—Full Paper—

Early Presence of Immune Cells in the Developing Gonad of the Gilthead Seabream (*Sparus aurata* Linnaeus, 1758)

Elena CHAVES-POZO³⁾, Sergio LIARTE¹⁾, Iván MULERO¹⁾, Emilia ABELLÁN²⁾, José MESEGUER¹⁾ and Alfonsa GARCÍA-AYALA¹⁾

¹⁾Department of Cell Biology and Histology, Faculty of Biology, University of Murcia, Campus Universitario de Espinardo, 30100 Murcia, ²⁾Oceanographic Center of Murcia, Spanish Oceanographic Institute (IEO), Carretera de la Azohía s/n. Puerto de Mazarrón, 30860 Murcia and ³⁾Centro de Investigación en Sanidad Animal (CISA-INIA), Carretera de Algete a El Casar Km. 8,1, Valdeolmos, 28130 Madrid, Spain

Abstract. A great deal is known regarding the process of sex differentiation in fish. However, little is known about the presence of immune cells and cytokines in this process. In the gilthead seabream, both immune cells and cytokines play an important role in the tissue reorganization of the gonads during the adult reproductive cycle. We have studied, using light microscopy and immunocytochemistry, the ontogenetic development of the gilthead seabream gonads, focusing on the presence of immune cells and cytokines. We show that the testicular area is quickly differentiated and becomes functional in specimens less than a year old, while the ovarian area differentiates later and continues to develop during the first two years of life. Throughout the morphogenesis process, acidophilic granulocytes were present in the gonad. Interleukin-1b (Il1b) is produced in the testicular area in juveniles and male fish, but not in the ovarian area. Macrophage-colony stimulating factor receptor (Mcsfr) is not produced in the undifferentiated gonad and is only found once the testicular area is well developed.

Key words: Cytokines, Gonadal development, Immune cells

(J. Reprod. Dev. 55: 440–445, 2009)

The process of gonad morphogenesis is of primary importance for precise understanding of the mechanisms of gonadal sex differentiation and could help to develop efficient methods of directing sexual development in aquaculture species. Teleosts are a group of vertebrates in which gonad ontogeny is very diverse. Gonadal differentiation can take many forms in teleosts, ranging from gonochoristic species, which directly develop one sex, to hermaphroditic species, which can produce mature male and female gametes during their lives; in turn, these can be divided into simultaneous or sequential hermaphrodite species. Sequential hermaphrodite specimens can initially mature either as males (protandrous) or females (protogynous) [1].

A great deal is known regarding the process of sex differentiation in fish, and the mechanisms involved in primary sex determination are now beginning to be defined. Thus, in teleost fish, primordial germ cells (PGCs) arise at extragonadal locations and migrate to the genital ridges (gonadal anlagen) during specific developmental phases [2]. The genital ridge forms as a longitudinal thickening of the mesoderm that protrudes into the coelomic cavity, ventral to the developing kidney and lateral to the dorsal mesentery [1]. Prior to differentiation, all somatic cells appear to be derived from a cortex epithelial layer, and are similar in presumptive males and females. Following migration of PGCs into the germinal ridge, cells divide to form oogonia and spermatogonia, while the differentiation and migration of somatic cells is concomitant with this process [1].

The gilthead seabream is a protandrous hermaphrodite seasonal breeding teleost of great interest in aquaculture. In the southwest Mediterranean area, the testicular area of the limited bisexual gonad of this species becomes functional during the first two years of life and then degenerates concomitantly with the sharply increasing development of the ovarian area, which becomes functional in a subsequent reproductive cycle [3–5].

