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Research paper

Sex hormone-binding globulin b expression in the rainbow trout ovary prior to sex differentiation

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ABSTRACT

Salmonids have two sex hormone-binding globulin (Shbg) paralogs. Shbga is mainly expressed in the liver, while Shbgb is secreted by the granulosa cells of the rainbow trout ovary. Coexpression of *shbgb* and the gonadal aromatase *cyp19a1a* mRNAs been observed in granulosa cells, suggesting a physiological coordination between Shbgb expression and estrogen synthesis. As estrogens are essential for female sex determination in the fish ovary, we propose that Shbgb participates in early ovarian differentiation, either by binding with estrogen or through another mechanism that remains to be discovered. To elucidate this potential role, monosex populations of female trout were studied during the molecular ovarian differentiation period (28–56 dpf). *shbgb* mRNA expression was measured using qPCR and compared with expression of genes for other ovarian markers (*cyp19a1a*, *foxl2*, *follistatin*, and *estrogen receptors*). *shbgb* transcript expression was detected during the final stages of embryonic development (21–26 dpf) and during molecular ovarian differentiation (32–52 dpf) after hatching (which occurred at 31 dpf). *In situ* hybridization localized *shbgb* transcription to the undifferentiated ovary at 42 dpf, and *shbgb* and *cyp19a1a* mRNA showed similar expression patterns. These results suggest that Shbgb is involved in early ovarian differentiation, supporting an important role for the salmonid *shbgb* gene in sex determination.

1. Introduction

Comparative studies of vertebrate ovarian differentiation have shown that the process is highly dependent on estrogens in fish, whereas in mammals, sex determination is entirely chromosomal (Cutting et al., 2013; Guiguen et al., 1999; Guiguen et al., 2010; Piferrer and Donaldson, 1994; Yamamoto, 1969). During early gonadal development of fish, estrogens stimulate *foxl2a* expression (Baron et al., 2004; Vizziano-Cantonnet et al., 2008) and upregulate transcription of the gonadal aromatase gene *cyp19a1a* (Wang et al., 2007), likely explaining the elevated *foxl2a* and *cyp19a1a* expression levels detectable at the onset of ovarian differentiation (Ijiri et al., 2008; Vizziano et al., 2007). Follistatin is another sexually-dimorphic factor that plays an important role in early ovarian differentiation (Vizziano-Cantonnet et al., 2008; Vizziano et al., 2007). Notably, expression of the genes for Follistatin and Cyp19a1a is upregulated in the same cells (Nicol et al., 2013), and both genes are stimulated *in vivo* by estrogens (Vizziano-Cantonnet et al., 2008).

Estrogens are critical to early ovarian development in trout (Guiguen et al., 2010). Estrogen production, in turn, depends on the activity of Cyp19a1a, which converts androgens to estrogens. A recent report describing the masculinization of zebrafish after gonadal aromatase knockout (Lau et al., 2016) confirmed the key role of aromatase and estrogens in the sexual differentiation of teleost fish. Therefore, understanding the regulation of intragonadal estrogen concentrations is important in elucidating the sex differentiation process of fish. Some authors suggest that steroid-binding proteins may regulate estrogen levels (Bobe et al., 2008; Hammond, 2011; Miguel-Queralt et al., 2009; Rosner et al., 2010). The SHBG protein, encoded by the *shbga* gene, is common to all vertebrates, including fish, and is secreted mainly in the liver to regulate circulating steroid levels (Hammond, 2011; Rosner et al., 2010). A SHBG ortholog is also expressed in the vertebrate testis, referred to as androgen-binding protein (Foucher and Le Gac, 1989). Salmonids, however, have two sex hormone-binding globulin genes, possibly attributable to an ancestral fourth whole-genome duplication (4R WGD or Ss4R) (Berthelot et al.

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(2014). These two genes encode the paralogous proteins Shbga and Shbgb (Bobe et al., 2010). Shbgb is unique to the Salmonidae family and is secreted principally in the follicular cells of the ovary (Bobe et al., 2010, 2008). Shbgb binds with both estradiol and testosterone in trout (Bobe et al., 2008) but is more selective for estradiol in Coho salmon (Miguel-Queralt et al., 2009). Coexpression of *shbgb* and *cyp19a1a* in ovarian follicular cells during and after vitellogenesis suggests an interaction between the two proteins (Bobe et al., 2008). Interestingly, *shbgb* and *cyp19a1a* are both stimulated during vitellogenesis, and expression of both genes is repressed during oocyte maturation, when estrogen production drops (Bobe et al., 2008). Moreover, *shbgb* expression is upregulated in the trout ovary during early gametogenesis (Marivin et al., 2014) in parallel with the proliferation of germ cells (Yoshizaki et al., 2010). Another study has suggested that estrogens play a key role in ovarian germ cell regulation during early gametogenesis (Miura et al., 2007), and results in the juvenile and adult trout ovary support a role for *shbgb* in local regulation of estrogens.

Estrogens are produced very early in trout, during the first steps of ovarian differentiation (Baron et al., 2004; Vizziano et al., 2007), and are known to regulate genes involved in sexual determination (Vizziano-Cantonnet et al., 2008). These data suggest that Shbgb may be involved in regulating early ovarian differentiation, in conjunction with Cyp19a1a. To elucidate the potential role of Shbgb, we studied the expression of *shbgb* and the gonadal aromatase gene *cyp19a1a* during early ovarian differentiation in genetic monosex female populations of rainbow trout. Because the activity of estrogens and their interactions with the nuclear estrogen receptor may activate *shbgb* transcription (Bobe et al., 2008), a complementary analysis of estrogen receptors was also performed.

2. Materials and methods

2.1. Animal rearing

The portions of the study involving animal experimentation were carried out in strict accordance with the recommendations of the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 85–23, revised 1996). The study was approved by the Institutional Animal Care and Use Committee of the Universidad de Chile and the CONICYT-Fondecyt Bioethics Advisory Committee in Chile. All-female populations of rainbow trout were reared under natural photoperiods and a temperature range of 8–12 °C at the Huililco fish farm (Pucón, Chile) until first reproduction, as previously described (Estay et al., 2012). All fish received a similar diet and were maintained under normal temperatures from fertilization until 20 days post-fertilization (dpf). At 20 dpf, about 1000 eyed larvae were transferred to laboratory test conditions and placed in 0.3 m^3 tanks. The embryos were transferred into a recirculated water system and kept under a controlled photoperiod at a temperature of 10 °C. Under these conditions, hatching occurred at 28–32 dpf. After complete yolk reabsorption (56 dpf), the fish were fed a commercial diet *ad libitum* (Estay et al., 2012) and grown to 85 dpf.

2.2. Animal sampling

Various experiments were performed to measure *shbgb* levels during early gonadal development. In the first experiment, the ovaries of female fish at 5, 7, and 12+ months of age and the gonads of one male fish at 12+ months of age were collected for histology (preserved in formaldehyde, 10%) and gene expression assessment (frozen at −80 °C until RNA extraction). One biological replicate was collected for each stage. The ovarian and testicular samples were used to validate previously-described *shbgb* primers (Bobe et al., 2008) and to confirm gene expression in the immature ovary. The amplified fragments were purified and sequenced to validate the products of qPCR.

Once the primers were validated, a second experiment was performed. The head and trunk of the fish from the genetic monosex female populations were collected during pre-eclosion (21–28 dpf), eclosion (31 dpf), post-eclosion (32–48 dpf), and first feeding (56 dpf) and frozen at −80 °C until RNA extraction. Differential expression patterns for sexually-dimorphic genes were expected to appear at around 32, 35, 40, 48, and 56 dpf at 10–11 °C (Vizziano et al., 2007). *shbgb* mRNA expression was analyzed using RT-PCR and qPCR in one biological replicate and two technical replicates. As temperature was not measured during embryo and larval rearing in this experiment, the molecular ovarian differentiation stage was confirmed by measuring *cyp19a1a* and *foxl2a* mRNA expression with qPCR (Vizziano et al., 2007). The presence of *shbgb* was confirmed by sequencing the products obtained by RT-PCR in an additional experiment.

During a third experiment, the larval trunks from the genetic monosex female populations reared at 10 °C were sampled during several stages of gonadal development spanning the sex differentiation period. Larvae were sampled at 28, 30, 32, 35, 37, 39, 42, 44, 46, 48, 52, and 56 dpf and frozen at −80 °C until RNA extraction. For each stage, 5 larvae were fixed in formaldehyde (10%) until use for classic histology; 10 larvae were fixed in paraformaldehyde (4%) in PBS, dehydrated in 100% methanol, and stored at −20 °C until use for *in situ* hybridization. *shbgb* and *cyp19a1a* mRNA expression was analyzed using qPCR in four biological replicates. Expression of estrogen receptor genes (*era1*, *era2*, *erb1*, and *erb2*) was also measured and compared with the *shbgb* expression levels.

