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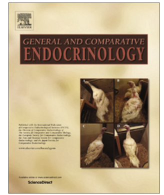

Research paper

Sex hormone binding globulin: Expression throughout early development and adult pejerrey fish, *Odontesthes bonariensis*

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A B S T R A C T

Sex hormone binding globulin (Shbg) is a plasma glycoprotein that binds and transports steroids in the blood of all vertebrate classes apart from birds. In the present study we characterized *shbg* from pejerrey, a fish species with a well characterized temperature-dependent sex determination. The pejerrey *shbg* mRNA comprises 1185 bp encoding for a 395 amino acid Shbg precursor protein that includes a leader sequence for secretion. Relative quantification of *shbg* transcript abundance revealed expression early in development coinciding with the sex-determining period and probably in association with temperature leading to male determination. The hepatopancreas was the main site of *shbg* expression, which varied according to the sex cycle in females. It was also expressed in gills, gonads, gut and taste buds during both larval stages and in adult fish. The presence of Shbg in organs in close contact with the environment such as gills, pseudobranchs, gut and taste buds suggests that these are potential sources of uptake or release of steroids/xenosteroids to and from the aquatic environment.

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1. Introduction

Sex hormone-binding globulins (SHBGs) are plasma glycoproteins, produced primarily in the liver, and they influence the metabolic clearance rates of sex steroids in vertebrates (Hammond, 2011, 2016). Thus, plasma SHBG levels influence the bioavailability of sex steroids and their access to target tissues (Hammond, 2016). Orthologues of human SHBG have been identified in elasmobranchs (Freeman and Idler, 1969; Ho et al., 1980), teleost fish (See Bobe et al., 2010 for details), amphibians (Caneguim et al., 2013), reptiles (Jennings et al., 2000) and in other mammals (Damassa et al., 1996; Selva and Hammond, 2006; Hammond et al., 2012), but there is no evidence of its presence in birds (Malisch and Breuner, 2010). These orthologues have several conserved characteristics: they share the same gene organization; they are all homodimeric glycoproteins with two laminin G-like (LG) domains, they have two sets of cysteines that form intramolecular disulphide bridges and, in the case of teleosts, three N-glycosylation consensus sites (Miguel-Queralt et al., 2004, 2005, 2009). In mammals the presence of alternatively spliced variants is well documented (Joseph, 1994; Hammond et al., 1989; Selva et al., 2005; Nakhla et al., 2009; Pinós et al., 2009), but little is known about this in other vertebrate groups.

In fish, the main site of *shbg* transcription is the liver, but it has also been detected in extra-hepatic sites, including the digestive tract, testis, spleen, stomach and brain (Miguel-Queralt et al., 2004, 2007, 2009; Bobe et al., 2010). In addition, Shbg was detected in the liver, gut, testes, the connective tissue around the ovary and skeletal muscle, during both reproductive and non-reproductive season (Miguel-Queralt et al., 2004, 2007). Moreover, plasma Shbg levels vary during the sex cycle (Foucher et al., 1992; Laidley and Thomas, 1997; Hobby et al., 2000).

In addition to its role as a steroid carrier protein, local functions of Shbg have been reported in specific tissues both in humans and fish. For example, in normal and cancer human cells, SHBG may participate in signal transduction at the cell membrane, where it has been reported to bind to a membrane receptor (R_{SHBG}, Hryb et al., 2002; Fortunati et al., 2010). In teleosts, Shbg protein and transcripts were found in the gills, suggesting it could have a local function probably related to the release of endogenous steroids or the uptake of natural or synthetic steroid ligands from the aquatic environment (Miguel-Queralt and Hammond, 2008).

The expression pattern of *shbg* and the plasma concentrations of Shbg have been studied in a few teleost species especially in the context of processes in which sex steroids have important roles, such as development and reproduction (Bobe et al., 2010). In all

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fish species studied, *shbg* was expressed early in development (Miguel-Queralt et al., 2004, 2007) and, it is presumed that its presence influences the distribution of sex steroids that are involved in gonadal differentiation. However, in adult fish, the results are highly dependent on the species; while Shbg fluctuated in blood in parallel to sex steroids levels in spotted weakfish (*Cynoscion nebulosus*, Laidley and Thomas, 1997) and Indian major carp (*Labeorohita*, Suresh et al., 2008), this was not the case in common carp (*Cyprinus carpio*, Chang and Chen, 1990), brown trout (*Salmo trutta*, Pottinger, 1988) or sea bass (*Dicentrachuslabrax*, Miguel-Queralt et al., 2007). Moreover, in sea bass, the latter authors assumed that Shbg levels responded to the changes in feeding and metabolic state, associated with the reproductive season in this species, rather than to changes in sex steroid levels (Miguel-Queralt et al., 2007).

Our model species, the pejerrey (*Odontesthes bonariensis*), is a fish native of Argentina (Somoza et al., 2008). Pejerrey has become a model fish to study the influence of temperature on the process of sex determination and differentiation (Fernandino et al., 2015; Yamamoto et al., 2014), and it has been demonstrated to be very sensitive to different pollutants including xenoestrogens (Carriquiriborde et al., 2009; Gasulla et al., 2016; Pérez et al., 2012). Since environmentally relevant concentrations of xenoestrogens have been detected in water bodies where this fish inhabits (Valdés et al., 2015) and because Shbg is considered to be a potential vector in the uptake of xenosteroids from the environment, our immediate goal has been to characterized the pejerrey fish *shbg*, to study its gene expression pattern throughout development and in sexually mature fish and to analyze its tissue distribution.

2. Materials and methods

2.1. Fish, source and handling

Pejerrey fish were obtained from the IIB-INTECH aquatic facility and from the Chascomús Lagoon (35°36′S58°02′W) depending on the experiment. All fish were handled in accordance with the UFAW Handbook on the Care and Management of Laboratory Animals (http://www.ufaw.org.uk) and IIB-INTECH internal institutional regulations.

2.2. Pejerrey shbg cDNA characterization

To characterize the pejerrey *shbg* coding sequence, total RNA was isolated from the hepatopancreas of captive adult pejerrey using TRIzol Reagent (InvitrogenTM, Life Technologies). RNA concentration and the quality of each sample were determined using a Sinergy H1 spectrophotometer (BioTek Instruments Inc, Winooski, Vermont, USA), and the purity of each sample was verified by 260/280 nm ratio. RNA samples were treated with DNase I (Invitrogen) and then reverse transcribed using SuperScript II, RNase OUT (Invitrogen) and oligo (dT) 12–18 following the manufacturer instructions. A pair of consensus forward and reverse primers, shbgdfwp and shbgdrvp (Table 1), were designed taking into consideration highly conserved regions of *shbg* from phylogenetically related species as *Dicentrarchus labrax* (AY700574.1), *Oryzias latipes* (XM_004079810.2) and *Verasper moseri* (AB243105.1) in order to amplify a fragment of approximately 1 kbp. The PCR reaction was performed using 1 μL of hepatopancreas cDNA as template using the following program: 5 min at 94 °C, 35 cycles with the following sequence: 94 °C for 20 s, 60 °C for 20 s and 72 °C for 45 s, and a final elongation step at 72 °C for 3 min. The resulting PCR products were then cloned in a bacterial vector using the pGEM-T Easy kit (Promega Corp.) and *Escherichia coli* OmniMAX competent cells were then transformed. White colonies were selected from X-Gal/IPTG ampicillin agar plates and grown in LB/ampicillin liquid media. Plasmid DNA was then extracted using the miniprep protocol (QIAGENE), sequenced and submitted to GenBank/EMBL for comparison to known accessible sequences. Once we had obtained the pejerrey *shbg* fragment sequence (KF680077.1), it was used to blast the pejerrey genome database (Campanella et al., 2013) to obtain the sequence of *shbg* gene and the presumptive pejerrey shbg mRNA sequence. To confirm the pejerrey shbg mRNA sequence two additional primers located in the presumptive exon 1 and exon 3 were designed (shbgspFw1-3 and shbgspRV1-3, Table 1). In addition, specific forward and reverse primers (shbgspFw and shbgspRv, Table 1) were designed to measure the abundance of *shbg* transcripts by Real Time quantitative PCR (RT-qPCR). The locations of all these primers within the pejerrey *shbg* gene are specified in Fig. 1.

