían estar implicados en el comportamiento de los leucocitos dentro y fuera de los órganos reproductores.

3.2 Los estrógenos modulan la respuesta inflamatoria de las células endoteliales de dorada

La evidencia acumulada en humanos y en diversos modelos de enfermedad cardiovascular ha demostrado la capacidad que tienen los estrógenos para regular la fisiología de las células endoteliales, alterando su capacidad de señalizar la inflamación y de mediar en el proceso de infiltración leucocitaria [Nilsson 2007; Straub 2007]. En esta línea, los resultados obtenidos en dorada sugieren la capacidad del E_2 para modular los procesos de infiltración leucocitaria en esta especie, ya que como se ha expuesto, el E_2 es capaz de promover la migración de granulocitos acidófilos *in vivo* hacia la gónada [Chaves-Pozo y col. 2007] así como de modular la actividad metaloproteasa de estas células y, por tanto, alterar su capacidad de infiltración [Chaves-Pozo y col. 2008b].

Para comprobar si las observaciones hechas en mamíferos tienen su correlato en teleósteos, en el siguiente trabajo [Liarte y col. 2011a] investigamos los efectos de dos estrógenos: i) la forma natural presente en dorada, el E_2 y ii) una forma sintética con gran actividad estrogénica, el EE_2, sobre las capacidades inflamatorias de las células endoteliales de dorada. El EE_2 está presente en la mayoría de las píldoras anticonceptivas y en los tratamientos hormonales sustitutorios y es un contaminante habitual en los ecosistemas acuáticos. El análisis de expresión de los tres genes, conocidos en dorada [Pinto y col. 2005, 2006], del receptor intracelular de estrógenos (*era, erb1, erb2*) en células endoteliales, demostró la presencia de *era* y *erb1* en estas células y que los estrógenos modulan su expresión. Así observamos aumentos o disminuciones de su expresión dependiendo del estrógeno utilizado, E_2 o EE_2, y de la concentración utilizada (5 o 50 ng/ml). Además, la expresión de estos receptores se altera en respuesta a diversos PAMPs. Estos datos demuestran que en peces, al igual que en mamíferos,

las células endoteliales responden a estrógenos y modulan su respuesta a estrógenos a lo largo de una respuesta inflamatoria [Nilsson 2007].

Una de las manifestaciones mejor caracterizadas de los tratamientos de E_2 en células endoteliales de mamíferos es el aumento de los niveles del vasodilatador ON [Nilsson 2007], fenómeno que no ocurre cuando las células endoteliales son tratadas con EE_2 [Arnal y col. 1996]. A este respecto, los resultados obtenidos en células endoteliales de dorada se corresponden con lo descrito en mamíferos, demostrando la proximidad existente en la regulación de la fisiología de las células endoteliales entre teleósteos y vertebrados superiores.

El análisis de la expresión génica de moléculas importantes para la respuesta inmunitaria de las células endoteliales tratadas con E_2 o EE_2 arrojó patrones de expresión distintos. Así, el E_2 parece tener un efecto pro-inflamatorio ya que es capaz de inducir la expresión de quimioquinas (*il 8* y *ccl4*) y moléculas de adhesión (*sele*) en las células endoteliales e incrementa, además, la expresión de citoquinas (*il1b, il6, tnfa*) y PRRs [*receptor similar a Toll (tlr) 5 anclada a membrana* (*tlr5mb), tlr9 y tlr22*)] cuando las células son co-estimuladas con ADN-bacteriano (ADN-b). Sin embargo, el EE_2 parece actuar en sentido contrario, ya que mientras sus efectos en solitario sobre la expresión génica son leves, el EE_2 reprime de forma dosis dependiente la expresión de citoquinas (*il1b* y *tnfa*), quimioquinas (*il8* y *ccl4*) y *sele* en las células endoteliales co-expuestas a ADN-b. Este comportamiento tan distinto, aún tratándose de dos compuestos estrogénicos, podría ser debido a que en dorada, de forma semejante a lo que ocurre en mamíferos, las células endoteliales presentan una mayor afinidad del EE_2 por *era* que por *erb1* mientras que la afinidad del E_2 es similar por ambos receptores [Barkhem y col. 1998; Kuiper y col. 1998].