In adult fish, the presence of immune cells in the interstitial tissue of the gonad and expression of several types of cytokine by germ and somatic cells have been demonstrated in several species [3–10] and have also been demonstrated in mammals [11]. Moreover, both immune cells and cytokines play an important role in post-spawned gonadal reorganization in the gilthead seabream [3–5, 8, 9]. In fact, our previous data demonstrate that an infiltration of acidophilic granulocytes, an immune cell type, is physiologically regulated by the gonad of the gilthead seabream during the post-spawning and testicular involution stages of the first two reproductive cycles [3–5, 9]. Moreover, the appearance of acidophilic granulocytes in the testicular area is essential for the tissue remodelling and renewal of the testicular germ cells that occurs during these stages [4, 5, 9, 12]. Interestingly, testicular acidophilic granulocytes, together with some germ cells, produce interleukin-1b (Il1b), a pro-inflammatory cytokine that, in mammals, is related to germ cell differentiation [8, 9, 13]. On the other hand, macrophage-colony stimulating factor receptor (Mcsfr) has recently been described as being present in somatic ovarian and testicular cells. All this together suggests that, as occurs in mammals, several immune molecules in fish gonad play an essential role in the orchestration of gametogenesis and maintenance of gonad tissue homeostasis [8]. However, little is known about the presence or

role of immune cells and cytokines in the process of sex differentiation.

In this paper, we report the ontogenetic development of the gilthead seabream gonads from hatching to adulthood, including the appearance of germ cells in the gonadal primordia, gonadal sex differentiation, gonad formation and pubertal development, focus on the presence of acidophilic granulocytes and some immune factors such as Il1b and Mcsfr and highlight the early interaction of acidophilic granulocytes within the gonad.

Material and Methods

Animals

Gilthead seabream (*Sparus aurata* L., Actinopterygii, Sparidae) fry were maintained at the Murcia Oceanographic Centre (IEO) using the "green water" technique in a 5000-l round tank with an initial density of about 60 eggs/l. Natural seawater (38‰ salinity) was heated to 17 ± 1 °C and filtered through mechanical and biological substrates. The temperature naturally increased and reached 26 °C by the end of the experiment. Water renewal was limited to 2% daily during the first 20 days of culture and was achieved by the addition of 70 ml/m³ of a microalgae concentrated solution (Phytobloom, Necton, Algarve, Portugal) containing 80% *Nannochloropsis oculata*. Subsequently, continuous water renewal (30%/h) and light aeration were provided in the tank. During the experiment, the light intensity was 1000 lx at the water surface, and the photoperiod was 16:8 (L:D). Larvae were successively fed with enriched (Selco, Inve Animal Health, Vigo, Spain) rotifers from 6 to 24 days post-hatching (dph), *Artemia nauplii* (Inve Animal Health) from 20 to 35 dph, enriched Instar II *Artemia* from 31 to 58 dph and a commercial dry pellet diet (Skretting, Burgos, Spain) from 54 dph onward. Larvae were sampled every two days from 1 to 83 dph, juveniles were sampled at 92, 103, 111, 131 and 180 dph and male fish were sampled at 210, 240, 270 and 300 dph. All animal studies were carried out in accordance with the European Union regulations for animal experimentation.

Light microscopy and immunohistochemistry

Larvae (1–76 dph) and juveniles at 92 to 111 dph (5 specimens of each age) were processed without further dissection, while juveniles at 180 dph and male specimens (210–300 dph) were sectioned, and the gonads were sampled. Samples were fixed overnight in Bouin-Hollande fluid or 4% buffered paraformaldehyde solution, embedded in Paraplast Plus (Sherwood Medical, Deland, FL, USA) and sectioned at 5 µm. After dewaxing and rehydration, sections fixed in Bouin-Hollande fluid were stained with haematoxylin-eosin (HE), while sections fixed in paraformaldehyde solution were subjected to an indirect immunocytochemical method [14] using i) G7 monoclonal antibody (mAb), which is specific to gilthead seabream acidophilic granulocytes [15], ii) purified rabbit polyclonal antiserum (pAb) raised against a synthetic peptide corresponding to an internal epitope of the extracellular domain of the gilthead seabream macrophage-colony stimulating factor receptor (Mcsfr, SLRVVRKEGEDYLLPC, Pacific Immunology, California, CA, USA) [16] or iii) purified rabbit polyclonal antiserum raised against a synthetic peptide corresponding to an internal

epitope corresponding to the C-terminus of the gilthead seabream Il1b (RRHRIFKFLPPKPEVEGEC, Pacific Immunology) [17, 18].