Table 1

Gene	Primer name	Primer sequence	Size
shbg	shbgdfwp	CTGATCCACACARCAGTCAACCTC	1060 pb
	shbgdrvp	AGGGCAGCTGTGAGAGGAGA	
shbg	shbgspFw1-3	GTGGCAGGGTATTTCTGCTG	400 pb
	shbgspRV1-3	CTGTCCCTTCAAGATGGCAT	
shbg	shbgspFw	CGGAGACACCAAAAATGGAG	75 pb
	shbgspRv	CCTCTGATGCAGATCAGCAA	
β-Actin	actinFw	CTCTGGTCGTACCACTGGTATCG	83 pb
	actinRv	GCAGAGCGTAGCCTTCATAGATG	

2.3. Thermal Manipulation of sex determination

Fertilized eggs were obtained by artificial insemination using gametes from captive-reared pejerrey brood stock from the IIB-INTECH aquatic facility and incubated at 18 ± 0.5 °C in flow-through brackish water (salinity: 5 g/L) incubators until hatching. Approximately 400 newly hatched larvae were stocked in two 60 L tanks set at 17 ± 0.5 °C (female-producing temperature: FPT) and 29 ± 0.5 °C (male-producing temperature: MPT) for 6 weeks after hatching (wah), and then reared at 25 ± 0.5 °C until the end of the experiment. These temperatures were chosen because 100% of females can be obtained when larvae are reared at FPT or 100% of males at MPT, as already described by Strüssmann et al. (1997). Larvae were reared at these temperatures in flowing brackish water (salinity: 15 g/L), under a 16L-8D light cycle. They were fed four times daily to satiation with *Artemia nauplii* and powdered fish food (Shulet, Argentina). For gene transcript abundance, larvae were sampled from each thermal treatment at 3 and 5 (sex determination period; Strüssmann et al., 1997), 7 and 9 (morphological gonadal differentiation period; Ito et al., 2005) weeks after hatching (n = 10 per week per group) and stored at −80 °C in TRIzol Reagent (Thermo Fisher Scientific). For histological and immunohistochemical analyses, larvae were sampled at 9 wah, where gonadal differentiation is clearly defined (Ito et al., 2005).

2.4. Pejerrey tissues/organs sampling

Five adult female and five male pejerrey fish were sampled in the Chascomús Lagoon (35°36′S58°02′W) in August 2013 (beginning of the reproductive season) and ten more were sampled in May 2014 (non-reproductive season), using a towing net. Fish were caught 100 m from the coast at approximately 1.2 m depth. They were immediately placed in tanks with aeration and moved to the laboratory where they were terminally anesthetized with benzocaine (ethyl 4-aminobenzoate) and then dissected. The fish and

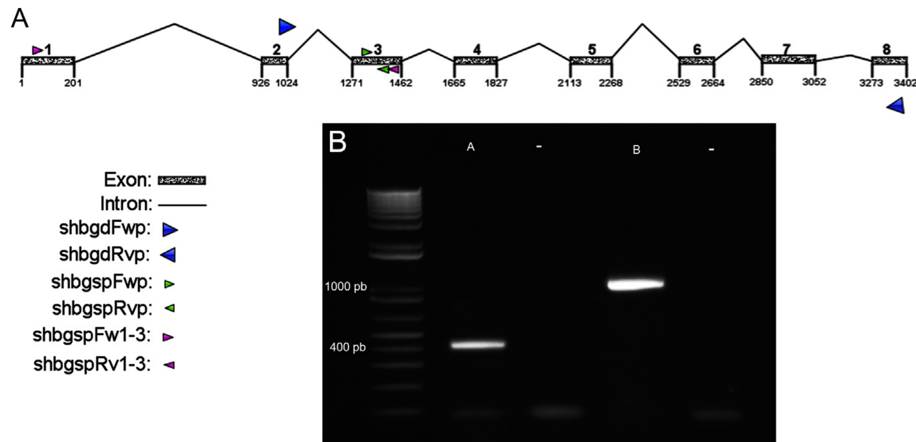

Fig. 1. A. Deduced structure of the *Odontesthes bonariensis shbg* gene. Primers used in this study are shown in the gene diagram. B. RT-PCR amplification of *shbg* cDNA from pejerrey hepatopancreas with two different primers sets. Lane A: amplicon obtained with primers shbgspFw1-3/ shbgspRV1-3 (from exon 1 to exon 3 coding regions). Lane B: amplicon obtained with primers shbgdfwp/shbgdrvp (from exon 2 to 8 coding regions) and their respective negative controls.

the gonads were weighed (TW and GW, respectively) to calculate gonadosomatic index (GSI% = 100GW/TW). A portion of the gills, liver, brain, heart, muscle, kidney, gut and gonads was dissected and immediately stored in TRIzol Reagent (Thermo Fisher Scientific) at −80 °C for total RNA extraction. A section of the contralateral gonad was also fixed in Bouińs fluid for 24 h, and stored in 70% ethanol for histological analysis. Ovarian and testicular stages were respectively defined according to the proportion of different oocyte developmental stages in the ovary and the number of the different types of germinal cells in the spermatogenic lobules respectively, following Elisio et al. (2014) and Elisio et al. (2015). Five individuals from each season were used to analyze the tissue/organ specific *shbg* transcript abundance.

2.5. Western blot analysis

Pejerrey hepatopancreas proteins (200 μg) and a Sea bass Shbg standard (50 ng) were heat-denatured in loading buffer and subjected to a discontinuous SDS-PAGE with 4% and 12% polyacrylamide in the stacking and resolving gels, respectively. Then, the proteins in the gel were transferred to an Immobilon–P PVDF membrane (Merck Millipore) and incubate for 1 h at room temperature with a Sea bass Shbg antiserum raised in rabbits against purified and deglycosylated recombinant sea bass Shbg. The full characterization of the antiserum is reported in Miguel-Queralt et al. (2005). The antiserum was diluted 1:2000 in Tris-Buffered Saline, 0.01% Tween 20 (TBS-T) with 5% skim milk powder. The membrane was then washed several times with TBS-T to remove excess of antiserum and specific antibody-antigen complexes were identified using secondary antibodies (alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG for 1 h at room temperature) and AP detection kit, using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP), as the chromogenic substrate, following the manufacturer's instructions (Promega Corporation, Madison, WI).

2.6. Immunohistochemistry

Pejerrey larvae (8 wah) and different adult pejerrey tissues/organs, taken from fish from IIB-INTECH aquatic facility stock, were fixed in Bouin solution for 24 h, and stored in 70% ethanol until used. Fixed samples were then dehydrated, embedded in Paraplast Plus and cut in 6 μm thick serial sections. The sections were then de-waxed and incubated at high power in a microwave oven for 5 min in citrate buffer (pH 6.0), then cooled at room temperature for 40 min, treated with 0.05% hydrogen peroxide solution for 45 min to inhibit endogenous peroxidase activity, and then blocked with Bovine Serum Albumin (Sigma-Aldrich), 5 mg/ml in Phosphate-Buffered Saline (PBS) at room temperature for one hour. The sections were then treated with Sea bass Shbg antiserum 1:2000 in PBS. The specificity of the reaction was first verified by preadsorbing the sea bass Shbg antiserum with 1 μM of recombinant European sea bass Shbg overnight at 4 °C before use, or by omission of the primary antiserum. Once the specificity of the immune reaction was established in the liver, the primary antiserum was omitted and substituted by PBS for further experiments. After an overnight incubation at 22 °C, immunoreactive Shbg (ir-Shbg) was revealed with a 0.5% 3.3-diaminobenzidine tetrahydrochloride in PBS containing 0.05% H_2O_2. In some cases we used immunofluorescent labeling, the sections were incubated at 37 °C for 90 min with the secondary antibody Alexa Fluor 488 (green) goat-anti-rabbit IgG (Invitrogen, Eugene, OR), diluted 1:100 in blocking solution. The sections were rinsed twice with PBS and mounted with mounting fluid (Sigma Aldrich, USA). When cell nucleus staining was required, sections were treated with 4′, 6-diamidino-2-phenylindole (DAPI, 5 μg/ml, Invitrogen^{TM}, Life Technologies) in PBS for 1 min, rinsed twice in PBS, and then mounted. Photographs sections were captured using the Nikon Eclipse E7000 and the Image Pro Plus (Media Cybernetcs, Bethesda, MD).