2.3. Comparison of shbgb expression in male rainbow trout larvae

As additional experiment, *shbgb* expression in males was also studied. One hundred twenty rainbow trout larvae were selected from a normal population (male and female sex ratio 1:1). In this experiment we analyzed 10 animals per stage of differentiation between 28 and 56 dpf. Genomic DNA was obtained from caudal fin and animals were sexed with the *sdY* gene as described by Yano et al. (2012). From the trunk of male fish total RNA was obtained and then cDNA synthesized. Subsequently, the levels of *shbgb* were analyzed by qPCR according to the protocol described in the following section. Expression levels were normalized according to the expression of 18S rRNA and the beta actin values.

2.4. RNA extraction and reverse transcription

Total RNA was extracted using TRIzol® (Thermo Fisher) according to manufacturer instructions. The RNA was quantified by spectrometry, and the ratio of absorbance at 260 nm and 280 nm was used to assess the purity of the RNA. All RNA was first treated with DNase to remove the presence of genomic DNA. cDNA synthesis was carried out using 2 μg of total RNA. The RNA was denatured in the presence of oligo(dT) (0.5 μg/μl) for 5 min at 70 °C, and then chilled on ice. Reverse transcription was performed at 42 °C for 1 h using RevertAid Reverse Transcriptase a according to manufacturer instructions (Thermo Fisher).

2.5. Real-time PCR

Real-time PCR was performed using an Eco™ Real-Time PCR instrument (Illumina). Reactions were performed in 20-μl samples with 300 nM of each primer (Table 1) and 2 μl of a 1:10 dilution of the RT reaction and the Power SYBR Green (Applied Biosystems) qPCR master mix, according to manufacturer instructions. The

Table 1
Primer sequences and list of genes assayed using qRT-PCR and sequencing.

Gene	Primer or probe sequence 5′-3′	Tm	bp	References
18SrRNA FW	5′-CGGAGGTTCGAAGACGATCA-3′	62	61	Vizziano et al. (2007)
18SrRNA RS	5′-TCGCTAGTTGGCATCGTTTAT-3′			
shbga FW	5′-CCCATTCTGGAACTTTGAGG-3′	60	106	Bobe et al. (2008)
shbga RS	5′-ACACCCCATAACCTGGTCAA-3′			
shbgb FW	5′-ACATGTGGGGGATGTTCATT-3′	57	117	Bobe et al. (2008)
shbgb RS	5′-GAGGCCATGTTACGGTTTTG-3′			
shbgb-11 FW	5′-ACAAGTATAGAACATAAACG-3′	58	1749	+
shbgb-1760 RS	5′-AATGCTTTATTGCCATTCAC-3′			
shbgb 56 FW	5′-AGCAAGTGCCCCAAAGGACCG-3′	65	1575	+
shbgb 1631 RS	5′-GAGGCCATGTTACGGTTTTGCAGT-3′			
foxl2a FW	5′-TGTGCTGGATTTGTTTTTTGTT-3′	60	93	Vizziano et al. (2007)
foxl2a RS	5′-GTGTCGTGGACCATCAGGGCCA-3′			
follistatin FW	5′-ACAAAGACGAGTGCGCGTTGCT-3′	60	91	Vizziano et al. (2007)
follistatin RS	5′-CGGCAGGTCTTCTTGCATTTGC-3′			
cyp19a1b 1323 FW	5′-TGAGGAAGGCACTGGAAGATGAC-3′	65	165	von Schalburg et al. (2010)
cyp19a1b 1488 RS	5′-GGCTGGAAGAAACGACTGGGC-3′			
cyp19a1a 243 FW	5′-GTTTGGCCCGGAGAGAAAGA-3′	58	1026	+
cyp19a1a 1269 RS	5′-AGACATGTCCTCCTGGAACA-3′			
cyp19a1a 4 FW	5′-TGCGTCAGTGTGTCTACCATGT-3′	58	1265	+
cyp19a1a 1269 RS	5′-AGACATGTCCTCCTGGAACA-3′			

+primer designed and used in this work based on the *shbgb* mRNA sequence from GenBank NM_001124673.1 (Bobe et al., 2008).

incubation steps were as follows: holding stage (95 °C for 5 min), cycling stage (95 °C for 15 s, 60 °C for 1 min), and melting curve stage (95 °C for 15 s, 60 °C for 1 min, 95° for 30 s, 60 °C for 15 s), followed by 40 PCR cycles. The validity of the qPCR was confirmed by analyzing the melting curves for each gene, checking amplified fragments in an agarose gel and through sequencing. PCR efficiency was calculated according to the equation $E = 10^{[-1/\text{slope}]}$ (Pfaffl, 2001). Data were normalized according to the mRNA expression of the housekeeping gene 18S rRNA, EF-1α, and beta-actin, according to the 2ΔΔCt method (Livak and Schmittgen, 2001). Relative expression was calculated as a percentage of the highest expression level recorded for each gene.

2.6. In situ hybridization

Digoxigenin-labeled antisense RNA probes were produced using the DIG RNA Labeling Kit (SP6/T7) from Roche Life Science according to manufacturer instructions. Two long fragments (1575 and 1749 bp) of *shbgb* cDNA were amplified (Table 1). The fragments were used as a template to amplify a specific *shbgb* probe with primers at the 5′ end containing RNA-polymerase promoters, allowing for incorporation of SP6 and T7 promoter elements into the DNA fragment at both ends of the probe (Ghafoory et al., 2012). Antisense RNA probes were synthesized using SP6-RNA-polymerase *in vitro* transcription, and T7-RNA-polymerase was used to produce sense cRNA probes (Table 2). After sample fixation with 4% paraformaldehyde (PAF) for 24 h, the larval trunks were stored in 100% methanol at −20 °C. The *in situ* hybridization assay was then performed (Vizziano-Cantonnet et al., 2011). Following fixation, the tissues were rehydrated for 5 min in 75% methanol and for 5 min in 50% methanol and finally incubated in PBS X 1 (Tween 0.1% v/v) for 15 min. Tissues were then cryoprotected in increasing concentrations of sucrose (5%, 15%, and 30%) in saline buffer phosphate (SBP, 13.68 mM NaCl, 0.268 mM KCl, 1.014 mM Na2HPO4, and 0.176 mM KH2PO4) overnight, at a pH of 7.4 and temperature of 4 °C. Tissues were cut to 10 μm and placed in a cryostat, and slides were coated with poly-lysine. The slides were washed in SBP at pH 7.4 at room temperature, re-fixed with PAF% at 4%, and permeabilized with proteinase K (3 μg ml^{-1}) for 5 min. After stopping the proteinase K treatment with 4% PAF, the samples were acetylated with buffer 0.1 M triethanolamine (pH 8) and acetic anhydride (2.5 μl ml^{-1}). Pre-hybridization was performed at 60–62 °C using a hybridization buffer (formamide 50%, SSC (75 mM standard saline citrate and 0.75 M NaCl; pH 7.4), Tween (0.1%), heparin (10 μg ml^{-1}), and ARNt (10 μg ml^{-1})). Hybridization was carried out using 3 ng ml^{-1} of the probe overnight at 60–62 °C. Incubation and elimination of non-specific binding were performed using SCC buffer (pH 7.4) with decreasing concentrations of formamide (50%, 25%, and 0%). The gonads were then washed with Tris buffer (0.1 M, pH 7.5) and saturated with Tris (0.1 M; pH 7.5) and 2% dehydrated milk. The digoxigenin was detected using an antibody to anti-digoxigenin coupled to a phosphatase alkaline and incubated overnight at 4 °C. The samples were then washed with Tris buffer (0.1 M; pH 7.5) and incubated with the buffer-based developer (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$; pH 9.5) and NBT-BCIP. The reaction was stopped using 0.1 M glycine (pH 2.4), PBS (pH 5.5) with 1 mM EDTA, and 4% PAF. The hybridization signal was observed with a binocular microscope, and macroscopic photos were captured. An Eclipse 80i Nikon microscope was used to capture images.

2.7. Histology

After fixation, the gonads were dehydrated, embedded in paraffin, and cut to 5-μm thick sections. The tissues were stained with Mayer's staining hematoxylin and eosin (Gabe, 1968).

2.8. Statistical analysis

ΔΔCt values were normalized to the 18S rRNA and the beta actin values obtained in the qPCR reactions. There were no significant statistical differences in expression levels when using either housekeeping to normalize. Results for the various larval stages were used calculate the ΔCt and ΔΔCt values, analyzed using the *stats* and *agricolae* packages from the R statistical environment (http://cran.r-project.org). In general, datasets were not distributed normally, according to the Shapiro-Wilks test. Therefore, differences between the respective mRNA expression values for the various larval stages were evaluated using a nonparametric, Kruskal-Wallis one-way ANOVA followed by a multiple comparisons test using the nonparametric Kruskal command in the R package *agricolae*. Each stage had a sample size of n = 4 for each transcript analyzed.

Table 2
Primer sequences for probes used in *in situ* hybridization and sequencing.