Los citados efectos del E_2 sobre la expresión génica de las células endoteliales parecen traducirse en una alteración de sus capacidades funcionales. Así, las células endoteliales expuestas a E_2 presentaron una mayor capacidad para adherir leucocitos que las células no tratadas. Sin embargo, a pesar de la alta inducción detectada en la expresión génica de factores pro-inflamatorios y quimioquinas, el medio condicionado de las células endoteliales tratadas con E_2 no indujo la migración de los leucocitos de riñón cefálico. Si bien estos datos sugieren que la inducción de quimioquinas en células endoteliales expuestas a E_2 no sería

suficiente para promover la infiltración de leucocitos, probablemente debido a la ausencia de factores quimiotácticos liberados por el tejido subyacente *in vivo* [Ebnet y col. 1996], todo lo anteriormente expuesto viene a confirmar que las células endoteliales y los estrógenos juegan un papel fundamental en la regulación del tráfico de leucocitos en la dorada.

3.3 El 17ß-estradiol modula la respuesta inflamatoria de los leucocitos de dorada

Además de sus funciones reproductoras, los datos obtenidos en mamíferos demuestran como los esteroides gonadales pueden interactuar con receptores intracelulares y de membrana presentes en las células efectoras del sistema inmunitario modulando su actividad [Benten y col. 1998, 2001; Zhang y col. 2001; Guo y col. 2002]. Aunque la bibliografía existente sobre la expresión de receptores de esteroides en leucocitos de peces es escasa y poco concreta [Slater y col. 1995; Law y col. 2001], ciertos datos obtenidos en dorada [Chaves-Pozo y col. 2005a, 2007; Cuesta y col. 2007] sugieren que los esteroides gonadales y en concreto el E_2 modulan directamente el comportamiento de los leucocitos.

Para confirmar dicha hipótesis, en este trabajo [Liarte y col. 2011b] analizamos la expresión de los tres genes que codifican para los receptores intracelulares de estrógenos (*era, erb1, erb2*) conocidos en dorada [Pinto y col. 2005, 2006], en aquellos tipos de leucocitos presentes en la gónada de dorada. Sorprendentemente, mientras los granulocitos acidófilos tanto de riñón cefálico como testiculares carecen de la expresión de algún receptor intracelular de estrógenos, los macrófagos y linfocitos de riñón cefálico expresan constitutivamente *era*. Estos datos contrastan con los resultados descritos en mamíferos, en los que macrófagos y linfocitos expresan constitutivamente los dos genes conocidos del ER (*erα y erβ*) y en los que se detecta un mayor nivel de expresión basal de *era* frente a *erb*, lo que se considera un mecanismo de control de los efectos del E_2 [Straub 2007]. Sin embargo hay que resaltar que en macrófagos y linfocitos de dorada estimulados con ADN-b se induce la expresión de *era, erb1* y *erb2* indicando la capacidad del sistema inmunitario de modular su sensibilidad a estrógenos en el transcurso de la respuesta inmunitaria.

Ensayos funcionales *in vitro* con leucocitos de riñón cefálico demostraron que distintas concentraciones de E_2 eran incapaces de alterar significativamente su comportamiento migratorio. Sin embargo, la mayor concentración ensayada inhibió ligeramente la fagocitosis y diversas concentraciones modificaron la producción de ROIs de los leucocitos cuando éstos fueron co-estimulados con ADN-b. Todos estos datos sugieren la existencia de una regulación indirecta del E_2 sobre los granulocitos acidófilos a través de su efecto sobre macrófagos y/o linfocitos, ya que, a pesar de no expresar receptores intracelulares de estrógenos, sus funciones se ven alteradas en presencia de E_2.