The sections used to localize acidophilic granulocytes were rinsed in Coons buffer (0.01 M sodium diethylbarbiturate, 0.1 M NaCl, pH 7.4) and in Coons buffer containing 0.01% bovine serum albumin (BSA) and 0.2% Triton X-100 (CBT). After incubation for 30 min with skimmed milk powder in Coons buffer to block the non-specific reaction, the sections were rinsed in CBT and incubated overnight at 4 °C with the G7 mAb at the optimal dilution of 1:100. After washing in CBT, the sections were exposed to swine anti-rabbit IgG (Dako, Glostrup, Denmark) diluted 1:20 with CBT for 1 h at room temperature, washed with CBT and incubated with rabbit peroxidase-antiperoxidase complex (Dako) diluted 1:100 with CBT for 1 h at room temperature. The sections were then washed in CBT and in 0.5 M Tris-HCl buffer (pH 7.6).

The sections immunostained with anti-Mcsfr serum were incubated in ethylenediaminetetraacetic acid (1 mM, pH 8.0) at 95 °C for 20 min to retrieve the antigen. After cooling, they were incubated for 40 min in peroxidase quenching solution (H₂O₂ in methanol, 1:9) to eliminate the endogenous peroxidase. After that, the sections used to localize both the Mcsfr and Il1b were rinsed in phosphate-buffered saline (PBS, pH 7.2–7.4) and in PBS containing 0.01% BSA and 0.2% Triton X-100 (PBT). After incubation for 30 min with PBS containing 0.5% BSA to block the non-specific reaction, they were rinsed in PBT and incubated for 2 h at room temperature with anti-Mcsfr serum (Pacific Immunology) at the optimal dilution of 1:100 or with anti-Il1b serum (Pacific Immunology) at the optimal dilution of 1:100. After washing in PBT, the sections were exposed to anti-rabbit IgG (whole molecule)-biotinylated antibody produced in swine (Dako) diluted 1:100 for 1 h at room temperature. Subsequently, the samples were washed in PBT and incubated for 1 h at room temperature with the avidin-biotinylated peroxidase complex (Dako) according to the manufacturer's instructions. The samples were then washed in PBT and in 0.5 M Tris-HCl buffer (pH 7.6).

The peroxidase activity of all immunocytochemical reactions was revealed by incubation with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) in Tris-HCl buffer (pH 7.6) containing 0.05% H₂O₂ for 15 min at room temperature. The specificity of the reactions was determined by omitting the first antiserum and by parallel incubation with the antiserum pre-adsorbed with a 10 molar excess of the synthetic peptide used to raise the corresponding serum. Slides were examined with an Axiolab (Zeiss, Madrid, Spain) light microscope.

Results

Gilthead seabream is a protandrous hermaphroditic teleost that first develops as male, although its gonads possess a non-developed ovarian area separated from the testicular area by connective tissue [3–5].

Gonad development: from hatching to spermatogenesis

On day 48 post-hatching when most of the organs of the fish are already developed, the primordia of the gonad appeared as a few

Fig. 1. Paraffin embedded sections from gilthead seabream larvae at 48 (a), 62 (b) and 83 (c) dph and from juveniles at 111 dph (d) and from the gonads of 180 dph juveniles (e, f) and 210 (g, h) and 240 (i) dph males stained with haematoxylin-eosin. In 48 (a) and 62 (b) dph larvae, germ cells are clearly distinguished as clusters in the coelomic mesoderm and are surrounded by small basophilic cells. In 83 (c) dph larvae and 111 (d) dph juveniles, the gonad primordia constitute a cord of cells. However, from 180 dph (e) onward, the testicular and ovarian areas are clearly distinguishable. Interestingly, in 111 (d) and 180 (e) dph juveniles, the gonad primordia are mostly occupied by numerous eosinophilic cells. In 180 dph juveniles, the testicular area is formed by mesenchymal cells and scarce germinal cells (inset Fig. 1e), while the ovarian tissue is mainly formed by mesenchymal cells (f). The acidophilic granulocytes remain in a different part of the gonad until they are completely developed. Thus, they are scarce in the well-developed testicular areas of 210 dph males (g, h) and numerous in the ovarian areas of 240 dph males (i). Scale bars=200 μ m (e), 100 μ m (g), 50 μ m (inset e, f, h), 25 μ m (d, i) and 10 μ m (a–c). K, kidney; G, gut; Pc, pancreas; P, peritoneal epithelium; Gp, gonadal primordium; T, testicular area; OC, ovarian cavity; O, ovarian area; ED, efferent duct; H&E, hematoxylin-eosin. Germ cells, somatic cells and oocytes are indicated by black arrows, white arrows and white arrowheads, respectively.