*** *shbgb* Probe 1; Tm 58 °C; 860 nt**

shbgb SP6 51 FW 5'-CAGTGAATTG ATTTAGGTGACACTATAGAAGTG AGAGAAGCAAGTGCCCCAAAGGA-3'

Extra nucleotides — SP6-RNA-Polymerase promoter — Upstream *shbgb*

shbgb T7 911 RS 5'-CAGTGAATTG TAATACGACTCACTATAGGG AGAAGAAAGCCTCTGGCTGAAACCGT-3'

Extra nucleotides — T7-RNA-Polymerase promoter — Downstream *shbgb*

*** *shbgb* Probe 2 ; Tm 58 °C; 817 nt**

shbgb FW 816 5'-CAGTGAATTG ATTTAGGTGACACTATAGAAGTG AGCCTTGCTGGGAAACTCTTCA-3'

Extra nucleotides — SP6-RNA-Polymerase promoter — Upstream *shbgb*

shbgb RS1633 5'-CAGTGAATTG TAATACGACTCACTATAGGG AGAAGAGGCCATGTTACGGTTTTGC-3'

Extra nucleotides — T7-RNA-Polymerase promoter — Downstream *shbgb*

*** *shbgb* Probe 3; Tm 58 °C; 1079 nt**

shbgb FW255 5'-CAGTGAATTG ATTTAGGTGACACTATAGAAGTG GGGGAATGTCCCCATTCTGG-3'

Extra nucleotides — SP6-RNA-Polymerase promoter — Upstream *shbgb*

shbgb RS1334 5'-CAGTGAATTG TAATACGACTCACTATAGGG AGCCATCTCTGATGTCGAGGGC-3'

Extra nucleotides — T7-RNA-Polymerase promoter — Downstream *shbgb*

*** *shbgb* Probe 4; Tm 58 °C; 1378 nt**

shbgb FW255 5'-CAGTGAATTG ATTTAGGTGACACTATAGAAGTG GGGGAATGTCCCCATTCTGG-3'

Extra nucleotides — SP6-RNA-Polymerase promoter — Upstream *shbgb*

shbgb RS1633 5'-CAGTGAATTG TAATACGACTCACTATAGGG AGAAGAGGCCATGTTACGGTTTTGC-3'

Extra nucleotides — T7-RNA-Polymerase promoter — Downstream *shbgb*

**** *cyp19a1a* probe; Tm 58 °C; 805 nt**

cyp19a1a SP6 270 FW 5'-CAGTGAATTG ATTTAGGTGACACTATAGAAGTG ACAGGAAAGGGGCAAAACTGGA-3'

Extra nucleotides — SP6-RNA-Polymerase promoter — Upstream *cyp19a1a*

cyp19a1a T7 1075 RS 5'-CAGTGAATTG TAATACGACTCACTATAGGG ACTTTTGACCAGAGCCCTGTGA-3'

Extra nucleotides — T7-RNA-Polymerase promoter — Downstream *cyp19a1a*

*Probes designed using SP6 and T7 promoter sequences (Ghafoory et al., 2012) and *shbgb* mRNA sequence NM_001124673.1 (Bobe et al., 2008).

**Probes designed using SP6 and T7 promoter sequences (Ghafoory et al., 2012), *cyp19a1a* expressed sequence tags for rainbow trout (GenBank; AM259371 ovary1; EST BX083177), and salmonDB (http://salmondb.cmm.uchile.cl) clone OM2U000484, length 1011 bp, 99% similar to rainbow trout *cyp19a1a* mRNA.

3. Results

3.1. Shbgb expression during embryonic and larval stages

In the first experiment, *shbgb* expression was measuring during the embryonic and larval stages using RT-PCR, covering the following periods: pre-eclosion, eclosion, post-eclosion, and first feeding. The *shbgb* gene was expressed at almost all embryonic and larval stages tested (Fig. 1). The housekeeping gene 18S rRNA was expressed in all stages tested. This finding represents the first evidence of very early *shbgb* expression in the developing trout ovary, including during the putative molecular sex differentiation period (31–55 dpf) (Vizziano et al., 2007).

3.2. Shbgb and cyp19a1a expression levels are elevated in the larval ovary

In the previous section, we demonstrated that *shbgb* is expressed during embryonic and larval development, including the putative molecular sex differentiation period (31–55 dpf), using RT-PCR. The kinetics of gonadal development depend on temperature. Because incubation temperature was not measured precisely in this phase of the study, *cyp19a1a* expression was used to confirm the molecular sex differentiation stage of the developing trout ovaries. *cyp19a1a* expression was low at 32 and 35 dpf, increasing at 38 dpf and then decreasing again at 56 dpf (Fig. 2). These results confirm that the sexual differentiation period spanned 38–56 dpf in this experiment, differing slightly from previous reports in trout (Vizziano et al., 2007). Interestingly, *shbgb* was highly expressed at the same time as *cyp19a1a* prior to ovarian differentiation. In this experiment, we also detected transcript expression for the gene for *foxl2* another key marker of the female sexual differentiation process (Vizziano et al., 2007). This experiment was performed with using one replicate each from five sampling times. Given this small sample size, results were descriptive but not suitable for a statistical analysis to confirm the gene expression trends. Additional data was therefore collected to further elucidate this process, described in the next section.

3.3. Shbgb and cyp19a1a expression levels are elevated in the ovary prior to sex differentiation

shbgb expression was studied in four biological replicates at 28, 30, 32, 35, 37, 39, 42, 44, 46, 49, 52, and 56 dpf. Morphology confirmed that the gonads were not sex-differentiated during these stages (Fig. 3), as there were no signs of meiosis in germ cells or special somatic arrangements. At 63 dpf, the ovaries were distinguished by somatic tissue invaginations inside the gonad, representing the primordium of the ovigerous lamellae (Fig. 3). To compare expression of the enzymes Cyp19a1a (which transforms androgens into estrogens) and Shbgb (which specifically binds

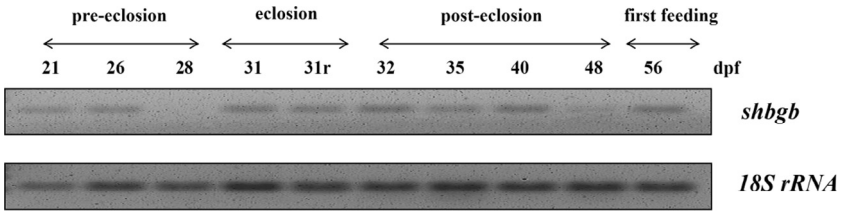

Fig. 1. *shbgb* mRNA transcript expression analyzed using RT-PCR in embryonic and larval tissue. An 18S rRNA transcript was amplified as a control.

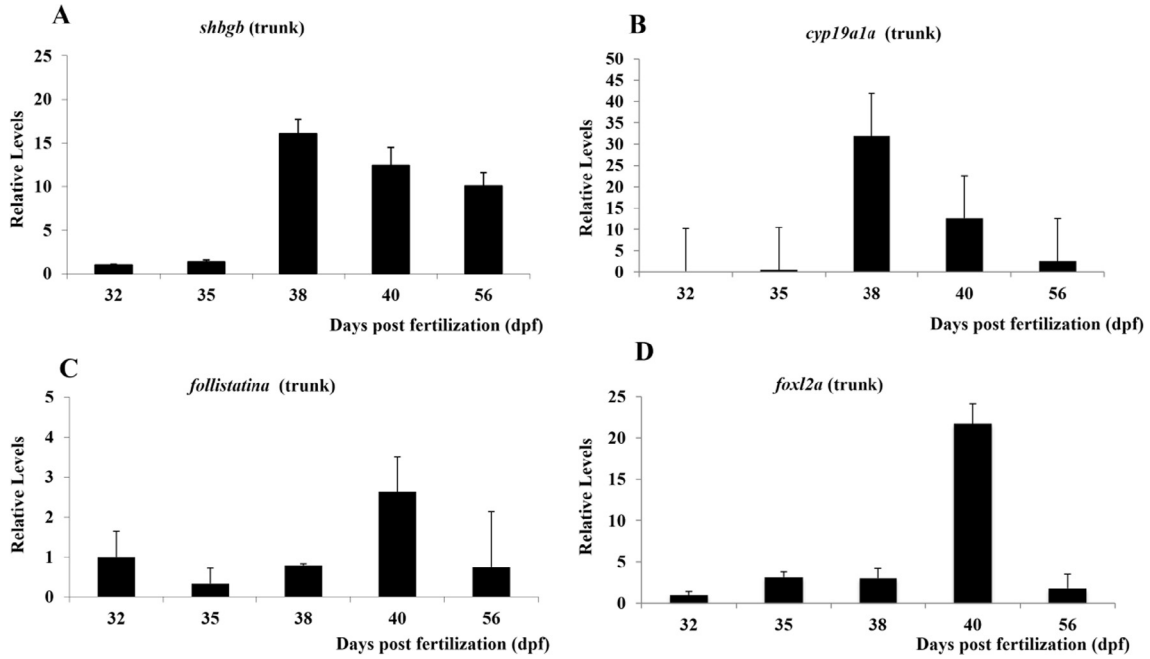

Fig. 2. Quantitative expression of ovarian mRNA markers and *shbgb* mRNA. We analyzed the relative quantitative expression of *shbgb* mRNA in the larval trunks of trout during the molecular sex differentiation period (32–56 dpf). A. *shbgb*, B. *cyp19a1a*, C. *follistatin*, and D. *foxl2a*. Transcript expression was normalized relative to 18S rRNA transcripts.