2.7. RNA extraction and quantification by RT-qPCR

Total RNA extracted using TRIzol reagent (Thermo Fisher Scientific) was used to synthetize cDNA from whole body larvae (whole body, from 3, 5, 7 and 9 wah) and eight different tissues/organs from adult fish: liver, gills, gonads, gut, kidney, heart, brain and muscle. The expression of *shbg* (Accession # KF680077) and β-actin (EF044319) as a reference gene was quantified by RT-qPCR. The transcript abundance of β-actin was already demonstrated to be constant in larvae and adult pejerrey fish (Fernandino et al., 2008, 2012). All primers used are given in Table 1. Each RT-qPCR reaction was performed in 15 μL, containing 7.5 μL of Fast Start Universal Master SYBR Green (Roche Applied Science), 1 μL of cDNA and 600 nM of each oligonucleotide. Samples were analyzed with Step One Plus Real-Time PCR System (Applied Biosystems, CA, USA). The amplification protocol consisted of an initial cycle of 1 min at 95 °C, followed by 10 s at 95 °C and 30 s at 60 °C for a total of 45 cycles. The subsequent quantification method was performed using the ΔΔCt method (threshold cycle, (www.appliedbiosystems.com/support/apptech).

2.8. Statistical analysis

Gene expression data was analyzed using fgStatistics software (Di Rienzo et al., 2009) based on the relative expression software tool by Pfaffl et al. (2002). In all cases statistical differences were considered to be significant when $p < 0.05$.

3. Results

3.1. Pejerrey shbg coding sequence

The deduced pejerrey Shbg gene structure has a conserved structure of 8 exons (Fig. 1A). The open reading frame of pejerrey *shbg* present 1185 bp and the deduced precursor polypeptide sequence has 395 amino acid residues (Supplementary Fig. 1). Based on the known amino-terminus of the mature Shbg sequence of the European sea bass (Miguel-Queralt et al., 2005), and the high level of identity between the amino-terminal regions of the pejerrey and European sea bass Shbg sequences, we can also deduce that the amino-terminus of the mature *secreted form of pejerrey Shbg is the Glu residue at position 36 in the* precursor polypeptide (Supplementary Fig. 1). Thus, the pejerrey Shbg precursor polypeptide sequence of 395 amino acid residues comprises a putative 35 residue signal polypeptide that could be removed prior to secretion, yielding a 360 residue mature pejerrey Shbg protein.

Among teleost species, the pejerrey Shbg amino acid sequence showed the highest identity with Percomorphaceans like European sea bass (73%), Turquoise killifish (*Nothobranchius furzeri*, 71%), topminnow (*Austrofundulus limnaeus*, 71%), medaka (*Oryzias latipes*, 69%), and Barfin flounder (*Verasper moseri*, 67%) and the lowest with Salmoniformes such as rainbow trout (*Oncorhynchus myskiss*, 52%), coho salmo (*Oncorhynchu skisutch*, 52%) and Cypriniformes, such as zebrafish (*Danio rerio*, 46%) and common carp (*Cyprinus carpio*, 46%). The identity was much lower when compared with mammalian SHBG sequences (32% against human and 30% against mouse).

To analyze the presence of alternatively spliced variants a PCR was performed with primers on exons 1 and 3, and 2 and 8 and mRNA from pejerrey fish hepatopancreas. In each case, only one band of the expected size was observed (Fig. 1).

3.2. shbg transcript abundance during pejerrey sex differentiation

Relative quantification of *shbg* transcript abundance in larvae was examined by RT-qPCR during early larval development. Pejerrey *shbg* mRNA was clearly detectable as early as 3 wah. In those larvae kept at FPT, *shbg* mRNA abundance was almost constant during this period, while larvae reared at MPT showed an increase of the relative abundance at 3, 7 and 9 wah compared to FPT (Fig. 2).

3.3. Localization of Shbg in larvae

The antiserum against European sea bass Shbg (Miguel-Queralt et al., 2005) was first validated for its use in pejerrey samples by western blotting. This antiserum recognized in pejerrey only one protein with an estimated molecular size corresponding to that of the fully glycosylated European sea bass Shbg (Supplementary Fig. 2).

The use of this antiserum in immunohistochemical studies in larvae, showed no immunoreactive Shbg (ir-Shbg) in hepatocyte cytoplasm, but a strong and granular ir-Shbg signal in the pancreas, which forms a composite organ with the liver, named the hepatopancreas (Fig. 3A). Some epithelial cells with ir-Shbg were also found in the gut (Fig. 3B). We also found a clear ir-Shbg signal in taste bud cells located at the epidermis (Fig. 3C). Interestingly, we detected an intense ir-Shbg staining in red blood cells within larval gills, particularly in the central blood capillary of the primary lamella either by labeling with diaminobenzidine or by immunofluorescense (Fig. 3D–E respectively). Similar labelling was observed in the larval pseudobranch (Fig. 3F). The specificity of this immnuo-reaction was verified by preadsorbing the primary antiserum with recombinant European sea bass Shbg (Figs. 3A′ and 5A′). Moreover, when the antiserum was omitted and substituted by PBS (Figs. 3B′-C′-D′-E′-F′, 5B′-C′-D′-E′), no reaction was observed in each case.

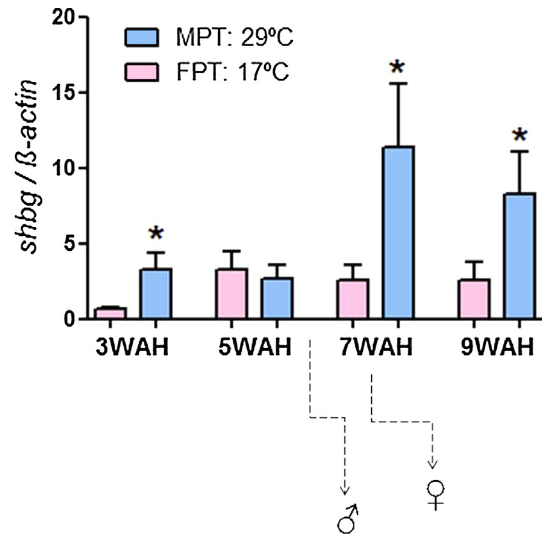

Fig. 2. Relative quantification of the *shbg* transcript abundance in pejerrey larvae during larval development at FPT (female producing temperature, 17 °C) and MPT (male producing temperature, 29 °C). The number of animals in each sampling point was 10. Asterisks mean significant differences between MPT and FPT at the same week. Arrows indicate the onset of morphological differentiation of the ovary or testis.

3.4. Tissue/organ shbg expression pattern

As pejerrey is a multiple spawner fish, female and male gonadal stages were characterized from fish sampled during reproductive and non-reproductive seasons by histology. Then the most characteristic stages from each season were further examined for both sexes: advanced vitellogenic females (VtgB, GSI% = 3.06 ± 0.4) in the reproductive and, females at the cortical alveoli stage (CA, GSI% = 1.28 ± 1.6) in non-reproductive season; and spermatocytary stage (SC, GSI% = 1.40 ± 0.5) in reproductive season and, spermatogonial stage (SG, GSI = 0.49 ± 0.3) in non-reproductive season in the case of males.