germ cells surrounded by a small amount of small basophilic cells. These primordia were located in the posterior region of the peritoneal cavity and were in contact with the peritoneal epithelium (Fig. 1a). The germ cells could be clearly distinguished by their large nuclei with a centrally positioned nucleoli and an eosinophilic cytoplasm. Some of the cells that surrounded the germ cells were in close proximity with them and had a triangular nuclei characteristic

of Sertoli cells (Fig. 1a). On day 62 post-hatching, no differences in the morphology of the gonad primordia were observed with respect to those previously described (Fig. 1b). On day 83 post-hatching, the gonad primordia were observed to be constituted by a cord of cells located in the dorsal region of the peritoneal cavity and separated from the peritoneal epithelium (Fig. 1c). Interestingly, in juveniles (111 dph) the gonad primordia were mostly occupied by numerous eosinophilic cells (Fig. 1d).

Both testicular and ovarian areas started to be clearly distinguished later in development (180 dph), although neither the testis nor the ovary was completely differentiated (Fig. 1e). The testis started to develop from the cephalic to caudal region of the gonad, showing a typical arrowhead shape (Fig. 1e) and spreading until it occupied the lateroventral part of the gonad. The testicular area was formed by mesenchymal cells, scarce germinal cells (Fig. inset 1e) and numerous eosinophilic cells (Fig. 1e). On the other hand, the ovarian cavity appeared in the caudal area of the gonad (Fig. 1e), and the ovarian tissue, formed by mesenchymal cells and numerous eosinophilic cells (Fig. 1f), was arranged around it. Later, the ovarian area occupied the mediadorsal area of the gonad.

Once the testicular area of the gonad was constituted by seminiferous tubules in which spermatogenesis had started (Fig. 1g), the specimens were considered to be males (from 210 dph). Thus, the testicular area showed the characteristic morphology previously described in adult fish [4]. In brief, the tubules consisted of spermatogonia stem cells (not surrounded by Sertoli 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 spermatozoa, which started to accumulate in the lumen of the tubules and the deferent duct (Figs. 1g and h), as occurs in the spermatogenesis stage of the first reproductive cycle of the one year old fish [4]. Moreover, the eosinophilic cells observed in the testicular area were scarce at this stage. Meanwhile, the ovarian area did not yet show the morphology seen in the one year old fish. Thus, although the folds of the germinal epithelium (called the ovigerous lamellae) were not developed in the ovarian area, the epithelial cells that limited the ovigerous lamellae were clearly distinguished in 240 dph fish (Fig. 1i). The germinal epithelium was formed by undifferentiated germ cells and numerous eosinophilic cells embedded in smooth connective tissue (Fig. 1i).

The eosinophilic cells observed in the developing testicular and ovarian areas are acidophilic granulocytes

All eosinophilic cells observed in the gonad of gilthead seabream juveniles were shown to be acidophilic granulocytes, since all of them specifically reacted with the G7 mAb (Figs. 2a and inset 2a). Interestingly, just as we observed in the case of eosinophilic cells, the acidophilic granulocytes appeared in the developing gonads of 111 dph fish and in the newly distinguished testicular and ovarian areas of 180 dph fish (Fig. 2a). However, once the testicular area was completely developed and spermatogenesis had started, the number of acidophilic granulocytes sharply decreased in the testis (Fig. 2b), and the cells appeared in the interstitial tissue as previously described in the spermatogenesis stage of the first reproductive cycle of the one year old fish [8]. In contrast,

