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Sex hormone binding globulin during an annual reproductive cycle in the hepatopancreas and ovary of pejerrey (*Odontesthes bonariensis)*

Anelisa González[a], Juan I. Fernandino[a], Mariano Elisio[a,1], Tomás Chalde[a,2], Leandro A. Miranda[a], Geoffrey L. Hammond[b], Gustavo M. Somoza[a,*]

[a] Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús "Dr. Raúl Alfonsín" (CONICET-UNSAM), Chascomús, Buenos Aires, Argentina
[b] Department of Cellular and Physiological Sciences, Faculty of Medicine, The University of British Columbia, British Columbia, Canada

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ABSTRACT

In the present study, we determined the hepatopancratic *shbg* transcript abundance and ovarian immunoreactive Shbg (ir-Shbg) localization in pejerrey females at different gonadal stages during an annual ovarian cycle. In the hepatopancreas, *shbg* expression remains was constant in pre-vitellogenic stages, decreased at final vitellogenesis to increase again in final maturation and atretic stages to previous levels at post-vitellogenic stages. Related to this, also we found a negative significant relation between sex steroid and *shbg* expression. On the other hand, in the ovary we found ir-Shbg inside of cortical alveoli, from previtellogenic stages to final maturation. This localization of Shbg in a teleost fish ovary suggests a new role for Shbg in oocytes, that may also extend the oocyte fertilization/development process.

1. Introduction

In fish females, oogenesis and oocyte maturation are triggered by environmental cues and are regulated by different hormones (Lubzens et al., 2010; Nagahama and Yamashita, 2008). Once oogenesis is triggered, gonadotropins are released from the pituitary gland into the bloodstream to induce oocyte growth and stimulate follicular cells to synthesize 17β-estradiol (E_2) (Lubzens et al., 2017). In turn, E_2 is transported in the plasma by carrier proteins to stimulate vitellogenin synthesis in the liver (Nagahama and Yamashita, 2008). Sex hormone-binding globulin (Shbg) is a carrier serum protein involved in the transport of sex steroids and it regulates their plasma metabolic clearance rates (Hammond 2011, 2016).

In teleosts, the main site of Shbg production is the liver, but there is little knowledge about its regulation, and particularly, about the involvement of sex steroids hormones on *shbg* expression (Bobe et al., 2010). In some species, a direct relationship between liver Shbg synthesis and E_2 levels has been assumed because of parallel changes in plasma Shbg and E_2 levels (Foucher et al., 1991; Hobby et al., 2000; Laidley and Thomas, 1997). However, in some species, Shbg plasma levels or *shbg* transcript levels decrease during the reproductive season when plasma sex steroid levels were elevated (González et al., 2017; Miguel-Queralt et al., 2007). Besides the liver, the presence of Shbg or *shbg* transcripts was documented in numerous other tissues of different vertebrates (González et al., 2017; Hryb et al., 2002; Joseph et al., 1997; Miguel-Queralt et al., 2004, 2007).

In some fish species, two *shbg* paralogs have been found and named *shbga* and *shbgb*. The Shbga is the ortholog of the mammalian SHBG, and it has been found in all teleost fish species studied (Bobe et al., 2010), while Shbgb was only identified in salmonids (Bobe et al, 2008; Miguel-Queralt et al., 2004). In this group of fish, *shbgb* transcripts have been detected mainly in the ovary, and their expression profile appears to decline with decreases in ovarian aromatase expression and E_2 serum levels, suggesting that Shbgb may somehow control ovarian function (Bobe et al, 2008; Miguel-Queralt et al., 2004). Also, in two non-salmonid fish, Shbga was found in the ovary (González et al., 2017; Miguel-Queralt et al., 2007). In Sea bass, immunoreactive Shbg (ir-Shbg) was observed in connective tissue surrounding the immature ovary and around post-vitellogenic oocytes (Miguel-Queralt et al., 2007), and in pejerrey, ir-Shbg is present in the cytoplasm of the oocytes and in the chorionic filaments (González et al., 2017).

Our model fish, the pejerrey *Odontesthes bonariensis*, is a multiple spawner with a seasonal reproductive cycle occurring during spring (Elisio et al., 2014). In this species, both E_2 and T plasma levels increase

* Corresponding author at: IIB-INTECH (CONICET-UNSAM), Av. Intendente Marino Km. 8.2 (B7130IWA), Chascomús, Buenos Aires, Argentina.
E-mail address: somoza@intech.gov.ar (G.M. Somoza).
[1] Present address: Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP), Mar del Plata, Argentina.
[2] Present address: Centro Austral de Investigaciones Científicas (CADIC), CONICET, Ushuaia, Argentina.

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in relation with gonadal development and vitellogenesis, and then decrease at final maturation and ovulation (Elisio et al., 2014). In this context, we therefore set out to study the variations of hepatopancreatic *shbg* expression during a reproductive cycle, the correlation with plasma E_2 levels as well as the localization of Shbg in the ovary during the ovarian cycle in pejerrey fish.