Transcript abundance of *shbg* in adult pejerrey was analyzed in hepatopancreas, gills, gonads, gut, kidney, heart, brain and muscle in reproductive and non-reproductive seasons. The hepatopancreas, from both sexes, irrespectively of reproductive status, presented the strongest abundance of *shbg* transcripts (Fig. 4A–B); however low transcripts levels were also found in the gills, gonads, heart, brain and muscle. Although males showed no differences in *shbg* hepatopancreas expression during the reproductive and non-reproductive seasons (Fig. 4B); females in the reproductive season showed a decreased liver *shbg* expression when compared to females in the non-reproductive season (Fig. 4A).

3.5. Localization of Shbg in adult fish

As in larvae, the strongest ir-Shbg was observed in the pancreas, with almost no signals in the hepatocytes (Fig. 5A). In the gut, a dif-

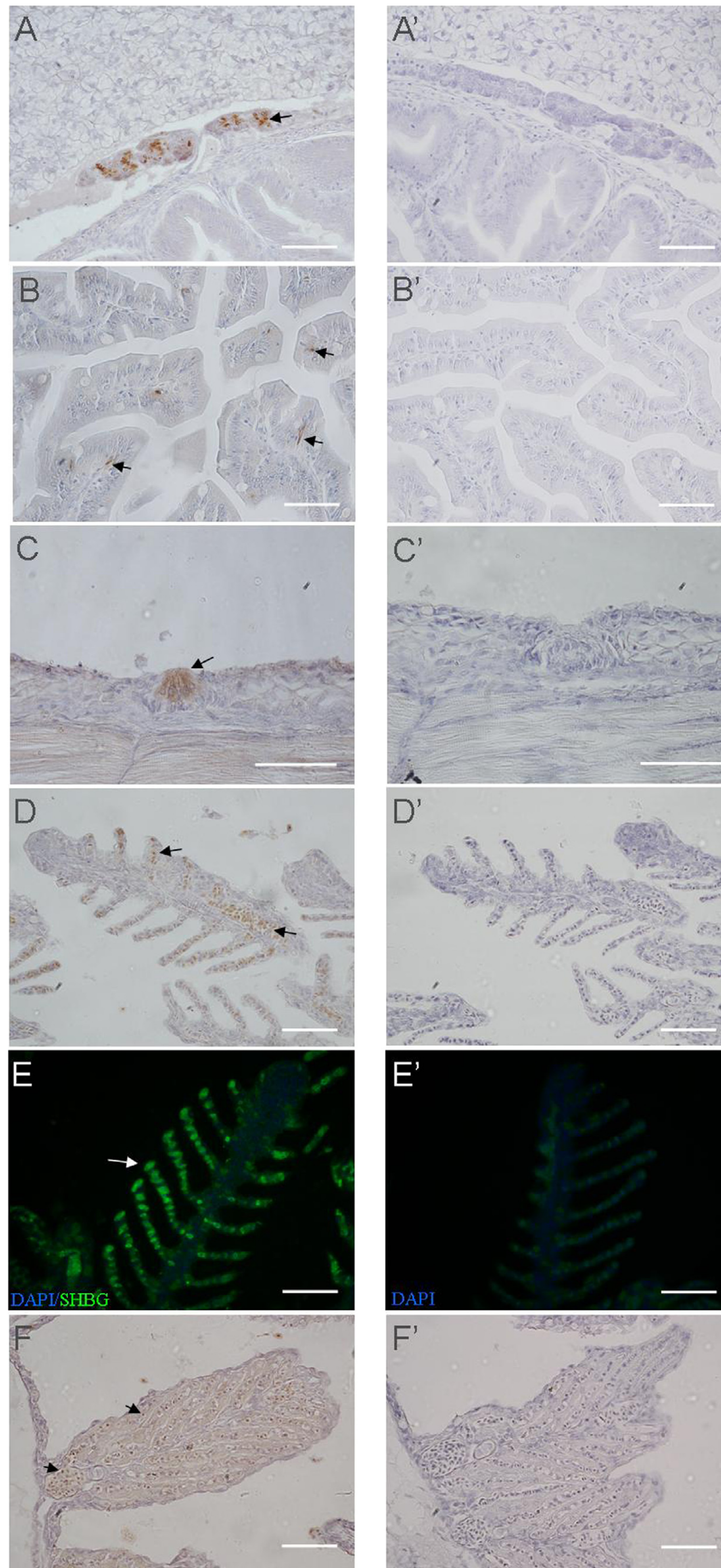

Fig. 3. Immunohistochemical localization of Shbg in pejerrey larvae (8wah) reared at 25 ± 0.5 °C. Serial sections were probed with rabbit anti-sea bass Shbg antiserum (A, B, C, D, E and F), with preadsorbed antiserum (A′) or without antiserum as negative controls (B′, C′, D′, E′ and F′). A: The arrow shows ir-Shbg in the pancreas that forms a composite organ with the liver. B: Scattered intestinal epithelial cells with ir-Shbg (arrow). C: ir-Shbg in taste buds within the epithelium (arrow). D–E: The arrows ir-Shbg in the central blood capillary of the primary lamella of larval gills, particularly in red blood cells (D with diaminobenzidine and E with immunofluorescense labelling). F: Larval pseudobranch showing ir-Shbg. Scales bars = 50 μm.

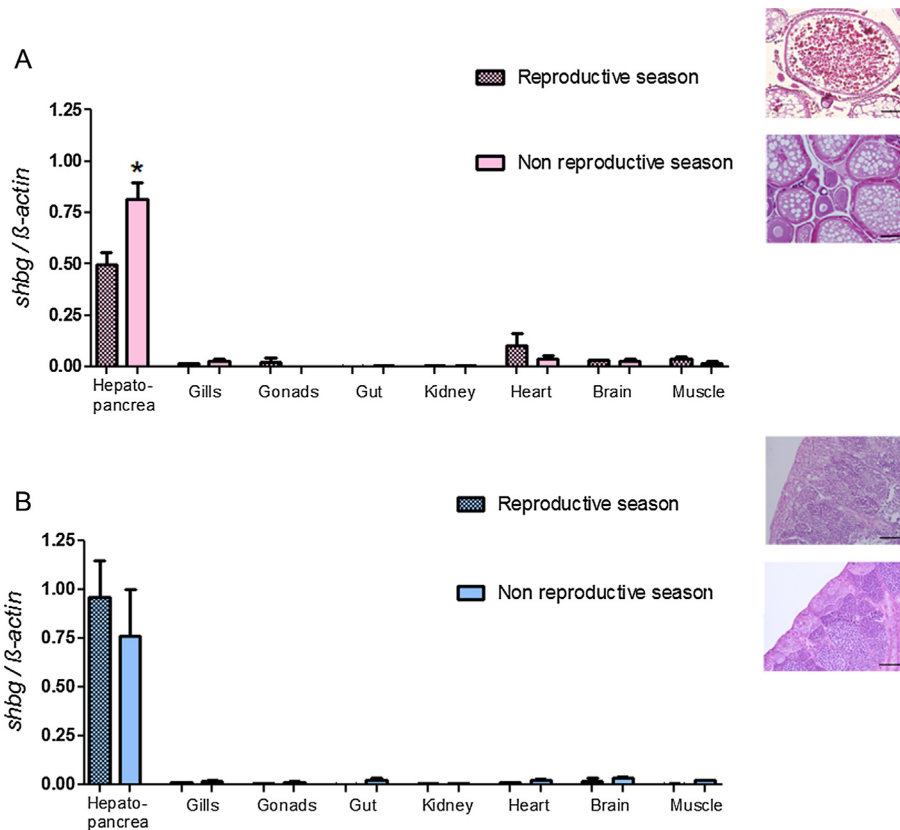

Fig. 4. Relative quantification of *shbg* transcripts by RTq-PCR in different organs/tissues from adult 5 pejerrey females (A) and 5 males (B) sampled during reproductive and non-reproductive seasons. Asterisk means significant differences between reproductive and non-reproductive seasons. The pictures represent histological sections of representative gonadal stages. Females: cortical alveoli (CA) stage in non-reproductive and advance vitellogenesis stage (VtgB) in reproductive season. Males: spermatogonial stage (SG) in non-reproductive and spermatocytary stage (SC) in reproductive seasons. Scale bars: 100 μm.