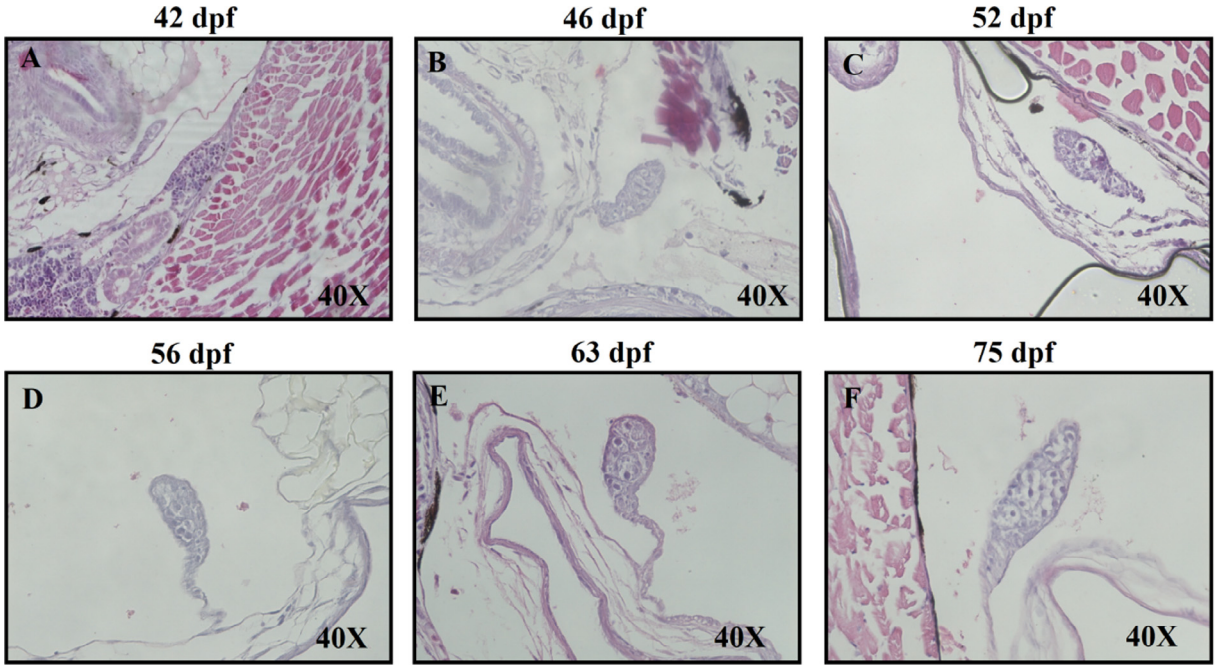

Fig. 3. Histology of undifferentiated gonads of female trout larvae. The figure shows undifferentiated gonads at 42–63 dpf (A–E) and a histologically differentiated ovary at 75 dpf (F). In the undifferentiated gonad, somatic cells are seen circling a few germ cells. Ovigerous lamellae are expected to appear at 63–75 dpf.

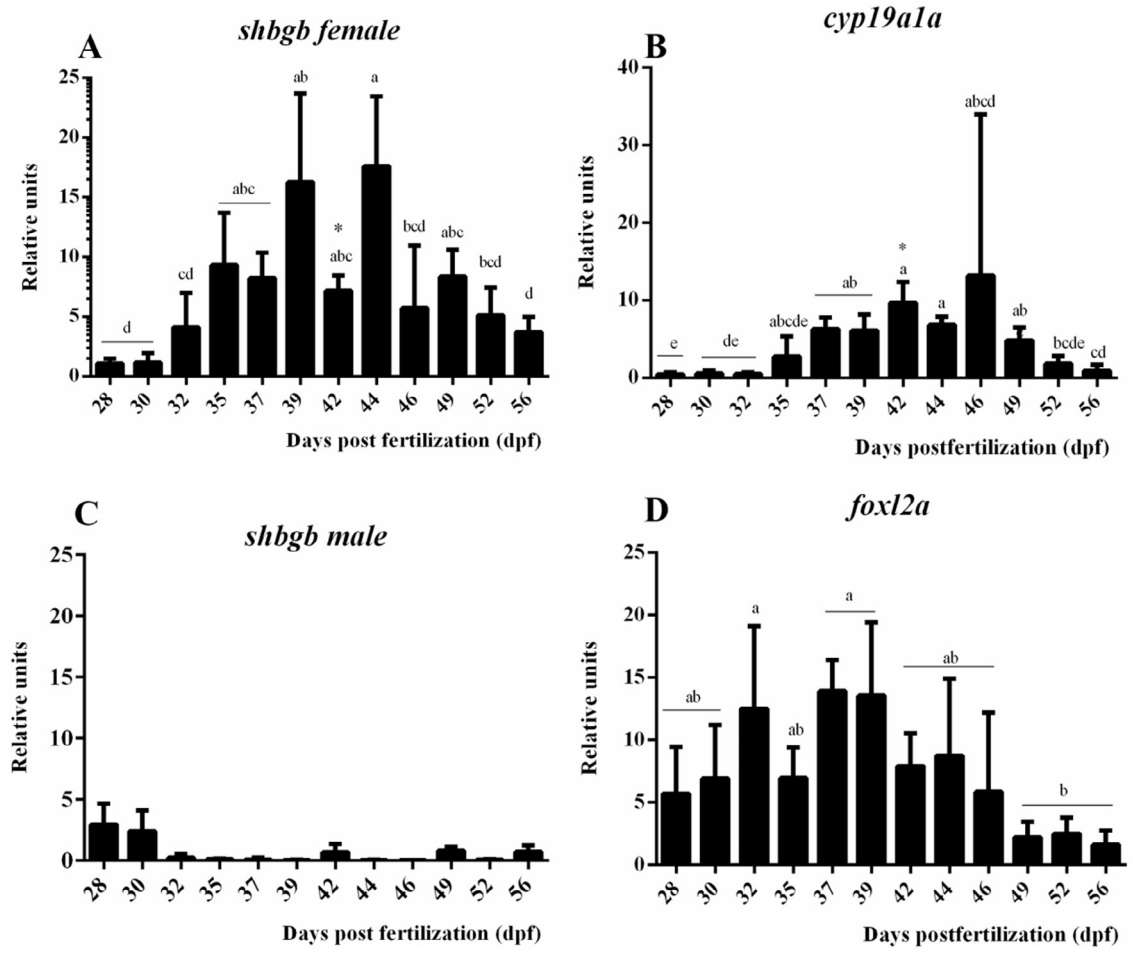

Fig. 4. Relative quantitative expression of *shbgb* mRNA and ovarian markers according to qPCR. The figure shows the expression of transcripts for (A) *shbgb in monosex female*, (B) *cyp19a1a*, (C) *shbgb* in male and (D) *foxl2a* in the larval trunks of trout during molecular differentiation (28–56 dpf). Expression was calculated relative to the 18S rRNA transcript. Different letters above bars indicate significantly different expression between stages ($p < .05$).

estrogens), transcript expression levels of the genes for both proteins were measured by qPCR (Fig. 4). Ovarian *shbgb* transcripts increased significantly ($p < .05$) during very early larval development (30–32 dpf) and again ($p > .05$) at 32–39/44 dpf. Expression was sustained and then decreased significantly at 52–56 dpf (Fig. 4A). *shbgb* peaked at 39–44 dpf. *cyp19a1a* expression increased significantly ($p < .05$) at 37 dpf, five days after the first significant increase in *shbgb* levels, reaching a significant ($p < .05$) maximum at 42 dpf. This *cyp19a1a* expression remained high until 49 dpf and then decreased significantly at 52–56 dpf (Fig. 4B). In order to compare expression of *shbgb* between sexes, we quantified the levels of transcript in male larvae. In Fig. 4C it is observed that the expression of *shbgb* in male during the stages of sexual differentiation between 28–56 dpf were extremely low. Lower levels of *shbgb* expression in males are maintained throughout the whole period of molecular sexual differentiation. In contrast, *shbgb* expression in genetic monosex females begins to increase before *cyp19a1a* expression (32 vs. 37 dpf), however the elevated expression levels for both genes were sustained simultaneously in females (37–49 dpf).

3.4. Expression of estrogen receptor mRNA during the sexual differentiation period

Transcripts for the estrogen receptor genes *era1*, *era2*, *erb1*, and *erb2* were also evaluated. Only two genes (*era1* and *erb1)* showed significant variation in expression levels. *era1* expression increased significantly ($p < .05$) at 37 dpf. Expression remained elevated from 37–42 dpf and then decreased significantly ($p < .05$) after 56 dpf (Fig. 5A). This expression pattern is similar to that of the aromatase gene *cyp19a1a*. Expression levels for *erb1*, on the other hand, were high during early development (28–42 dpf), decreasing significantly ($p < .05$) after 48 dpf (Fig. 5C).