Analizando el efecto del E_2 sobre la expresión génica de macrófagos demostramos que el E_2, solo o acompañado de ADN-b, induce la expresión de *erb2* y estimula la expresión de *era* así como de la mayoría de los genes de relevancia inmunitaria estudiados: citoquinas pro-inflamatorias (*il1b, il6, tnfa*), quimioquinas (*ccl4, il8*), metaloproteasas de la matriz extracelular (*mmp) (mmp9*, *mmp13*), receptores inmunitarios (*tlr5mb, receptor similar a Toll 5 soluble (tlr5s), tlr9, tlr22*) y moléculas efectoras [proteína de resistencia de *myxovirus influenza* (*mx), ciclooxigenasa 2 (cox2*)]. En este sentido, la utilización de medio condicionado de macrófagos expuestos a E_2 nos permitió comprobar como los efectos del E_2 sobre la expresión génica de macrófagos se traducen en la capacidad de estos medios para inhibir la habilidad y la capacidad fagocítica de los leucocitos de dorada así como su producción de ROIs a tiempo corto, tal y como ocurre al tratar leucocitos totales con E_2. Sorprendentemente, estos medios no fueron efectivos para alterar significativamente el comportamiento migratorio *in vitro* o la producción de ROIs a largo plazo de los leucocitos de dorada.

3.4 El 17ß-estradiol altera la expresión génica y la fisiología de los macrófagos de dorada

La gran habilidad demostrada por el E_2 para estimular la expresión de multitud de genes involucrados en la respuesta inflamatoria en macrófagos (punto 3.3) sugiere la capacidad de esta hormona para modular un amplio espectro de rutas moleculares en este tipo celular. Para comprobar esta hipótesis, en este último trabajo [Liarte y col. 2011c] decidimos realizar un análisis transcripcional en macrófagos expuestos a E_2 empleando para ello la técnica de supresión mediante hibridación substractiva (SSH), técnica que permite el análisis a gran escala de la

expresión diferencial existente entre muestras control (macrófagos) y muestras sometidas a tratamiento (macrófagos tratados con E_2). Así, empleando SSH conseguimos aislar 363 secuencias únicas (uni-gen) candidatas a ser elementos de respuesta a estrógenos. De estos uni-genes, tras realizar un análisis comparativo con las bases de datos de secuencias existentes encontramos dos grupos: (1) 205 uni-genes son huérfanos de ortólogo significativo y que representan elementos, potencialmente, desconocidos de la fisiología de los macrófagos y (2) 162 uni-genes se identificaron significativamente con 159 secuencias previamente descritas, algunas de las cuales fueron analizadas mediante reacción en cadena de la polimerasa (PCR) a tiempo real para comprobar la validez de los resultados obtenidos.

El análisis del conjunto de ortólogos identificados siguiendo las reglas del *Gene Onthologies Consortium* (GO) y de la *Kyoto Enciclopedia of Genes and Genomics* (KEGG) permitió la clasificación funcional de éstos, proporcionándonos una visión integral del efecto que el E_2 tiene sobre la modulación transcripcional en macrófagos. Mediante el análisis GO, que se centra en las capacidades funcionales de cada uni-gen, se identificaron 95 términos estadísticamente representativos que se agruparon en 3 categorías (compartimiento celular, función molecular y procesos biológicos). Así, en la categoría *Compartimento Celular* se agruparon mayoritariamente en los términos *citoplasma* y *núcleo*, en la categoría *Función Molecular* lo hicieron mayoritariamente en el término *unión a proteínas* y en la categoría *Proceso Biológico* los términos más representados fueron *regulación biológica, proceso del sistema inmunitario, respuesta a estímulo* e *interacción fisiológica*. Es de destacar que todos los procesos biológicos mencionados tienen una elevada relación con la respuesta ante un estímulo inmunitario, observación que refuerza el papel previamente sugerido del E_2 como actor relevante en la regulación de la respuesta inmunitaria de la dorada. Para comprobar la veracidad de estas observaciones se estudió mediante el análisis KEGG la participación de cada uni-gen en rutas moleculares caracterizadas, revelando la activación de diversas rutas de marcado carácter inmunitario como *procesado y presentación de antígenos* o *infección por bacterias enterotoxigénicas*, así como otras rutas de carácter fisiológico relacionadas con la inmunidad como *proteosoma* o *regulación del citoesqueleto*.