fuse ir-Shbg was observed in the cytoplasm of all epithelial cells (Fig. 5B). Adult pejerrey gills also presented ir-Shbg, however, unlike in larvae, it was mainly concentrated in endothelial cells of the primary lamella and in interlamellar cells. However no ir-Shbg was observed in red blood cells, which are nucleated in all fish species (Fig. 5C). Immunoreactive signals were also found in the gonads of both sexes. In the case of females, ir-Shbg was observed inside the oocyte, like dispersed drops near the plasma membrane, in the cytoplasm of primary oocytes, as well as on the chorionic filaments of vitellogenic oocytes (Fig. 5D). In males, ir-Shbg was mainly present surrounding the seminiferous lobules (Fig. 5E). However, no ir-Shbg positive material was found in the brain and muscle either in larvae or adult fish (data not shown).

4. Discussion

In all vertebrates, gonadal steroids fluctuate throughout the life cycle and are involved in the regulation of many processes such as embryonic development, sex differentiation, immune responses, circadian rhythms, stress and reproduction (Tokarz et al., 2015). Studies of SHBG are important because of the bioavailability of sex steroids depends on its actions, and more studies are needed to unravel basic aspects of its physiology in teleost species because of its possible role as portal of environmental xenobiotics (Miguel-Queralt and Hammond, 2008).

The pejerrey Shbg sequence reveals that it corresponds to the typical Shbgα, according to Bobe et al. (2010), and is distinct from the Shbgβ that seems to be specific to the salmonid lineage (Bobe et al., 2008). Moreover, a search in the pejerrey genome database (Campanella et al., 2013) did not reveal a paralog *shbgβ* sequence. The deduced pejerrey Shbg precursor sequence of 395 amino acids therefore resembles other teleost orthologues that comprise 380 to 400 residues (Bobe et al., 2010), and presents the highest identity when compared to other fish from the group Ovalentariae (Betancur-R et al., 2013).

The deduced pejerrey Shbg gene structure has a conserved structure of 8 exons, as in other fish species (Bobe et al., 2010). The initial characterization of pejerrey *shbg* mRNA from hepatopancreas shows single bands of the expected size using different primers and no clear evidence of alternative spliced variants were observed. These data, together with the lack of reports of *shbg* alternative variants in fish, suggest the absence of *shbg* alternative splicing in this species, contrary to what has been reported in mammals (Joseph, 1994; Hammond et al., 1989; Selva et al., 2005; Nakhla et al., 2009; Pinós et al., 2009).

In teleosts, sex steroids play important roles in the gonadal differentiation process (Nakamura, 2010; Tokarz et al., 2015). In pejerrey, estrogens and androgens are both important hormones in a critically sensitive period during the differentiation of the gonads (Fernandino et al., 2008, 2012, 2013; Karube et al., 2007). In this species the sensitive period to steroids overlaps the temperature sensitive window and expands from 1 to 5 wah depending on rearing temperature (Strüssmann et al., 1997). During this early developmental period, *shbg* transcripts were detected at both rearing temperatures consistent with previous reports in zebrafish and European sea bass, in which *shbg* was also observed early during development (Miguel-Queralt et al., 2004, 2007). Our results also suggested a temperature- and/or sex-bias expression, with a higher amount of *shbg*

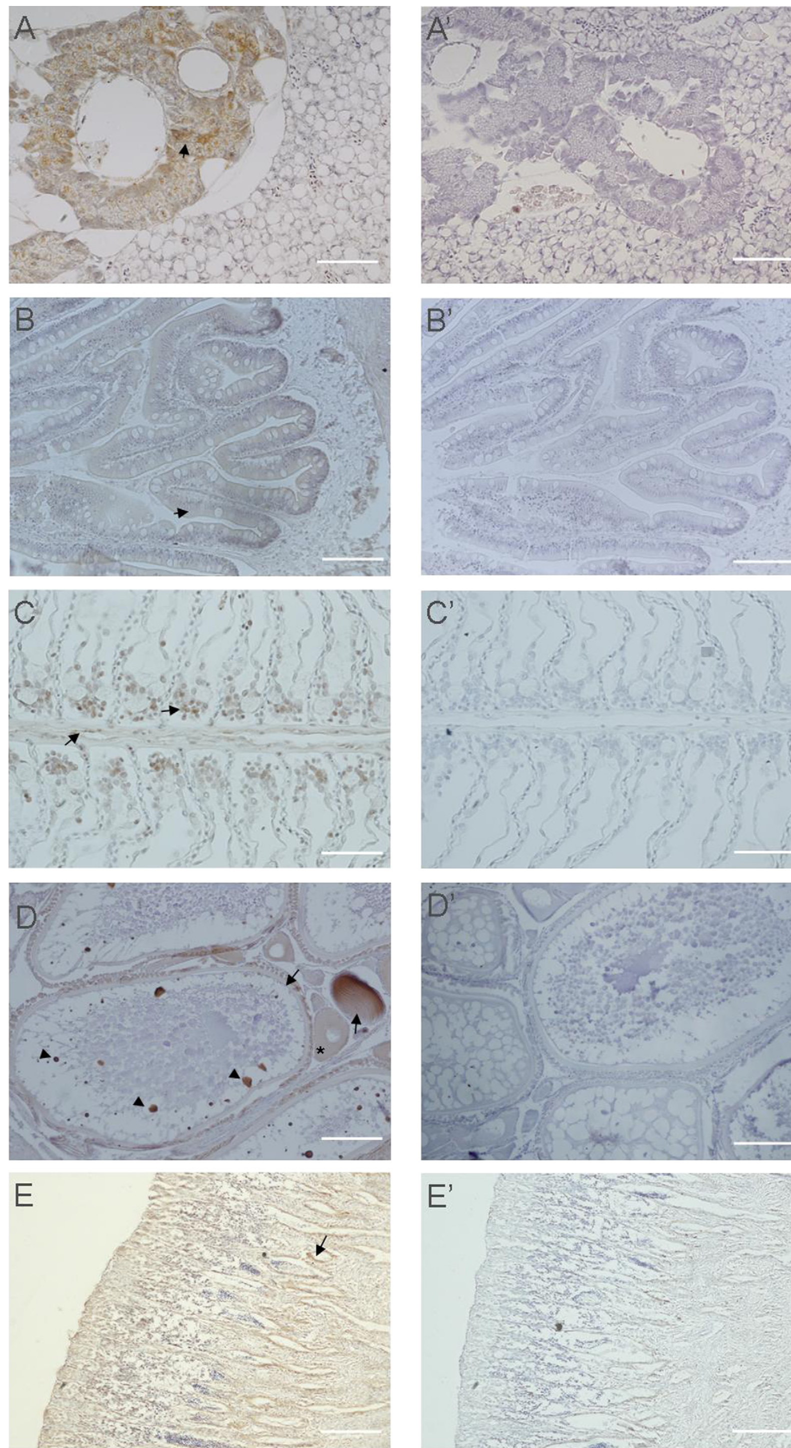

Fig. 5. Immunohistochemical localization of Shbg in adult pejerrey. Serial sections were probed with rabbit anti-sea bass Shbg antiserum (A, B, C, D and E), with preadsorbed antiserum (A′) or without antiserum as negative controls (B′, C′, D′ and E′). A: Hepatopancreas with ir-Shbg in the pancreas (arrow). B: Gut with a diffuse ir-Shbg staining in all epithelial cells (arrow). C: Pejerrey gill with ir-Shbg mainly concentrated in the epithelial cells both in secondary lamella as filaments (arrows). D: Section of the ovary of a vitellogenic female showing ir-Shbg in the cytoplasm of primary oocytes (*), near the plasmatic membrane as scattered drops (arrow heads) as well as in the chorionic filaments (arrows). E: Section of a testis at spermatocytary stage with ir-Shbg surrounded the seminiferous lobules (arrow). Scale bars: 100 μm.