Fig. 2. Paraffin embedded sections of the gonads of juvenile gilthead seabream at 180 (a, b, g) dph and 210 (d, 270 (h, i) and 300 (c, e, f) dph males immunostained with G7, an mAb against gilthead seabream acidophilic granulocytes (a, c, d), with serum against the gilthead seabream Mcsfr (e, f) or with pAb against gilthead seabream II1b (g–i). The acidophilic granulocytes correspond to the eosinophilic cells (a, b) previously described. Once the testicular area has initiated the spermatogenic process, the acidophilic granulocytes are scarce (c). However, numerous acidophilic granulocytes remain in the ovarian area of the gonad (d). The Mcsfr is located in the Sertoli cells that limit the lumen of the tubules and the efferent duct once the cysts have released the spermatozoa (e). Notice that all the Sertoli cell branches that limit the different cyst types are stained with anti-Mcsfr (f). In 180 (g) dph juveniles, the II1b-positive cells include mesenchymal cells, germ cells and acidophilic granulocytes. However, in 270 (h, i) dph male fish, most of the spermatocyte cysts and endothelial cells are also II1b positive. Scale bars=50 μ m (a–f, h, i) and 25 μ m (g). T, testicular area; OC, ovarian cavity; ED, efferent duct; G7, immunostained with G7 mAb; Mcsfr, immunostained with the serum against Mcsfr; H&E, hematoxylin-eosin; SC, spermatocytes; II1b, immunostained with serum against II1b. Germ cells, Sertoli cells branches that evolve the cysts, acidophilic granulocytes, mesenchymal cells, endothelial cells and ovarian areas are indicated by black arrows, black dashed arrows, white arrows, white arrowheads, black arrowheads and asterisks, respectively.

they were still numerous in the ovarian area of 210 dph fish (Figs. 2c and d).

Mcsfr appeared in the cells that line the tubules and efferent duct in the testicular area

When we investigated whether Mcsfr was present in the developing gonad, the protein was only detected in the Sertoli cells that formed cysts and in the Sertoli cells that limited the efferent duct after spermatozoa were released from the cysts of the newly developed testicular area in 240–300 dph fish (Figs. 2e and f). Interestingly, no immunostaining was detected in the interstitial tissue or in the ovarian area of the gonad in any of the stages analyzed (data not shown). The controls incubated with the preadsorbed antiserum were all negative (data not shown).

II1b mainly appeared in the cytoplasm of germ and mesenchymal cells in the testicular area

II1b was only detected in the testicular area of the gonads of juveniles in which the testicular and ovarian areas could be clearly distinguished at 180 dph (Fig. 2g). In those fish, both mesenchymal and germ cells were immunostained with anti-II1b (Fig. 2g). However, in 270 dph male fish, most of the spermatocyte cysts and endothelial cells were immunostained with anti-II1b (Figs. 2h and i). II1b was also detected in the acidophilic granulocytes of both juvenile and male fish (Figs. 2g–i). Interestingly, no immunostaining was detected in the ovarian area of the gonad in any of the stages analyzed (data not shown). The controls incubated with the preadsorbed antiserum were all negative (data not shown).

Discussion

In this paper, we have focused our attention on the appearance of acidophilic granulocytes and some immune molecules, such as II1b and Mcsfr, and correlated this with the ontogenetic process of the gonad. Immune cells as well as immune factors have been described to be present in the adult gonad and to be involved in the physiology of this organ in several fish species; specifically they are mainly involved in tissue remodelling and cell renewal [3–10].

In fish, the appearance of the first germ cells in the primordia of the gonad varies between species. Thus, in some species germ cells are already present in the gonadal primordia at day 1 after hatching, while in others, they appear around day 100 after hatching [1, 19, 20]. In the gilthead seabream, germ cells appeared as discontinuous clusters of cells in the coelomic mesoderm at day 48 post-hatching. The presence of germ cells in cord-like as discontinuous clusters intercalated with germ cell-free spaces during early gonadal development has also been reported in other teleosts [19, 21]. At day 83 post-hatching, a well formed cord structure was observed located ventral to the kidney in the dorsal region of the coelomic cavity. Later, at day 180 dph, both cephalic testicular and caudal ovarian areas were clearly distinguished in the gonad. Interestingly, the spermatogenic process started early (210 dph) in the testicular area of the gonad of the gilthead seabream. However, the ovarian area of these specimens was much less undifferentiated than the ovarian area of the gonad of the one year old fish, at which point it is formed by folds of the germinal epithelium that contain nests of oogonia and immature oocytes (pre-perinucleolar and perinucleolar) embedded in a smooth connective tissue and delimited by epithelial cells [4]. Although the ovary of the 210–300 dph fish