El escrutinio de los ortólogos identificados permitió identificar la activación de elementos involucrados en diversos procesos que escapan al análisis computacional y entre los que destacan los procesos relacionados con i) supervivencia [genes de proteínas anti-apoptóticas como *proteína de la familia "célula B CCL/linfoma 7", miembro B* (*bcl7b)* y proteína 14-3-3 β/α1 (*143b1)*], ii) la detección de estímulos inmunitarios [genes codificantes de PRRs como *receptor similar a NOD C-3* (*nlrc3)* y *helicasa específica de tejido linfoide* (*hells)*], iii) el procesamiento y la presentación de antígenos [genes codificantes de proteínas que participan del proteasoma inmunitario y de los complejos de histo-compatibilidad como *subunidad 26s del proteasoma* (*psmd8), ubiquitina (ubiq), proteína modificadora de bajo tamaño similar a ubiquitina (sumo), proteína del complejo principal de histocompatibilidad 1α* (*mhc1a)* y microglobulina β2 (*b2m)*] o iv) la respuesta inmunitaria e inflamación [genes codificantes para proteínas de la fase aguda como *proteína bactericida incrementadota de la permeabilidad* (*bpi), perforina* 1 (*perf)* y la isoforma hepática de la subunidad media de la ferritina (*friml)*].

Por último, el estudio pormenorizado de los distintos uni-genes identificados y de sus interacciones transcriptómicas nos permitió la identificación del factor nuclear κB (NF-κB), considerado uno de los principales factores de transcripción involucrados en la regulación de la respuesta inmunitaria, como elemento común en la regulación inicial de la mayoría de procesos y factores identificados. El sistema inmunitario se vale de complejos mecanismos para el control de la magnitud de las respuestas celulares y pequeñas variaciones en la intensidad de las señales que las células perciben resultan en comportamientos distintos [Abbas y col. 2001]. En dorada hemos demostrado que un reto inmunológico produce la sobre-expresión drástica de *era* y la inducción de la expresión de *erb1* y *erb2* en macrófagos, incrementando así su sensibilidad al E_2 durante la respuesta inmunitaria [Liarte y col. 2011b]. Además los datos obtenidos en el análisis transcriptómico sugieren que el E_2 puede jugar un papel crítico en la regulación de procesos inmunológicos de relevancia, como la activación del inflamasoma o la presentación de antígenos, por medio de una regulación directa de la actividad del NF-κB en macrófagos. Todos estos datos concuerdan con la evidencia existente en mamíferos [Straub 2007] y aporta, aún más si cabe, un mayor peso al E_2 y a su capacidad para modular la respuesta inmunitaria de la dorada y señalan a los macrófagos como un tipo celular relevante en la modulación de la respuesta inmunitaria por estrógenos.

En conjunto, los resultados presentados en esta Tesis Doctoral demuestran la existencia de una estrecha interacción entre los sistemas reproductor e inmunitario de dorada. Así hemos comprobado que los leucocitos participan en los procesos que tienen lugar en la gónada a la vez que factores humorales secretados por ésta, tales como el E_2, son capaces de modular de manera efectiva las capacidades de las células que participan en la respuesta inmunitaria innata.

La presencia de granulocitos acidófilos, macrófagos y linfocitos en la gónada de dorada a lo largo de los ciclos reproductores en los que los ejemplares son machos queda demostrada. Estas células tienen una relevancia especial durante las etapas de post-puesta e involución testicular en las que los granulocitos acidófilos en respuesta a factores liberados por la gónada, participan activamente en los procesos de remodelación tisular que ocurren de forma previa al cambio de sexo.

Tomando como punto de partida los resultados anteriormente descritos así como datos sobre los niveles plasmáticos de E_2 y la capacidad de esta hormona para provocar la migración de leucocitos *in vivo*, analizamos la habilidad de dos compuestos estrogénicos, E_2 y EE_2, para modular la fisiología, la expresión génica y la capacidad de adherir leucocitos de las células endoteliales de dorada. Así hemos demostrado que los estrógenos modulan el tráfico de leucocitos a través de las células endoteliales durante el desarrollo de los procesos inflamatorios, procesos que se pueden ver comprometidos por la exposición a contaminantes ambientales estrogénicos tales como el EE_2, con el consecuente riesgo que ello representa para el mantenimiento de las especies.