3.5. Shbgb is expressed in the trout gonad prior to sex differentiation

In the first *in situ* hybridization experiment, four *shbgb* probes (Table 2) were tested in an adult ovary as a positive control. The probe sizes were as follows: probe 1 = 860 nt, probe 2 = 817 nt, probe 3 = 1079, and probe 4 = 1378 nt. *shbgb* expression was evident in the granulosa cells of the ovarian follicles when probes 1, 2, and 3 were used (Fig. 6), but only a slight reaction was observed for the 1378nt probe (probe 4) (Fig. 6). The same four probes were also tested in larval ovaries at 56 dpf, prior to sex differentiation (Fig. 6). The 56 dpf stage was selected because the gonad was large (Fig. 6), and *shbgb* was well-expressed according to qPCR (Fig. 4A). A slight, non-conclusive reaction was observed for all four probes at 56 dpf.

In the second *in situ* hybridization experiment, probe 4 was hydrolyzed and tested in the future ovaries of monosex female larvae prior to sex differentiation (42–75 dpf). The specific signal for the antisense probe was observed in the ovaries at 42, 44, 46, 49,

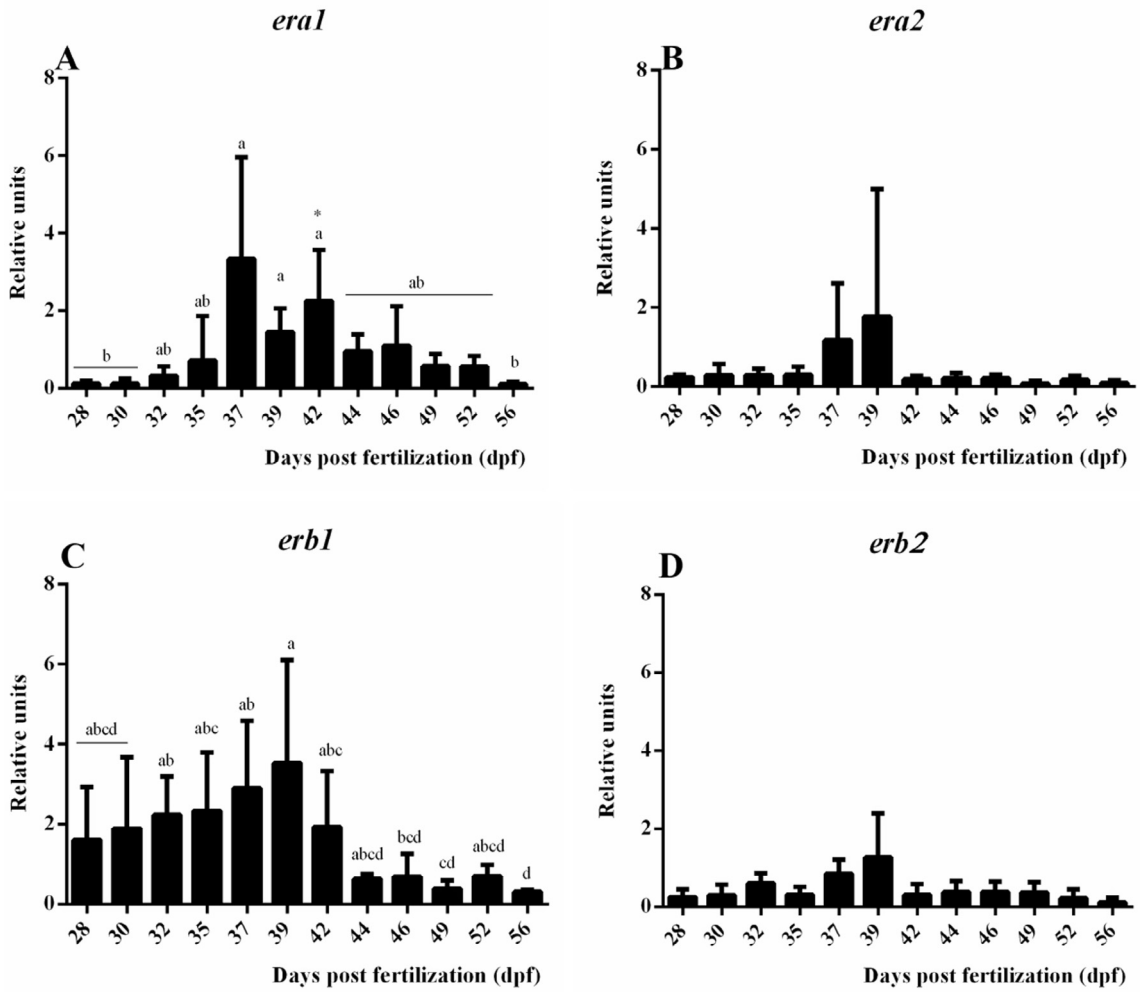

Fig. 5. Relative quantitative expression of estrogen receptor mRNAs. The figure shows expression of *era1* (A), *era2* (B), *erb1* (C), and *erb2* (D) transcripts in larval trunks during molecular sex differentiation (28–56 dpf) is observed comparatively. Expression was calculated relative to the 18S rRNA transcript. Different letters above bars indicate significantly-different expression between stages ($p < .05$).

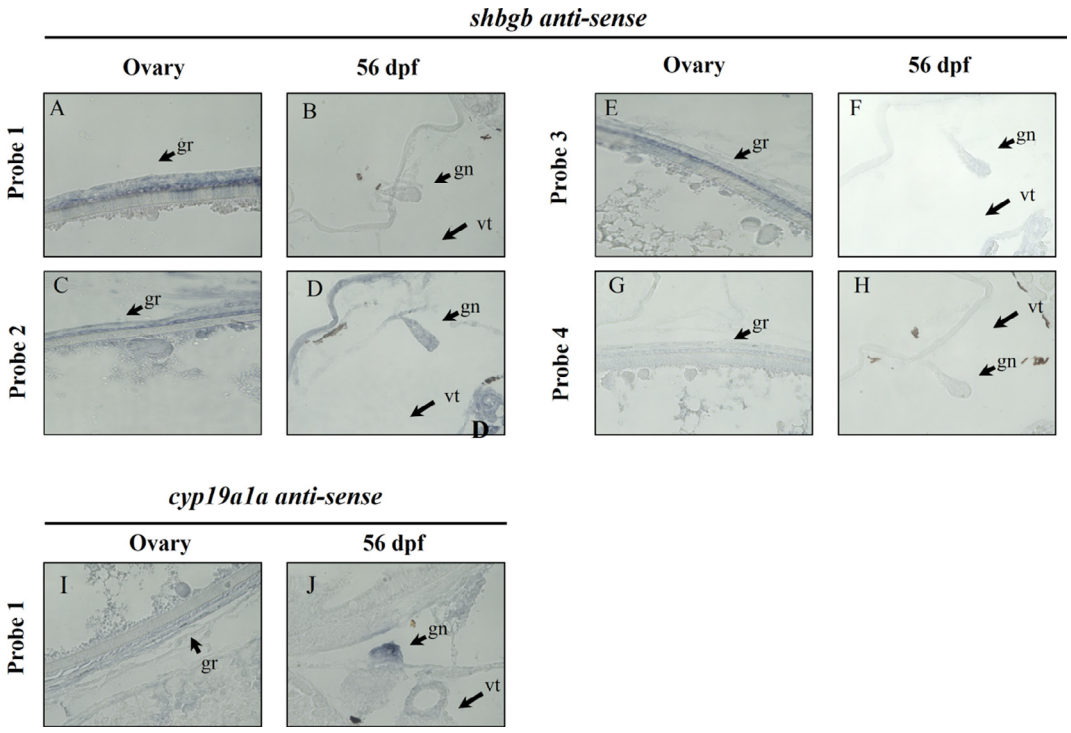

Fig. 6. *In situ* hybridization with *shbgb* and *cyp19a1a* antisense probes. *shbgb* probes were as follows: probe 1 (860 nt) (A-B), probe 2 (817 nt) (C-D), probe 3 (1079 nt) (E-F), and probe 4 (1378 nt) (G-H). The *cyp19a1a* antisense probe 1 was 805 nt (I-J). Cryostat sections of adult ovaries in post-vitellogenesis and undifferentiated ovaries at 56 dpf were used. gn: gonad, gr: granulosa cells, vt: vitellogenin.