still lacks the ovigerous lamellae and immature oocytes, it contains numerous acidophilic granulocytes. However, in spite these differences compared with one year adult fish [4], the ovarian area responds to hormonal treatment, and it is possible to induce sex change in these fish using hormonal treatments [22]. In addition, the ovarian area of the gonad of gilthead seabream males continues to grow during the first two reproductive cycles in each post-spawning stage [4, 5].

Although the overall pattern and timing of gonad sex differentiation have been established in a considerable number of teleosts, no attention had been paid to the presence of immune cells or immune molecules in the gonadal primordia. Interestingly, our data demonstrate that acidophilic granulocytes and II1b are essential for renewal of the gonad after each spawning stage [3, 4, 8, 9, 12]. As gonad ontogeny involves tissue remodelling and cell renewal, we investigated whether acidophilic granulocytes and/or II1b were present in the gonadal primordia. Moreover, we also studied the pattern of appearance of Mcsfr, as we have previously demonstrated that this molecule is exclusively produced by differentiated somatic cells in the gonad of the gilthead seabream and can be used as a marker of the differentiation of these cells. In the present study, we describe the presence of numerous acidophilic granulocytes in the gonadal primordia of juveniles (from 111 to 180 dph), as also occurs in *Cichlasoma dimerus* testis [19], and in the ovarian area of the newly developed male gonad (from 210 to 300 dph). However, once the spermatogenesis started in the testicular area, the acidophilic granulocytes became scarce in this area as occurs in the spermatogenesis stage of adult males [4, 9]. In the ovarian area of the larvae and juvenile fish, the acidophilic granulocytes remained numerous, while in one year old fish, the ovarian area is much more developed, and acidophilic granulocytes are scarce [3]. Taking all these data into account, we suggest that acidophilic granulocytes are involved in gonadal morphogenesis.

Interestingly, these acidophilic granulocytes, together with mesenchymal cells, and a few germ cells were observed to produce II1b in 180 dph juveniles, whose testicular areas were not fully formed. Furthermore, in 270 dph males, in which the spermatogenesis process is active, II1b was located in acidophilic granulocytes, spermatocytes, as occurs in one year old fish [8], and in endothelial cells. Our observations of the gonadal primordia support the hypothesis that II1b is a germ cell growth factor, as is also the case in mammals [23–25]. Interestingly, in the developing gonad of the gilthead seabream, II1b appeared in mesenchymal and germ cells as well as acidophilic granulocytes, while in the adult gonads, II1b only appeared in germ cells and acidophilic granulocytes [8]. However, the prepubertal testes of mammals produce neither II1a nor II1b without previous stimulation by LPS, while the adult testis only produces constitutive II1a [26]. Interestingly, II1b is not produced in the developing ovarian area, although the oocytes present in the immature ovary areas of adult males produce this cytokine [8].

We also analyzed the appearance of Mcsfr during gonad development and only observed it in the well-developed testicular areas of 240–300 dph males. This molecule was observed in the Sertoli cells that surrounded the lumen of the tubules and the efferent duct, and this has also been observed in the adult testis [8]. Moreover, in

one year old adult fish, Mcsfr is also detected in the follicle cells of the immature ovary [8]. However, no immunoreaction was observed during early development of the gonad (either in undifferentiated Sertoli cells or in undifferentiated follicle cells). All this suggests that Mcsf only plays a role in the physiology of the adult gonad. Interestingly, neither II1b nor Mcsf were detected in the ovaries of young adult fish; however, these molecules were present in the ovarian area of the gonads of one year old adults. All these data demonstrated that the ovarian area was not completely developed in the male specimens analysed in the present study and that it needs a couple of years to really gain complete functionality. However, this ovarian area is able to respond to hormonal treatment and develops precociously [22].