Confirmada la capacidad de los estrógenos para modular la fisiología de las células endoteliales proseguimos nuestro análisis comprobando la capacidad del estrógeno endógeno, E_2, para alterar la fisiología de los leucocitos de dorada. Así hemos sido los primeros en comprobar que estos tipos celulares expresan receptores intracelulares de estrógenos. Además, demostramos que el E_2 altera las actividades propias de los leucocitos en esta especie así como la expresión génica de los macrófagos, tipo celular que juega un papel importante en la regulación de la modulación de las funciones de los leucocitos ejercida por el E_2.

Por último, el análisis de los efectos que la exposición a E_2 tiene sobre el transcriptoma de los macrófagos ha permitido constatar que los estrógenos modulan la expresión génica de esta población celular, afectando globalmente su fisiología

pero de forma especial a aquellos procesos relacionados con el desarrollo de la respuesta inmunitaria. En este sentido, debido a su papel fundamental en la regulación inmunitaria, la identificación de NF-κB, como elemento involucrado en la mediación de los efectos del E_2, otorga relevancia a estos resultados y desvela el potencial científico que, el estudio de la interacción inmuno-reproductora en teleósteos, puede tener.

4 CONCLUSIONES

4.1. Granulocitos acidófilos, macrófagos y linfocitos están presentes en la gónada de dorada a lo largo del segundo ciclo reproductor.

4.2. Los granulocitos acidófilos infiltran el testículo de dorada e intervienen en los procesos de regeneración tisular que ocurren tras la puesta.

4.3. Macrófagos, linfocitos y células endoteliales de dorada expresan alguno de los tres receptores intracelulares de estrógenos descritos mientras que su expresión no se detecta en los granulocitos acidófilos.

4.4. Los estrógenos, naturales y sintéticos, modulan, de forma diferencial, la expresión génica de moléculas relevantes en la respuesta inmunitaria y el efecto inflamatorio de la exposición a ADN bacteriano de las células endoteliales y la capacidad de éstas para adherir leucocitos.

4.5. El 17ß-estradiol modula la fagocitosis y la producción de especies reactivas de oxígeno de los leucocitos de dorada.

4.6. El 17ß-estradiol modula la expresión génica y potencia los efectos pro-inflamatorios de la exposición a ADN bacteriano de los macrófagos de dorada.

4.7. El 17ß-estradiol provoca una alteración del transcriptoma en macrófagos de dorada que potencia la activación de diversas rutas moleculares con compromiso inmunitario.

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ANEXOS

1 Índice de impacto de las publicaciones de que consta la Tesis

- **Reproductive Biology and Endocrinology**

Abbreviated Journal Title *(linked to journal information)*	ISSN	JCR Data					
		Total Cites	Impact Factor	5-Year Impact Factor	Immediacy Index	Articles	Cited Half-life
BIOL REPROD	1477-7827	1487	2.077		0.169	154	4.9

Full Journal Title: REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY

ISO Abbrev. Title: Reprod. Biol. Endocrinol.

JCR Abbrev. Title: REPROD BIOL ENDOCRIN

ISSN: 1477-7827

Issues/Year: 0

Language: ENGLISH

Journal Country/Territory: UNITED STATES

Publisher: BIOMED CENTRAL LTD

Publisher Address: 236 GRAYS INN RD, FLOOR 6, LONDON WC1X 8HL, ENGLAND

Subject Categories: ENDOCRINOLGY & METABOLISM

REPRODUCTIVE BIOLOGY

Category Name	Total Journals in Category	Journal Rank in Category	Quartile in Category
ENDOCRINOLOGY & METABOLISM	105	70	Q3
REPRODUCTIVE BIOLOGY	26	15	Q3

- **Moecular Immunology**

Abbreviated Journal Title *(linked to journal information)*	ISSN	JCR Data					
		Total Cites	Impact Factor	5-Year Impact Factor	Immediacy Index	Articles	Cited Half-life
MOL IMMUNOL	0161-5890	7153	3.202	3.233	0.528	432	3.8

Full Journal Title: MOLECULAR IMMUNOLOGY

ISO Abbrev. Title: Mol. Immunol.