52, 56, 63, and 75 dpf, and the *shbgb* probe was negative in the sense assay (Fig. 7). The signal increased at 42 and 49 dpf, prior to sex differentiation, and at 75 dpf, during early gametogenesis. At 42 dpf, *shbgb* mRNA expression was observed and localized to the somatic cells of the undifferentiated gonad (Fig. 7). At this stage, the *in situ* signal was slightly masked by a non-specific

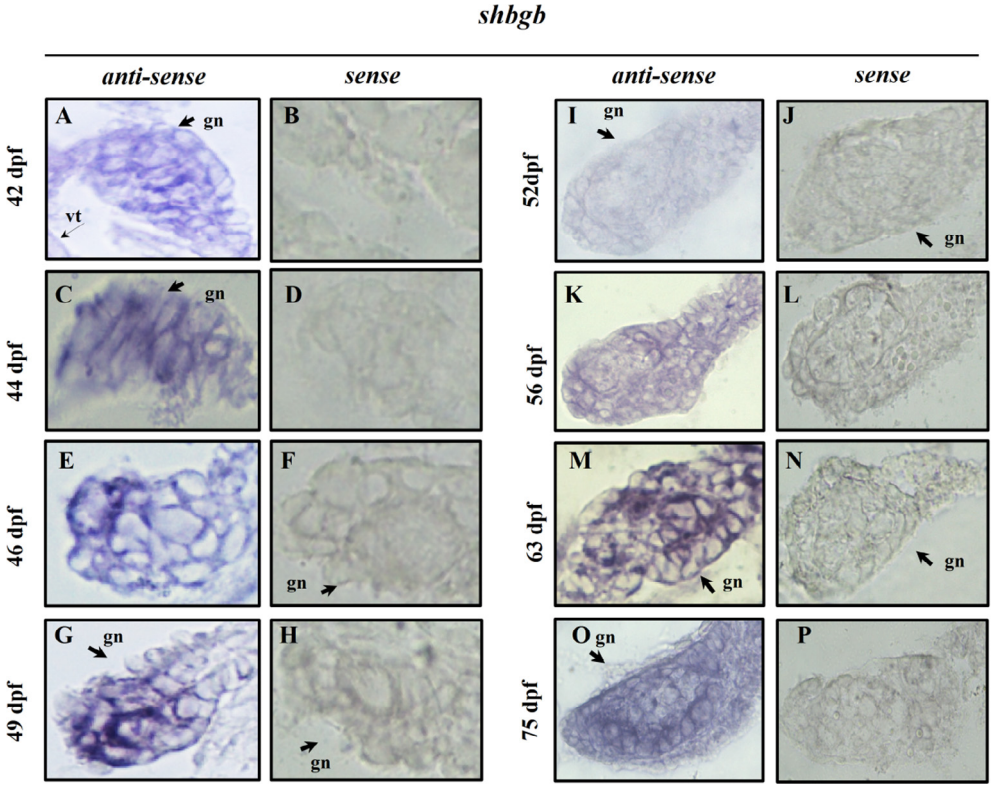

Fig. 7. *In situ* hybridization of *shbgb* (probe 4, hydrolyzed) in various stages of sexual differentiation. A&B-undifferentiated ovaries (42 dpf). C&D-undifferentiated ovaries (44 dpf). E&F-undifferentiated ovaries (46 dpf). G&H-undifferentiated ovaries (49 dpf). I&J-undifferentiated ovaries (52 dpf). K&L-undifferentiated ovaries (56 dpf). M&N-undifferentiated ovaries (63 dpf). O&P-differentiated ovaries (75 dpf). Arrow indicates the location of the developing gonad. gn: gonad, vt: vitellogenin.

reaction present in the yolk surrounding the gonad. From 44–63 dpf, the undifferentiated gonad showed clear expression of *shbgb* (Fig. 7), especially as compared to the corresponding negative control panel (Fig. 7). At 49 dpf, the reaction became stronger, decreasing again between 52–63 dpf, coinciding with the decline in expression levels as assessed by qPCR (Fig. 4). The number of somatic cells expressing *shbgb* increased in the ovary again at 75 dpf, during early gametogenesis.

4. Discussion

These results show that *shbgb* mRNA in monosex female is expressed prior to hatching, throughout the molecular differentiation period proposed by Vizziano et al. (2007) as well as at the beginning of morphological differentiation. The morphological differentiation stage was defined based on histological criteria in rainbow trout 5 weeks post-hatching at 10 °C (67 dpf) (Lebrun et al., 1982). Early expression of *shbgb* during pre-hatching may serve to delimit the physiological effects of estrogens during a highly vulnerable stage of embryo development. *shbgb* expression in unhatched embryos may be attributable to small concentrations of estrogens that are optimized or stored during embryonic development to be used during larval development (Antila, 1984; Feist and Schreck, 1996; van den Hurk et al., 1982; Yeoh et al., 1996a, b). Other studies have indicated that estrogens are harmful or detrimental to the maintenance and differentiation of various cellular precursors and that SHBG proteins may protect against excessive concentrations of sex steroid during development (Becchis et al., 1996; Becker and Iles, 1985). A recent study indicated that survival rates were lower for rainbow trout larvae treated with high concentrations of estradiol during the embryonic stage and that the surviving fish showed abnormal growth and adult length (Marlatt et al., 2014). In our work, *shbgb* mRNA levels in monosex females increased at the beginning of the molecular differentiation period, peaking at 49 dpf. At 52 dpf, *in situ* hybridization of the *shbgb* signal was no longer detectable. This finding could be attributable to a depletion of nutrient reserves, resulting in a deprivation-like effect. At 75 dpf, *shbgb* expression increased again, in agreement with a prior report by Marivin et al. (2014). Although our study of *shbgb* expression was focused on the physiological role of this protein in the stages of sexual differentiation of the ovary in rainbow trout, we also studied *shbgb* expression in males. The results of *shbgb* expression in males indicate that the levels of these transcripts were very low throughout the period of molecular differentiation (28–56 dpf). These levels of *shbgb* in males reported here are in agreement with the data obtained by Marivin et al. (2014) and they indicate that both mRNAs maintain consistently lower levels during early gonadal development in males.

It should be emphasized that *shbgb* mRNA in monosex female may show a "biphasic" expression pattern. Some authors suggest that during the initial increase in expression, Shbgb proteins may serve to regulate estrogen levels, in close coordination with the synthesis of estradiol and the expression of aromatase and foxl2 transcription factors (Baron et al., 2004; Vizziano et al., 2007). During these stages, interactions between Shbgb, Cyp19a1a and Foxl2 may also be associated with maintenance of granulosa cell differentiation (Vizziano et al., 2007). During the second period of increased expression at 63–75 dpf, Shbgb may modulate levels of 17β-estradiol, which acts directly in oogonial proliferation during early oogenesis (Lubzens et al., 2010).

The absence of *shbgb* mRNA expression at 52–56 dpf in monosex female could be due to a depletion of nutrient reserves, as mentioned above. Several studies on the transcriptional regulation of the SHBG gene in mammals have presented strong evidence that this gene is highly sensitive to metabolic disturbances and lowered concentrations of growth factors (Selva et al., 2007). The transcriptional inhibition of the *shbgb* gene in female observed in this work seems critical and may be an essential factor for morphological differentiation of the ovary. The lack of Shbgb protein secretion during this period may allow for the release of estrogen reserves that are bound to the protein, in turn allowing for redirection of hormone activity towards other tissues or cells.

In terms of the expression and early localization of *shbgb* mRNA during the molecular differentiation period of the ovary, our work differs from the results reported by Marivin et al. (2014). The sensitivity of our *in situ* methodology and the type of histological sections used may explain the differences in early detection and localization of *shbgb* transcripts. Marivin et al. (2014) performed hybridizations of *shbgb* in female whole larval tissue, observing an incipient localization of these transcripts only at 68 dpf, during an advanced stage of ovarian morphological differentiation. To the above result it should be added that in the *in situ* hybridization assays of *shbgb* in males they did not observe hybridization signal of these transcripts. Which is consistent with the low levels of these transcripts, detected by qPCR assays in the non-differentiated male gonad in this work. It is important to emphasize that they did not include control of sense probes in the *shbgb in situ* hybridization experiments, which are fundamental for this type of study. Moreover, whole-mount *in* situ hybridization of rainbow trout larval tissue has a clear disadvantage, in that this method does not have the capacity to localize transcripts under conditions of low or minimal expression. Therefore, the conclusions from the report by Marivin et al. (2014) must be interpreted with caution. Our *shbgb* hybridization results in monosex female, indicating a spike in expression during the molecular differentiation period, strongly support our initial hypothesis that physiological coordination among Cyp19a1a expression, estrogen synthesis, and Shbgb localization may serve to regulate hormone levels in cellular precursors during the molecular differentiation process leading to ovarian development.

4.1. Estrogen signals and role of shbgb proteins during ovarian sexual differentiation

The three known targets of estrogens are estrogen receptors, GPR30, and SHBG proteins (Barton et al., 2017; Ge et al., 2012; Ijiri et al., 2008; Thomas, 2017). To understand the action of estrogens during molecular differentiation of the trout ovary, it is crucial to know which factors are produced to support maintenance and proliferation of germinal and somatic cellular precursors. In birds, for example, germline precursors do not express estrogen receptors during the gonadal differentiation period prior to morphological differentiation, but rather GPR30 proteins that are activated by estrogens (Ge et al., 2012). Stimulation of the GPR30 receptor induces cAMP synthesis, in turn stimulating cell proliferation (Ge et al., 2012). In tilapia, the estrogen receptors expressed (esr1, esr2a, and esr2b) do not show dimorphic expression during gonadal molecular differentiation (Ijiri et al., 2008). In another study, *era1* receptor expression was observed in the undifferentiated trout ovary only after 55 dpf (Guiguen et al., 1999). In this same study, *era1* expression was found to increase slightly at 63 dpf. *era1* expression was similar in males and females during morphological differentiation of the ovary and testis (Guiguen et al., 1999). Later studies indicated that rainbow trout and salmonid estrogen genes include duplicates (Nagler et al., 2007). Our qPCR analysis of the larval trunk was aimed at determining whether estrogen receptor expression was correlated with *shbgb* transcript expression. Our qPCR results did not indicate significant differences in the expression of the four types of estrogen receptors expressed during molecular differentiation. Our results for estrogen receptor expression levels are similar to previous findings

(Boyce-Derricott et al., 2010). Although we were unable to document differential expression of estrogen receptors, the abrupt drop in expression of the estrogen receptor after 42 dpf is striking. It is unclear whether this receptor participates directly in molecular differentiation events in rainbow trout.