transcripts in larvae reared at MPT compared to FPT at 3, 7 and 9 wah. It is important to note that differences between FPT and MPT, observed either at 3 and 9 wah, correlate with an increased expression of two enzymes that plays a key role in 11-oxygenated androgens synthesis, *hds11β2* at 3 wah (Fernandino et al., 2012) and *cyp11b2* at 9 wah (Blasco et al., 2010). However, although *cyp19a1a* or gonadal aromatase, is differentially expressed in FPT at 6 wah, with the first histological signal of the ovarian sex differentiation (Karube et al., 2007; Fernandino et al., 2008), no differences in *shbg* expression were observed. Whether early *shbg* expression in pejerrey development responds first to androgen stimulation is an unresolved question, because these differences could also be related to the exposure to different temperatures.

In pejerrey, as in other fish species, the liver (or the hepatopancreas) was the main source of Shbg (Bobe et al., 2010), but it was also weakly expressed in different tissues/organs like gills and gonads. As pejerrey is a multiple spawner and an asynchronous spawning fish (Somoza et al., 2008), it is common to observe different oocyte developmental stages in one ovary in a given period. For this reason the most representative gonadal stages were selected either for females and males for comparison. Transcripts of *shbg* were detected in the hepatopancreas of both females and males during the reproductive and non-reproductive seasons. However, *shbg* transcripts showed a clear decrease in abundance when females with vitellogenic were compared to those with previtellogenic oocytes at the cortical alveoli stage. It is important to note that, pejerrey females at the final vitellogenic stage (VtgB) are characterized by the highest plasma levels of both testosterone and estradiol (Elisio et al., 2014). A similar decrease of Shbg plasma levels during reproduction was also observed in both sexes of the European sea bass (Miguel-Queralt et al., 2007). However this behavior was observed in immature and triploid fish and, the latter authors, concluded that these variations were not related to the plasma levels of gonadal sex steroids, but in European sea bass it might be related to a reduction in food intake during the reproductive period (Zanuy and Carrillo, 1985). Moreover, long-term fasting has been reported to induce significant reductions in plasma Shbg levels in rainbow trout while castration had no effect (Foucher et al., 1992). In pejerrey there are some metabolic differences between sexes during the reproductive season. For example, mesenteric fat, which is inversely proportional to zooplankton availability, a feeding resource for pejerrey, is lower in males than in females during the reproductive season (Freyre et al., 2009), but it is also possible that these differences are related to male reproductive behavior because males are continuously active during prolonged periods of time during the reproductive season (Freyre et al., 2009). In this regard, it is known that lipogenesis regulates *SHBG* gene expression in the human liver (Selva et al., 2007), but further studies are needed to establish if *shbg* expression in the hepatopancreas is related to differences in nutrition and/or metabolic status in pejerrey during the reproductive season.

While the hepatopancreas was the organ with the highest *shbg* expression in pejerrey, lower levels of expression were also found in other tissues/organs by RT-qPCR and immunocytochemistry. The hepatopancreas of both, larvae and adult fish, showed almost no ir-Shbg material in the hepatocytes, while intense ir-Shbg staining was observed in the pancreas. Low ir-Shbg in hepatocytes was also observed in other fish species (Miguel-Queralt et al., 2004, 2007) and has been attributed to the fact that Shbg is rapidly secreted by the hepatocytes into the blood (Jänne et al., 1998). We did not measure *shbg* expression in the pancreas directly because it is almost impossible to dissect it from the hepatic tissue. However, if Shbg is not produced by pancreatic cell types, they may somehow sequester and retain it. We are currently analyzing this possibility using different approaches.

We also found, Shbg immunoreactivity in pejerrey ovaries like dispersed drops near the plasma membrane as well as on the chorionic filaments of vitellogenic oocytes and in the cytoplasm of primary oocytes. This location is somewhat similar to that reported in post-vitellogenic oocytes of zebrafish (Miguel-Queralt et al., 2004) and European sea bass (Miguel-Queralt et al., 2007); however the cytoplasmatic presence of ir-Shbg in previtellogenic oocytes of pejerrey suggests that variations in the presence of Shbg along the gonadal cycle, probably regulate the steroid bioavailability in and/or around the oocytes. In the testes, ir-Shbg was confined to the outer margins of the seminiferous lobules and was not restricted to a particular cell type, as already reported in zebrafish (Miguel-Queralt et al., 2004). In this respect it seems that this is a common pattern in teleosts and, as already discussed by the latter authors, this localization may help to regulate androgen access to both germinal cells and developing sperm.

As in zebrafish (Miguel-Queralt and Hammond, 2008), we observed ir-Shbg in the gills of adult pejerrey fish, mainly concentrated in the epithelial cells. This observation led these authors to propose the gills serve as a portal of xenosteroids in fish. We also found ir-Shbg in larval gills and pseudobranchs, where surprisingly it was clearly detected in red blood cells by both diaminobenzidine staining and immunofluorescence as in other organs. However in adults, ir-Shbg was never found in red blood cells. The physiological significance of this is unknown but, the ir-Shbg in the gills of both adults and larvae was clear, and it does not reflect the low transcript abundance in this organ. One possible explanation is that the gills accumulate plasma Shbg originating from the hepatopancreas but additional studies should be conducted to clarify this point.

In pejerrey, ir-Shbg was found in the gut, as observed in zebrafish (Miguel-Queralt et al., 2004). We also observed ir-Shbg for the first time in taste buds within the epidermis. It is known that the skin of teleosts contains specialized cells to detect mechanical and/or chemical changes in the microenvironment (Hara, 2000). The taste buds are cutaneous chemosensory cells (Hansen et al., 2002; Kotrschal et al., 1997) that enable fish to identify food by detecting different chemical substances at short distances (Buddington and Kuz'mina, 2000). The function of Shbg in these cells is not known, but estrogens have been involved in neuromasts development in amphibians (Hamilton et al., 2014) and all these sensory systems may have a local estrogenic system that involves the participation of Shbg. Whatever the case, the presence of Shbg in the gut and taste buds could indicate that these two locations are also involved in detection and/or release of sex steroids and the uptake of xenosteroids from the environment. In this context, these organs, together with the gills, can account for of the enormous capacity of fish to rapidly capture specific steroids from water (Maunder et al., 2007; Miguel-Queralt and Hammond, 2008).

In sum, our studies demonstrate that *shbg* showed a sexually dimorphic expression. It is expressed early in pejerrey development, with significant differences between FPT and MPT, as well as between females and males during the reproductive season. Also, while we found that the hepatopancreas is the main site of *shbg* expression, shbg was found in the gonads and other tissues including the gills, gut and taste buds, where it may act to control the release and/or uptake of steroids/xenosteroids from the aquatic environment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2017.02.004.

References

Betancur-R, R., Broughton, R.E., Wiley, E.O., Carpenter, K., López, J.A., Li, C.-H., Holcroft, N.I., Arcila, D., Sanciangco, M., Cureton II, J.C., Zhang, F.-F., Buser, T., Campbell, M.A., Ballesteros, J.A., Roa-Varon, A., Willis, S., Borden, W.C., Rowley, T., Reneau, P.C., Hough, D.J., Lu, G.-Q., Grande, T., Arratia, G., Ortí, G., 2013. The tree of life and a new classification of bony fishes. PLoS Curr. Tree Life. http://dx.doi.org/10.1371/currents.tol.53ba26640df0ccaee75bb165c8c26288. Ed. 1.