JCR Abbrev. Title: MOL IMMUNOL

ISSN: 0161-5890

Issues/Year: 18

Language: MULTI-LANGUAGE

Journal Country/Territory: ENGLAND

Publisher: PERGAMON-ELSEVIER SCIENCE LTD

Publisher Address: THE BOULEVARD, LANGFORD LANE, KIDLINGTON, OXFORD OX5 1GB, ENGLAND

Subject Categories: BIOCHEMISTRY & MOLECULAR BIOLOGY
IMMUNOLOGY

Category Name	Total Journals in Category	Journal Rank in Category	Quartile in Category
BIOCHEMISTRY & MOLECULAR BIOLOGY	283	104	Q2
IMMUNOLOGY	128	45	Q2

- **Developmental and Comparative Immunology**

Abbreviated Journal Title *(linked to journal information)*	ISSN	JCR Data					
		Total Cites	Impact Factor	5-Year Impact Factor	Immediacy Index	Articles	Cited Half-life
DEV COMP IMMUNOL	0145-305X	3751	3.290	3.454	1.065	138	5.7

Full Journal Title: DEVELOPMENTAL AND COMPARATIVE IMMUNOLOGY
ISO Abbrev. Title: Dev. Comp. Immunol.
JCR Abbrev. Title: DEV COMP IMMUNOL
ISSN: 0145-305X
Issues/Year: 12
Language: ENGLISH
Journal Country/Territory: ENGLAND
Publisher: ELSEVIER SCI LTD
Publisher Address: THE BOULEVARD, LANGFORD LANE, KIDLINGTON, OXFORD OX5 1GB, OXON, ENGLAND
Subject Categories: IMMUNOLOGY
ZOOLOGY

Category Name	Total Journals in Category	Journal Rank in Category	Quartile in Category
IMMUNOLOGY	128	42	Q2
ZOOLOGY	129	4	Q1

2 Publicaciones correspondientes a colaboraciones durante el periodo de formación predoctoral

- 17Beta-estradiol triggers postspawning in spermatogenically active gilthead seabream (*Sparus aurata* L.) males.

Chaves-Pozo E, Liarte S, Vargas-Chacoff L, García-López A, Mulero V, Meseguer J, Mancera JM, García-Ayala A (2007) Biol Reprod 76, 142-148.

- Pattern of expression of immune-relevant genes in the gonad of a teleost, the gilthead seabream (*Sparus aurata* L.).

Chaves-Pozo E, Liarte S, Fernández-Alacid L, Abellán E, Meseguer J, Mulero V, García-Ayala A (2008) Mol Immunol 45, 2998-3011.

- Early presence of immune cells in the developing gonad of the gilthead seabream (*Sparus aurata* Linnaeus, 1758).

Chaves-Pozo E, Liarte S, Mulero I, Abellán E, Meseguer J, García-Ayala A. (2008) J Reprod Endocrinol 55, 440-445.

- Oestrogen-induced androgen insufficiency results in a reduction of proliferation and differentiation of spermatogonia in the zebrafish testis.

de Waal PP, Leal MC, García-López A, Liarte S, de Jonge H, Hinfray N, Brion F, Schulz RW, Bogerd J. (2009) J Endrocrinol 202, 287-297.

- Immune and reproductive interaction: An essential clue for understanding gonad functions in gilthead seabream.

E Chaves-Pozo, S Liarte-Lastra, A García-Ayala (2009) En: Recent Advances in Fish Reproductive Biology. Research Signpost, India.