In terms of other receptors, GPR30 has been identified in limited numbers of fish species, including Atlantic croaker (*Micropogonias undulates*), zebrafish (*Danio rerio*), orange-spotted grouper (*Epinephelus coioides*), and channel catfish (*Ictalurus punctatus*) (Liu et al., 2016; Pang et al., 2008; Thomas, 2017). The participation of GPR30 receptors in the context of gonadal differentiation of fish has received very little attention. However, a study in *E. coioides* revealed a significant increase in GPR30 expression at 110 days post-hatching in the forebrain and midbrain during gonadal sex differentiation (Nagarajan et al., 2011). There are no sequences in GenBank for this type of receptor in rainbow trout or other salmonids, suggesting either that these receptors have been lost during salmonid evolution or that they are under-represented in cDNA libraries. It is important to continue the search for non-classical estrogen receptors in salmonids, given their functional relevance and potential contributions to the physiological process of gonadal differentiation in fish. Studies in mammals indicate that SHBG alone or SHBG plus estradiol may activate membrane receptors, triggering the activation of cAMP synthesis (Fortunati et al., 2010; Rosner et al., 2010). In fish, there are no reports on the molecular properties of Shbga and Shbgb in terms of binding to membrane receptors to stimulate the synthesis of this type of second messenger. Although we have identified *shbgb* transcripts at the level of cellular somatic precursors during molecular differentiation, it remains unclear whether Shbgb proteins are also directly involved in estrogen signal transduction mechanisms, possibly through activation of G-protein-coupled receptors. In rainbow trout, there is no biochemical evidence of effective endogenous estrogen synthesis during very early gonadal differentiation. It is difficult to quantify estrogen levels in extremely small gonads, and mixtures of gonadal extracts complicate discrimination of the sexual phenotype of origin (Hines et al., 1999; Rowell et al., 2002; van den Hurk et al., 1982). Therefore, we cannot rule out the possibility that Shbgb proteins interact with a specific membrane receptor during early gonadal differentiation of fish, independent of estrogen availability in the extracellular medium. In later stages of molecular differentiation, Shbgb proteins may bind to the local estrogens contributed by incipient Cyp19a1a activity.

Findings from this work support two important concepts posed by Yamamoto (1969) regarding the mechanisms underlying sexual differentiation of teleost fish. This author elegantly elucidated the importance of identifying 1) the precursors of sex hormones (sex inducers) and the manner in which these precursors influence the indifferent gonia (protogonia) to develop into oocytes or spermatocytes; and 2) the biosynthesis routes for estrogens (sexogens) acting on cells surrounding the indifferent gonad, which may be active or inactive (Yamamoto, 1969). In this article, we reinterpret these concepts to elucidate the function of testosterone and estrogen mobilization as well as the nature of the transport of these hormones. Shbg proteins may transport these hormones from non-reproductive tissues towards the incipient gonads during early ovarian molecular differentiation. The yolk sac in trout is an important reservoir of hormones, steroid precursors, and nutrients vital for embryonic and larval development (Yeoh et al., 1996a,b). The need to transport hormones from these reserve tissues to the undifferentiated gonads in trout may be explained by the biosynthetic limitations of steroidogenic cell precursors and the absence of key enzymes such as 17beta-HSD, which transforms androstenedione into testosterone. Almost no studies in fish other than tilapia (Ijiri et al., 2008; Kobayashi and Nagahama, 2009) have provided evidence regarding 17beta-HSD expression during ovarian molecular differentiation. Partial or limited steroidogenesis during the early differentiation stages in trout creates a need to transport testosterone to the germ cell precursors, possibly inducing the cellular proliferation of these precursors. The transport and input of testosterone (or estrogens), mediated by hepatic Shbga and Shbgb proteins, may represent an important cooperative mechanism that contributes to ovarian molecular differentiation and that is essential for subsequent Cyp19a1a activity and estrogen synthesis in the undifferentiated gonad. Once estrogens are synthesized, levels of these hormones are finely modulated by Shbgb proteins secreted by steroidogenic somatic cell precursors. Therefore, we cannot rule out the possibility that extragonadal Shbga proteins, along with local Shbgb in undifferentiated gonads, contribute in some measure to molecular differentiation of the ovary.

5. Conclusion

The results in this study performed by qPCR experiments and *in situ* hybridization of *shbgb* transcripts demonstrate that during the molecular differentiation period of the rainbow trout ovary, a group of cells express these mRNAs. Perhaps the function of expression and secretion of Shbgb to the external environment could be related to regulation of estrogen and testosterone levels during the period of sexual differentiation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2017.11.021.

References

Antila, E., 1984. Steroid conversion by oocytes and early embryos of Salmo gairdneri. Ann. Zool. Fenn. 21, 465–471.
Baron, D., Cocquet, J., Xia, X., Fellous, M., Guiguen, Y., Veitia, R.A., 2004. An evolutionary and functional analysis of FoxL2 in rainbow trout gonad differentiation. J. Mol. Endocrinol. 33, 705–715.
Barton, M., Filardo, E.J., Lolait, S.J., Thomas, P., Maggiolini, M., Prossnitz, E.R., 2017. Twenty years of the G protein-coupled estrogen receptor GPER: historical and personal perspectives. J. Steroid Biochem. Mol. Biol.
Becchis, M., Sullivan, P.M., Ordronneau, P., Petrusz, P., Joseph, D.R., 1996. Distribution of immunoreactive androgen-binding protein/sex hormone-binding globulin in tissues of the fetal rat. Steroids 61, 392–400.
Becker, R.R., Iles, D.J., 1985. Developmental pattern of androgen-binding protein secretion during the critical period of sexual differentiation. Arch. Androl. 14, 107–114.
Berthelot, C., Brunet, F., Chalopin, D., Juanchich, A., Bernard, M., Noel, B., Bento, P., Da Silva, C., Labadie, K., Alberti, A., Aury, J.M., Louis, A., Dehais, P., Bardou, P., Montfort, J., Klopp, C., Cabau, C., Gaspin, C., Thorgaard, G.H., Boussaha, M., Quillet, E., Guyomard, R., Galiana, D., Bobe, J., Volff, J.N., Genet, C., Wincker, P., Jaillon, O., Roest Crollius, H., Guiguen, Y., 2014. The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates. Nat. Commun. 5, 3657.
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.