Blasco, M., Fernandino, J.I., Guilgur, L.G., Strussmann, C.A., Somoza, G.M., Vizziano-Cantonnet, D., 2010. Molecular characterization of *cyp11a1* and *cyp11b1* and their gene expression profile in pejerrey (*Odontesthes bonariensis*) during early gonadal development. Comp. Biochem. Physiol. 156A, 110–118.

Bobe, J., Guiguen, Y., Fostier, A., 2010. Diversity and biological significance of sex hormone-binding globulin in fish, an evolutionary perspective. Mol. Cell. Endocrinol. 316, 66–78.

Bobe, J., Mahe, S., Nguyen, T., Rime, H., Vizziano, D., Fostier, A., Guiguen, Y., 2008. A novel, functional, and highly divergent sex hormone-binding globulin that may participate in the local control of ovarian functions in salmonids. Endocrinology 149, 2980–2989.

Buddington, R.K., Kuz'mina, V., 2000. Digestive system. In: Ostgrander, G.K. (Ed.), The Laboratory Fish. The Handbook of Experimental Animal. Academic Press, New York, pp. 379–384.

Campanella, D., Caler, E., Miller, J., Lorenzi, H., Fernandino, J., Valenzuela, N., Somoza, G.M. Ortí, G., 2013. Phylogenetic context, whole genome sequencing, assembly and annotation of a new model species with Temperature-dependent Sex Determination. In: The 8th International Conference on Genomics. Shenzhen, China.

Caneguim, B.H., Beltrame, F.L., da Luz, J.S., Valentini, S.R., Cerri, P.S., Sasso-Cerri, E., 2013. Primordial germ cells (spermatogonial stem cells) of bullfrogs express sex hormone-binding globulin and steroid receptors during seasonal spermatogenesis. Cells Tissues Organs 197, 136–144.

Carriquiriborde, P., Díaz, J., López, G.C., Ronco, A.E., Somoza, G.M., 2009. Effects of cypermethrin chronic exposure and water temperature on survival, growth, sex differentiation, and gonadal developmental stages of *Odontesthes bonariensis* (Teleostei). Chemosphere 76, 374–380.

Chang, C.F., Chen, M.R., 1990. Fluctuation in sex steroids and sex steroid-binding protein during the development and annual cycle of the male common carp, *Cyprinus carpio*. Comp. Biochem. Physiol. 97A, 565–568.

Damassa, D.A., Gagin, G.A., Gustafson, A.W., 1996. Purification and characterization of the sex hormone-binding globulin in serum from Djungarian hamsters. Comp. Biochem. Physiol. 113B, 593–599.

Di Rienzo, J.A., Gonzalez, L.A., Tablada, E.M., 2009. FgStatistics – User Manual. Electronic Edition, Argentina. http://sites.google.com/site/fgstatistics.

Elisio, M., Chalde, T., Miranda, L.A., 2014. Seasonal changes and endocrine regulation of pejerrey (*Odontesthesbonariensis*) oogenesis in the wild. Comp. Biochem. Physiol. 175A, 102–109.

Elisio, M., Chalde, T., Miranda, L.A., 2015. Seasonal changes and endocrine regulation of pejerrey (*Odontesthes bonariensis*) spermatogenesis in the wild. Gen. Comp. Endocrinol. 221, 236–243.

Fernandino, J.I., Hattori, R.S., Kimura, H., Strüssmann, C.A., Somoza, G.M., 2008. Dimorphic expression of dmrt1 and cyp19a1 (ovarian aromatase) during early gonadal development in pejerrey, *Odontesthes bonariensis*. Sex Dev. 2, 316–324.

Fernandino, J.I., Hattori, R.S., Kishii, A., Strussmann, C.A., Somoza, G.M., 2012. The cortisol and androgen pathways cross talk in high temperature-induced masculinization: the 11beta-hydroxysteroid dehydrogenase as a key enzyme. Endocrinology 153, 6003–6011.

Fernandino, J.I., Hattori, R.S., Moreno Acosta, O.D., Strüssmann, C.A., Somoza, G.M., 2013. Environmental stress-induced testis differentiation: androgen as a by-product of cortisol inactivation. Gen. Comp. Endocrinol. 192, 36–44.

Fernandino, J.I., Hattori, R.S., Strüssmann, C.A., Yamamoto, Y., Somoza, G.M., 2015. Sex determination in fish: *Odontesthes spp*. (Atherinopsidae) as experimental models. Anim. Reprod. 12, 24–27.

Fortunati, N., Catalano, M.G., Boccuzzi, G., Frairia, R., 2010. Sex Hormone-Binding Globulin (SHBG), estradiol and breast cancer. Mol. Cell. Endocrinol. 316, 86–92.

Foucher, J.L., Le Bail, P.Y., Le Gac, F., 1992. Influence of hypophysectomy, castration, fasting, and spermiation on SBP concentration in male rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. 85, 101–110.

Freeman, H.C., Idler, D.R., 1969. Sex hormone binding proteins. II. Isolation from serum of an elasmobranch (*Raja radiata*). Gen. Comp. Endocrinol. 13, 83–91.

Freyre, L.R., Colautti, D.C., Maronas, M.E., Sendra, E.D., Remes-Lenicov, M., 2009. Seasonal changes in the somatic indices of the freshwater silverside, *Odontesthesbonariensis* (Teleostei, Atheriniformes) from a Neotropical shallow lake (Argentina). Braz. J. Biol. 69, 389–395.

Gasulla, J., Picco, S.J., Carriquiriborde, P., Dulout, F.N., Ronco, A.E., de Luca, J.C., 2016. Genotoxic effects induced by Cd^{+2}, Cr^{+6}, Cu^{+2} in the gill and liver of *Odontesthes bonariensis* (Piscies, Atherinopsidae). Bull. Environ. Contam. Toxicol. 96, 591–595.

Hamilton, C.K., Navarro-Martin, L., Neufeld, M., Basak, A., Trudeau, V.L., 2014. Early expression of aromatase and the membrane estrogen receptor GPER in neuromasts reveals a role for estrogens in the development of the frog lateral line system. Gen. Comp. Endocrinol. 205, 242–250.

Hammond, G.L., 2011. Diverse roles for sex hormone-binding globulin in reproduction. Biol. Reprod. 85, 431–441.

Hammond, G.L., 2016. Plasma steroid-binding proteins: primary gatekeepers of steroid hormone action. J. Endocrinol. 230, R13–R25.

Hammond, G.L., Miguel-Queralt, S., Yalcinkaya, T.M., Underhill, C., Place, N.J., Glickman, S.E., Drea, C.M., Wagner, A.P., Siiteri, P.K., 2012. Phylogenetic comparisons implicate sex hormone-binding globulin in "masculinization" of the female spotted hyena (*Crocutacrocuta*). Endocrinology 153, 1435–1443.

Hammond, G.L., Underhill, D.A., Rykse, H.M., Smith, C.L., 1989. The human sex hormone-binding globulin gene contains exons for androgen-binding protein and two other testicular messenger RNAs. Mol. Endocrinol. 3, 1869–1876.

Hansen, A., Reutter, K., Zeiske, E., 2002. Taste bud development in the zebrafish, *Danio rerio*. Dev. Dyn. 223, 483–496.

Hara, T.J., 2000. Chemoreception. In: Ostgrander, G.K. (Ed.), The Laboratory Fish. The Handbook of Experimental Animal. Academic Press, New York, pp. 245–249.

Ho, S.M., Tsang, P., Callard, I.P., 1980. Some properties of a steroid-binding protein in the plasma of an ovoviviparous dogfish, *Squalus acanthias*, at different stages of the life cycle. Biol. Reprod. 23, 281–289.

Hobby, A.C., Geraghty, D.P., Pankhurst, N.W., 2000. Differences in binding characteristics of sex steroid binding protein in reproductive and non-reproductive female rainbow trout (*Oncorhynchus mykiss*), black bream (*Acanthopagrus butcheri*), and greenback flounder (*Rhombosolea tapirina*). Gen. Comp. Endocrinol. 120, 249–259.