In conclusion, the present study shows that the testicular area of the gonad of the gilthead seabream is quickly differentiated and that spermatozoa are produced in less than one year old specimens, while the ovarian area differentiates later and continues to develop during the first two years of life, as we described previously [4, 5]. Moreover, we also described for the first time, the timing of the appearance of an immune cell type, acidophilic granulocytes, and some immune molecules (II1b and Mcsfr) that have been demonstrated to be important in gonad remodelling in adult fish. These acidophilic granulocytes, together with other cells, produce II1b in juveniles and male fish throughout the process of morphogenesis of the testicular area; this supports the idea that II1b may be a proliferative factor for testicular germ cells, as suggested by our previous data in the adult gilthead seabream [8, 9]. However, the ovarian area does not produce this cytokine during gonadal morphogenesis. On the other hand, Mcsfr is not produced in the undifferentiated gonad and is only present once the testicular area is well developed. Although further studies are needed to clearly understand the role of acidophilic granulocytes in the gonad, taking into account our previous data in adults and the data described here in larvae and juveniles, we hypothesize that acidophilic granulocytes are essential for testicular tissue formation, remodelling and cell renewal because they are an important source of II1b, a potential testicular germ cell growth factor, as described in mammals [13]. On the other hand, Mcsfr seems not to be important in gonad differentiation, although it is produced by gonad somatic cells in adult tissue, so it may be useful as marker for differentiated somatic cell.

Acknowledgments

This work was supported by The Fundación Séneca, Coordination Centre for Research, CARM (GERM/04538 to A García Ayala and fellowships to S Liarte and I Mulero) and the Spanish Ministry of Science and Innovation (Programa Juan de la Cierva's contract to E Chaves-Pozo).

References

1. Devlin RH, Nagahama Y. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture* 2002; 208: 191–364.
2. Braat AK, Speksnijder JE, Zivkovic D. Germ line development in fishes. *Int J Dev Biol* 1999; 43: 745–760.
3. Chaves-Pozo E, Mulero V, Meseguer J, García-Ayala A. Professional phagocytic granulocytes of the bony fish gilthead seabream display functional adaptation to tes-