3 Participación en congresos y comunicaciones correspondientes al periodo de formación predoctoral

- Cytokine and cell adhesion molecule expression pattern in the gilthead seabream (Sparus aurata L.) testis. E CHAVES POZO, S LIARTE, L FERNÁNDEZ ALACID, I CABAS, A GARCÍA ALCAZAr, J MESEGUER, V MULERO, A GARCÍA AYALA. 8th International Symposium on Reproductive Endocrinology of Fish, Saint-Malo, Francia, 2007. Comunicación oral.

- Los granulocitos acidófilos de dorada infiltran el testículo en la etapa de involución testicular. S LIARTE, E CHAVES POZO, V MULERO, J MESEGUER, A GARCÍA AYALA. XII Congreso de la Sociedad Española de Biología Celular, Pamplona, España, 2007. Comunicación oral.

- Leukocytes and cytokines in the gilthead seabream (Sparus aurata L.) testis. J MESEGUER, E CHAVES POZO, S LIARTE, A GARCÍA ALCÁZAR, P CASTILLO, L FERNÁNDEZ, V MULERO, A GARCÍA AYALA. XIII International Congress of Immunology, Rio de Janeiro, Brazil, 2007. Poster.

- Factores inmunitarios implicados en el desarrollo de los machos de dorada (Sparus aurata L.). E CHAVES POZO, S LIARTE, I MULERO, p ACSTILLO-BRICEÑO, V MULERO, J MESEGUER, A GARCÍA AYALA. VI Congreso de la Asociación Ibérica de Endocrinología Comparada, Cádiz, España, 2007. Poster.

- 17β-estradiol modulates immune activities in the Gilthead seabream (Sparu aurata L.). LIARTE S, CABAS I, CHAVES POZO E, MESEGUER J, MULERO V, GARCÍA AYALA A. 6th International Symposium in Fish Endocrinology, Calgary, Alberta, Canadá, 2008. Comunicación oral.

- Estrogens modulate cytokine and chemokine expression of primary macrophages in the gilthead seabream (Sparus aurata L.). LIARTE S, CABAS I, CHAVES POZO E, MESEGUER J, MULERO V, GARCÍA AYALA A. 16th International Congress of Comparative Endocrinology, Hong Kong, República Popular China. 2009. Póster.

- Expression of extracellular matrix mollecules in immune cells and tissues of the Gilthead Seabream. CASTILLO-BRICEÑO P, LIARTE S, CABAS I, MESEGUER J, GARCÍA-AYALA A, MULERO V. 11th Congress of the International Society of Developmental and Comparative Immunology. Praga, República Checa. 2009. Comunicación oral

- 17α-ethinylestradiol modulates activation of the professional phagocytes of the bony fish gilthead seabream. CABAS I, LIARTE S, MESEGUER J, MULERO V, GARCÍA-AYALA A. XIII Congreso de la Sociedad Española de Biología Celular. Murcia, España. 2009. Comunicación oral.

- Estradiol modulate leukocyte activities and alter inflammatory response gene expression profile on primary macrophages of the gilthead seabream. LIARTE S, CABAS I, CHAVES POZO E, MESEGUER J, MULERO V, GARCÍA AYALA A. First Symposium of the European Organisation of Fish Immunology, Viterbo, Itália, 2010. Poster.

- Estradiol modulates leukocyte activities and alters inflammatory response gene expression profile on primary Endothelial cells of the gilthead seabream (Sparus aurata L.). LIARTE S, CABAS I, CHAVES POZO E, MESEGUER J, MULERO V, GARCÍA AYALA A. 9th International Congress on the Biology of Fish, Barcelona, España, 2010. Póster.

- Identification of estrogen-responsive genes in primary macrophage cultures of the gilthead seabream (Sparus aurata L.) using suppression subtractive hybridization). LIARTE S, PINTO PI, MESEGUER J, MULERO V, CANARIO AV, GARCÍA AYALA A. 9th International Congress on the Biology of Fish, Barcelona, España, 2010. Comunicación oral.

- **17α-ethinylestradiol in vivo treatments alter imflammatory gene expression profile in the gilthead seabream (Sparus aurata L.).** CABAS I, LIARTE S, MESEGUER J, MULERO V, GARCÍA-AYALA A. 9th International Congress on the Biology of Fish, Barcelona, España, 2010. Póster.