Hryb, D.J., Nakhla, A.M., Kahn, S.M., St George, J., Levy, N.C., Romas, N.A., Rosner, W., 2002. Sex hormone-binding globulin in the human prostate is locally synthesized and may act as an autocrine/paracrine effector. J. Biol. Chem. 277, 26618–26622.

Ito, L.S., Yamashita, M., Takashima, F., Strüssmann, C.A., 2005. Dynamics and histological characteristics of gonadal sex differentiation in pejerrey (*Odontesthes bonariensis*) at feminizing and masculinizing temperatures. J. Exp. Zool. 303A, 504–514.

Jänne, M., Deol, H.K., Power, S.G., Yee, S.P., Hammond, G.L., 1998. Human sex hormone-binding globulin gene expression in transgenic mice. Mol. Endocrinol. 12, 123–136.

Jennings, D.H., Moore, M.C., Knapp, R., Matthews, L., Orchinik, M., 2000. Plasma steroid-binding globulin mediation of differences in stress reactivity in alternative male phenotypes in tree lizards, *Urosaurus ornatus*. Gen. Comp. Endocrinol. 120, 289–299.

Joseph, D.R., 1994. Structure, function, and regulation of androgen-binding protein/sex hormone-binding globulin. Vitam. Horm. 49, 197–280.

Karube, M., Fernandino, J.I., Strobl-Mazzulla, P., Strüssmann, C.A., Yoshizaki, G., Somoza, G.M., Patiño, R., 2007. Characterization and expression profile of the ovarian cytochrome P-450 aromatase (*cyp19A1*) gene during thermolabile sex determination in Pejerrey, *Odontesthes bonariensis*. J. Exp. Zool. 307A, 625–636.

Kotrschal, K., Krautgartner, W.D., Hansen, A., 1997. Ontogeny of the solitary chemosensory cells in the zebrafish, *Danio rerio*. Chem. Sens. 22, 111–118.

Laidley, C.W., Thomas, P., 1997. Changes in plasma sex steroid-binding protein levels associated with ovarian recrudescence in the spotted seatrout (*Cynoscion nebulosus*). Biol. Reprod. 56, 931–937.

Malisch, J.L., Breuner, C.W., 2010. Steroid-binding proteins and free steroids in birds. Mol. Cell. Endocrinol. 316, 42–52.

Maunder, R.J., Matthiessen, P., Sumpter, J.P., Pottinger, T.G., 2007. Rapid bioconcentration of steroids in the plasma of three-spined stickleback Gasterosteus aculeatus exposed to waterborne testosterone and 17β-oestradiol. J. Fish Biol. 70, 678–690.

Miguel-Queralt, S., Avvakumov, G.V., Blazquez, M., Piferrer, F., Hammond, G.L., 2005. Sea bass (*Dicentrarchus labrax*) sex hormone binding globulin: molecular and biochemical properties and phylogenetic comparison of its orthologues in multiple fish species. Mol. Cell. Endocrinol. 229, 21–29.

Miguel-Queralt, S., Blazquez, M., Piferrer, F., Hammond, G.L., 2007. Sex hormone-binding globulin expression in sea bass (*Dicentrarchus labrax* L.) throughout development and the reproductive season. Mol. Cell. Endocrinol. 276, 55–62.

Miguel-Queralt, S., Hammond, G.L., 2008. Sex hormone-binding globulin in fish gills is a portal for sex steroids breached by xenobiotics. Endocrinology 149, 4269–4275.

Miguel-Queralt, S., Knowlton, M., Avvakumov, G.V., Al-Nouno, R., Kelly, G.M., Hammond, G.L., 2004. Molecular and functional characterization of sex hormone binding globulin in zebrafish. Endocrinology 145, 5221–5230.

Miguel-Queralt, S., Underhill, C., Devlin, R.H., Hammond, G.L., 2009. Characterization and measurement of the plasma alpha- and beta-sex hormone-binding globulin paralogs in salmon. Endocrinology 150, 366–375.

Nakamura, M., 2010. The mechanism of sex determination in vertebrates-Are sex steroids the key-factor? J. Exp. Zool. 313A, 381–398.

Nakhla, A.M., Hryb, D.J., Rosner, W., Romas, N.A., Xiang, Z., Kahn, S.M., 2009. Human sex hormone-binding globulin gene expression- multiple promoters and complex alternative splicing. BMC Mol. Biol. 10, 37.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, e36.

Pérez, M.R., Fernandino, J.I., Carriquiriborde, P., Somoza, G.M., 2012. Feminization and altered gonadal gene expression profile by ethynylestradiol exposure to pejerrey, *Odontesthes bonariensis*, a South American teleost fish. Environ. Toxicol. Chem. 31, 941–946.

Pinós, T., Barbosa-Desongles, A., Hurtado, A., Santamaría-Martínez, A., de Torres, I., Morote, J., Reventós, J., Munell, F., 2009. Identification, characterization and expression of novel Sex Hormone Binding Globulin alternative first exons in the human prostate. BMC Mol. Biol. 10, 59.

Pottinger, T.G., 1988. Seasonal variation in specific plasma- and target-tissue binding of androgens, relative to plasma steroid levels, in the brown trout, *Salmo trutta* L. Gen. Comp. Endocrinol. 70, 334–344.

Selva, D.M., Bassas, L., Munell, F., Mata, A., Tekpetey, F., Lewis, J.G., Hammond, G.L., 2005. Human sperm sex hormone-binding globulin isoform: characterization and measurement by time-resolved fluorescence immunoassay. J. Clin. Endocrinol. Metab. 90, 6275–6282.

Selva, D.M., Hammond, G.L., 2006. Human sex hormone-binding globulin is expressed in testicular germ cells and not in Sertoli cells. Horm. Metab. Res. 38, 230–235.

Selva, D.M., Hogeveen, K.N., Innis, S.M., Hammond, G.L., 2007. Monosaccharide-induced lipogenesis regulates the human hepatic sex hormone-binding globulin gene. J. Clin. Invest. 117, 3979–3987.
Somoza, G.M., Miranda, L.A., Berasain, G.E., Colautti, D., Remes Lenicov, M., Strüssmann, C.A., 2008. Historical aspects, current status, and prospects of pejerrey aquaculture in South America. Aquac. Res. 39, 784–793.
Strüssmann, C.A., Saito, T., Usui, M., Yamada, H., Takashima, F., 1997. Thermal thresholds and critical period of thermolabile sex determination in two atherinid fishes, *Odontesthes bonariensis* and *Patagonina hatcheri*. J. Exp. Zool. 278, 167–177.
Suresh, D.V., Baile, V.V., Prasada Rao, P.D., 2008. Annual reproductive phase-related profile of sex steroids and their carrier, SHBG, in the Indian major carp, *Labeo rohit*a. Gen. Comp. Endocrinol. 159, 143–149.
Tokarz, J., Moller, G., Hrabe de Angelis, M., Adamski, J., 2015. Steroids in teleost fishes: a functional point of view. Steroids 103, 123–144.
Valdés, M.E., Marino, D.J., Wunderlin, D.A., Somoza, G.M., Ronco, A.E., Carriquiriborde, P., 2015. Screening concentration of E1, E2 and EE2 in sewage effluent and surface waters of the "Pampas" region and the "Río de la Plata" estuary (Argentina). Bull. Environ. Contam. Toxicol. 94, 29–33.
Yamamoto, Y., Zhang, Y., Sarida, M., Hattori, R.S., Strüssmann, C.A., 2014. Coexistence of genotypic and temperature-dependent sex determination in pejerrey *Odontesthes bonariensis*. PLoS One 9, e102574.
Zanuy, S., Carrillo, M., 1985. Annual cycles of growth, feeding rate, gross conversion efficiency and hematocrit levels of sea bass (*Dicentrarchus labrax L.*) adapted to two different osmotic media. Aquaculture 44, 11–25.