- ticular microenvironment. *J Leukoc Biol* 2005; 78: 345–351.
4. **Chaves-Pozo E, Mulero V, Meseguer J, García-Ayala A.** An overview of cell renewal in the testis throughout the reproductive cycle of a seasonal breeding teleost, the gilthead seabream (*Sparus aurata* L.). *Biol Reprod* 2005; 72: 593–601.
 5. **Liarte S, Chaves-Pozo E, García-Alcazar A, Mulero V, Meseguer J, García-Ayala A.** Testicular involution prior to sex change in gilthead seabream is characterized by a decrease in DMRT1 gene expression and by massive leukocyte infiltration. *Reprod Biol Endocrinol* 2007; 5: 20.
 6. **Besseau L, Faliex E.** Resorption of unemitted gametes in *Lithognathus mormyrus* (Sparidae, Teleostei): a possible synergic action of somatic and immune cells. *Cell Tissue Res* 1994; 276: 123–132.
 7. **Bruslé-Sicard S, Fourcault B.** Recognition of sex-inverting protandric *Sparus aurata*: ultrastructural aspects. *J Fish Biol* 1997; 50: 1094–1103.
 8. **Chaves-Pozo E, Liarte S, Fernández-Alácid L, Abellán E, Meseguer J, Mulero V, García-Ayala A.** Pattern of expression of immune-relevant genes in the gonad of a teleost, the gilthead seabream (*Sparus aurata* L.). *Mol Immunol* 2008; 45: 2998–3011.
 9. **Chaves-Pozo E, Pelegrín P, Mulero V, Meseguer J, García-Ayala A.** A role for acidophilic granulocytes in the testis of the gilthead seabream (*Sparus aurata* L., Teleostei). *J Endocrinol* 2003; 179: 165–174.
 10. **Lo Nostro FL, Antoneli FN, Quagio-Grassiotto I, Guerrero GA.** Testicular interstitial cells, and steroidogenic detection in the protogynous fish, *Synbranchus marmoratus* (Teleostei, Synbranchidae). *Tissue Cell* 2004; 36: 221–231.
 11. **Hedger MP.** Macrophages and the immune responsiveness of the testis. *J Reprod Immunol* 2002; 57: 19–34.
 12. **Chaves-Pozo E, Liarte S, Vargas-Chacoff L, García-López A, Mulero V, Meseguer J, Mancera JM, García-Ayala A.** 17Beta-estradiol triggers postspawning in spermatogenically active gilthead seabream (*Sparus aurata* L.) males. *Biol Reprod* 2007; 76: 142–148.
 13. **Hedger MP, Meinhardt A.** Cytokines and the immune-testicular axis. *J Reprod Immunol* 2003; 58: 1–26.
 14. **Sternberger LA.** Immunocytochemistry. New York: Wiley; 1986.
 15. **Sepulcre MP, Pelegrín P, Mulero V, Meseguer J.** Characterisation of gilthead seabream acidophilic granulocytes by a monoclonal antibody unequivocally points to their involvement in fish phagocytic response. *Cell Tissue Res* 2002; 308: 97–102.
 16. **Roca FJ, Sepulcre MA, López-Castejón G, Meseguer J, Mulero V.** The colony-stimulating factor-1 receptor is a specific marker of macrophages from the bony fish gilthead seabream. *Mol Immunol* 2006; 43: 1418–1423.
 17. **López-Castejón G, Sepulcre MP, Roca FJ, Castellana B, Planas JV, Meseguer J, Mulero V.** The type II interleukin-1 receptor (IL-1RII) of the bony fish gilthead seabream *Sparus aurata* is strongly induced after infection and tightly regulated at transcriptional and post-transcriptional levels. *Mol Immunol* 2007; 44: 2772–2780.
 18. **Mulero I, Pilar Sepulcre M, Roca FJ, Meseguer J, García-Ayala A, Mulero V.** Characterization of macrophages from the bony fish gilthead seabream using an antibody against the macrophage colony-stimulating factor receptor. *Dev Comp Immunol* 2008; 32: 1151–1159.
 19. **Meijide FJ, Lo Nostro FL, Guerrero GA.** Gonadal development and sex differentiation in the cichlid fish *Cichlasoma dimerus* (Teleostei, Perciformes): a light- and electron-microscopic study. *J Morphol* 2005; 264: 191–210.
 20. **Shimizu A, Hamaguchi M, Ito H, Ohkubo M, Udagawa M, Fujii K, Kobayashi T, Nakamura M.** Appearances and chronological changes of mummichog *Fundulus heteroclitus* FSH cells and LH cells during ontogeny, sexual differentiation, and gonadal development. *Gen Comp Endocrinol* 2008; 156: 312–322.
 21. **Parmentier HK, Timmermans LP.** The differentiation of germ cells and gonads during development of carp (*Cyprinus carpio* L.). A study with anti-carp sperm monoclonal antibodies. *J Embryol Exp Morphol* 1985; 90: 13–32.
 22. **Condeca JB, Canario AV.** The effect of estrogen on the gonads and on *in vitro* conversion of androstenedione to testosterone, 11-ketotestosterone, and estradiol-17beta in *Sparus aurata* (Teleostei, Sparidae). *Gen Comp Endocrinol* 1999; 116: 59–72.
 23. **Khan SA, Khan SJ, Dorrington JH.** Interleukin-1 stimulates deoxyribonucleic acid synthesis in immature rat Leydig cells *in vitro*. *Endocrinology* 1992; 131: 1853–1857.
 24. **Parvinen M, Soder O, Mali P, Froysoa B, Ritzen EM.** *In vitro* stimulation of stage-specific deoxyribonucleic acid synthesis in rat seminiferous tubule segments by interleukin-1 alpha. *Endocrinology* 1991; 129: 1614–1620.
 25. **Pollanen P, Soder O, Parvinen M.** Interleukin-1 alpha stimulation of spermatogonial proliferation *in vivo*. *Reprod Fertil Dev* 1989; 1: 85–87.
 26. **Jonsson CK, Setchell BP, Martinelle N, Svechnikov K, Soder O.** Endotoxin-induced interleukin 1 expression in testicular macrophages is accompanied by downregulation of the constitutive expression in Sertoli cells. *Cytokine* 2001; 14: 283–288.