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.
Boyce-Derricott, J., Nagler, J.J., Cloud, J.G., 2010. The ontogeny of nuclear estrogen receptor isoform expression and the effect of 17beta-estradiol in embryonic rainbow trout (Oncorhynchus mykiss). Mol. Cell. Endocrinol. 315, 277–281.
Cutting, A., Chue, J., Smith, C.A., 2013. Just how conserved is vertebrate sex determination? Dev. Dyn. 242, 380–387.
Estay, F., Colihueque, N., Araneda, C., 2012. Comparison of oogenesis and sex steroid profiles between twice and once annually spawning of rainbow trout females (Oncorhynchus mykiss). ScientificWorldJournal 2012, 986590.
Feist, G., Schreck, C.B., 1996. Brain-pituitary-gonadal axis during early development and sexual differentiation in the rainbow trout, Oncorhynchus mykiss. Gen. Comp. Endocrinol. 102, 394–409.
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 Gac, F., 1989. Evidence for an androgen binding protein in the testis of a teleost fish (Salmo gairdneri R.): a potential marker of Sertoli cell function. J. Steroid Biochem. 32, 545–552.
Gabe, M., 1968. Techniques Histologiques. Masson, Paris.
Ge, C., Yu, M., Zhang, C., 2012. G protein-coupled receptor 30 mediates estrogen-induced proliferation of primordial germ cells via EGFR/Akt/beta-catenin signaling pathway. Endocrinology 153, 3504–3516.
Ghafoory, S., Breitkopf-Heinlein, K., Li, Q., Dzieran, J., Scholl, C., Dooley, S., Wolfl, S., 2012. A fast and efficient polymerase chain reaction-based method for the preparation of in situ hybridization probes. Histopathology 61, 306–313.
Guiguen, Y., Baroiller, J.F., Ricordel, M.J., Iseki, K., McMeel, O.M., Martin, S.A., Fostier, A., 1999. Involvement of estrogens in the process of sex differentiation in two fish species: the rainbow trout (Oncorhynchus mykiss) and a tilapia (Oreochromis niloticus). Mol. Reprod. dev. 54, 154–162.
Guiguen, Y., Fostier, A., Piferrer, F., Chang, C.F., 2010. Ovarian aromatase and estrogens: a pivotal role for gonadal sex differentiation and sex change in fish. Gen. Comp. Endocrinol. 165, 352–366.
Hammond, G.L., 2011. Diverse roles for sex hormone-binding globulin in reproduction. Biol. Reprod. 85, 431–441.
Hines, G.A., Boots, L.R., Wibbels, T., Watts, S.A., 1999. Steroid levels and steroid metabolism in relation to early gonadal development in the tilapia Oreochromis niloticus (Teleostei: cyprinoidei). Gen. Comp. Endocrinol. 114, 235–248.
Ijiri, S., Kaneko, H., Kobayashi, T., Wang, D.S., Sakai, F., Paul-Prasanth, B., Nakamura, M., Nagahama, Y., 2008. Sexual dimorphic expression of genes in gonads during early differentiation of a teleost fish, the Nile tilapia Oreochromis niloticus. Biol. Reprod. 78, 333–341.
Kobayashi, T., Nagahama, Y., 2009. Molecular aspects of gonadal differentiation in a teleost fish, the Nile tilapia. Sex. Dev. 3, 108–117.
Lau, E.S., Zhang, Z., Qin, M., Ge, W., 2016. Knockout of zebrafish ovarian aromatase gene (cyp19a1a) by TALEN and CRISPR/Cas9 leads to all-male offspring due to failed ovarian differentiation. Sci. Rep. 6, 37357.
Lebrun, C., Billard, R., Jalabert, B., 1982. Changes in the number of germ cells in the gonads of the rainbow trout (Salmo gairdneri) during the first 10 post-hatching weeks. Reprod. Nutr. Dev. 22, 405–412.
Liu, Z., Liu, S., Yao, J., Bao, L., Zhang, J., Li, Y., Jiang, C., Sun, L., Wang, R., Zhang, Y., Zhou, T., Zeng, Q., Fu, Q., Gao, S., Li, N., Koren, S., Jiang, Y., Zimin, A., Xu, P., Phillippy, A.M., Geng, X., Song, L., Sun, F., Li, C., Wang, X., Chen, A., Jin, Y., Yuan, Z., Yang, Y., Tan, S., Peatman, E., Lu, J., Qin, Z., Dunham, R., Li, Z., Sonstegard, T., Feng, J., Danzmann, R.G., Schroeder, S., Scheffler, B., Duke, M.V., Ballard, L., Kucuktas, H., Kaltenboeck, L., Liu, H., Armbruster, J., Xie, Y., Kirby, M.L., Tian, Y., Flanagan, M.E., Mu, W., Waldbieser, G.C., 2016. The channel catfish genome sequence provides insights into the evolution of scale formation in teleosts. Nat. Commun. 7, 11757.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.
Lubzens, E., Young, G., Bobe, J., Cerda, J., 2010. Oogenesis in teleosts: how eggs are formed. Gen. Comp. Endocrinol. 165, 367–389.
Marivin, E., Yano, A., Guerin, A., Nguyen, T.V., Fostier, A., Bobe, J., Guiguen, Y., 2014. Sex hormone-binding globulins characterization and gonadal gene expression during sex differentiation in the rainbow trout, Oncorhynchus mykiss. Mol. Reprod. dev. 81, 757–765.
Marlatt, V.L., Sun, J., Curran, C.A., Bailey, H.C., Kennedy, C.K., Elphick, J.R., Martyniuk, C.J., 2014. Molecular responses to 17beta-estradiol in early life stage salmonids. Gen. Comp. Endocrinol. 203, 203–214.
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.
Miura, C., Higashino, T., Miura, T., 2007. A progestin and an estrogen regulate early stages of oogenesis in fish. Biol. Reprod. 77, 822–828.
Nagarajan, G., Tsai, Y.J., Chen, C.Y., Chang, C.F., 2011. Developmental expression of genes involved in neural estrogen biosynthesis and signaling in the brain of the orange-spotted grouper Epinephelus coioides during gonadal sex differentiation. J. Steroid Biochem. Mol. Biol. 127, 155–166.
Nagler, J.J., Cavileer, T., Sullivan, J., Cyr, D.G., Rexroad 3rd, C., 2007. The complete nuclear estrogen receptor family in the rainbow trout: discovery of the novel ERalpha2 and both ERbeta isoforms. Gene 392, 164–173.
Nicol, B., Yano, A., Jouanno, E., Guerin, A., Fostier, A., Guiguen, Y., 2013. Follistatin is an early player in rainbow trout ovarian differentiation and is both colocalized with aromatase and regulated by the wnt pathway. Sex. Dev. 7, 267–276.
Pang, Y., Dong, J., Thomas, P., 2008. Estrogen signaling characteristics of Atlantic croaker G protein-coupled receptor 30 (GPR30) and evidence it is involved in maintenance of oocyte meiotic arrest. Endocrinology 149, 3410–3426.
Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucl. Acids Res. 29, e45.
Piferrer, F., Donaldson, E.M., 1994. Uptake and clearance of exogenous estradiol-17beta and testosterone during the early development of coho salmon (Oncorhynchus kisutch), including eggs, alevins and fry. Fish Physiol. Biochem. 13, 219–232.
Rosner, W., Hryb, D.J., Kahn, S.M., Nakhla, A.M., Romas, N.A., 2010. Interactions of sex hormone-binding globulin with target cells. Mol. Cell. Endocrinol. 316, 79–85.
Rowell, C.B., Watts, S.A., Wibbels, T., Hines, G.A., Mair, G., 2002. Androgen and estrogen metabolism during sex differentiation in mono-sex populations of the Nile tilapia, Oreochromis niloticus. Gen. Comp. Endocrinol. 125, 151–162.
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.
Thomas, P., 2017. Role of G-protein-coupled estrogen receptor (GPER/GPR30) in maintenance of meiotic arrest in fish oocytes. J. Steroid Biochem. Mol. Biol. 167, 153–161.
van den Hurk, R., Lambert, J.G., Peute, J., 1982. Steroidogenesis in the gonads of rainbow trout fry (Salmo gairdneri) before and after the onset of gonadal sex differentiation. Reprod. Nutr. Dev. 22, 413–425.
von Schalburg, K.R., Motoshige, Y., Davidson, W.S., Koop, B.F., 2010. Regulation, expression and characterization of aromatase (*cyp19b1*) transcripts in ovary and testis of rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 155, 118–125.
Vizziano, D., Randuineau, G., Baron, D., Cauty, C., Guiguen, Y., 2007. Characterization of early molecular sex differentiation in rainbow trout, Oncorhynchus mykiss. Dev. Dyn. 236, 2198–2206.
Vizziano-Cantonnet, D., Baron, D., Mahe, S., Cauty, C., Fostier, A., Guiguen, Y., 2008. Estrogen treatment up-regulates female genes but does not suppress all early testicular markers during rainbow trout male-to-female gonadal transdifferentiation. J. Mol. Endocrinol. 41, 277–288.
Vizziano-Cantonnet, D., Anglade, I., Pellegrini, E., Gueguen, M.M., Fostier, A., Guiguen, Y., Kah, O., 2011. Sexual dimorphism in the brain aromatase expression and activity, and in the central expression of other steroidogenic enzymes during the period of sex differentiation in monosex rainbow trout populations. Gen. Comp. Endocrinol. 170, 346–355.
Wang, D.S., Kobayashi, T., Zhou, L.Y., Paul-Prasanth, B., Ijiri, S., Sakai, F., Okubo, K., Morohashi, K., Nagahama, Y., 2007. Foxl2 up-regulates aromatase gene transcription in a female-specific manner by binding to the promoter as well as interacting with ad4 binding protein/steroidogenic factor 1. Mol. Endocrinol. 21, 712–725.
Yamamoto, T.-O., 1969. Sex differentiation. Fish Physiol. 3, 117–175.
Yano, A., Guyomard, R., Nicol, B., Jouanno, E., Quillet, E., Klopp, C., Cabau, C., Bouchez, O., Fostier, A., Guiguen, Y., 2012. An immune-related gene evolved into the master sex-determining gene in rainbow trout, Oncorhynchus mykiss. Curr. Biol. 22, 1423–1428.
Yeoh, C.G., Schreck, C.B., Feist, G.W., Fitzpatrick, M.S., 1996a. Endogenous steroid metabolism is indicated by fluctuations of endogenous steroid and steroid glucuronide levels in early development of the steelhead trout (Oncorhynchus mykiss). Gen. Comp. Endocrinol. 103, 107–114.
Yeoh, C.G., Schreck, C.B., Fitzpatrick, M.S., Feist, G.W., 1996b. In vivo steroid metabolism in embryonic and newly hatched steelhead trout (Oncorhynchus mykiss). Gen. Comp. Endocrinol. 102, 197–209.
Yoshizaki, G., Ichikawa, M., Hayashi, M., Iwasaki, Y., Miwa, M., Shikina, S., Okutsu, T., 2010. Sexual plasticity of ovarian germ cells in rainbow trout. Development 137, 1227–1230.