2. Materials and methods

2.1. Animal sampling

All animals used in this study were captured in the Chascomús Lagoon (35°36′S 58°02′W). The cDNA and histological samples were previously obtained by Elisio et al. (2014). Briefly, hepatopancreas, gonads and blood of pejerrey females were sampled monthly. The hepatopancreas and gonads were weighed (GW and HW ± 0.1 g, respectively) for gonadosomatic (GSI = 100 × gonad weight/total weight) and hepatopancreatic-somatic index (HSI = 100 × hepatopancreas weight/total weight) calculations. A portion of each hepatopancreas was stored in TRIzol Reagent (ThermoFisher Scientific, Waltham, USA) at −80 °C prior to RNA extraction. Thereafter *shbg* transcripts were measured by quantitative Real Time PCR (RT-qPCR) as already described in González et al. (2017). A section of each gonad was fixed in Bouin's fluid and processed for histological analysis, embedded in Paraplast® (Leica Biosystems, Wetzlar, Germany) and then cut at 6 µm sections, stained with hematoxylin-eosin and mounted. Oogenic stages were classified according to the proportion of different oocyte development in the ovary and classified into five gonadal stages: primary growth (PG), cortical alveoli (CA), initial vitellogenesis (VtgA), final vitellogenesis (VtgB), final maturation (FM) and atretic (AT), as defined by Elisio et al. (2014). All fish were handled and sacrificed in accordance with the UFAW (Use and Care Committee Handbook on the Care and Management of Laboratory Animals; http://www.ufaw.org.uk/pubs.htm#Lab) and local regulations.

2.2. RNA extraction and quantification by RT-qPCR

Total RNA was extracted using Trizol reagent to synthetize cDNA from female pejerrey hepatopancreas and hepatopancreatic slices from e*x vivo* experiments as already described (González et al., 2017). Briefly, RNA samples were treated with DNaseI (ThermoFisher Scientific) and then reverse transcribed using SuperScript II, RNase OUT (ThermoFisher Scientific) and oligo (dT) 12–18 following the manufacturer instructions. The expression of the following genes was quantified by RT-qPCR: *shbg* (GenBank Accession KF680077.1), and *b-actin* (EF044319) as a reference gene. The *b-actin* as a reference gene was select considering the lack of significative variance between control and treatment (Fig. Supplementary 1). All primers used are shown in Table 1. Each RT-qPCR reaction was performed in a volume of 15 µL, containing 7.5 µL of FastStart Universal Master SYBR Green (Roche Applied Science, Penzberg, Germany), 1 µL of cDNA and 600 nM of each oligonucleotide. Each primers concentration used was optimized with a PCR reaction efficiency between 95 and 105% by standard dilution curve as previously established (González et al., 2017). Samples were analyzed with Step OnePlus Real-Time PCR System (Applied Biosystems, Foster City, USA). The amplification protocol consisted of an initial cycle of 1 m 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).

Table 1
Gene-specific primers used for RT-qPCR analysis.

Gene	*Name*	*Sequence*	*Size*	*ACC#*
shbg	RQshbgspFw	CGGAGACACCAAAAATGGAG	75 pb	KF680077.1
	RQshbgspRv	CCTCTGATGCAGATCAGCAA		
b-actin	RQactinFw	CTCTGGTCGTACCACTGGTATCG	83 pb	EF044319
	RQactinRv	GCAGAGCGTAGCCTTCATAGATG		

2.3. Immunocytochemistry

Ovaries from pejerrey at different gonadal stages were fixed in Bouin solution for 24 h at 4 °C, and then stored in 70% ethanol until processed. Tissues were then dehydrated, embedded in Paraplast® and cut in 6 µm thick serial sections. The sections were de-waxed and incubated at high power in a microwave oven for 5 min in citrate buffer (pH 6.0). They were then cooled to room temperature for 40 min, treated with 0.05% hydrogen peroxide solution for 45 min, and blocked for 1 h at room temperature with BSA (5 mg/ml) in PBS. An antiserum specific for sea bass Shbg raised in rabbits (Miguel-Queralt et al., 2005), previously validated using pejerrey hepatopancreas by Western blotting and immunocytochemistry was used (González et al., 2017). Briefly, the antiserum was used at a 1:2000 dilution in PBS or preabsorbed with purified sea bass-Shbg peptide as control. After an overnight incubation at 22 °C immunoreactivity was revealed with a 0.5% of 3.3-diaminobenzidine tetrahydrochloride in PBS, containing 0.05% H_2O_2. Photographs were taken using a Nikon Eclipse E7000 and the Image Pro Plus (Media Cybernetics, Rockville, USA).

2.4. Statistical analyses

All experiments related to gene expression profiles were analyzed using fgStatistics software (Di Rienzo et al., 2010). The relationship between E_2 plasma levels (data taken from Elisio et al., 2014 and *shbg* expression was assessed by a linear regression model using a previous logarithmic transformation of the response variable (*shbg* expression levels). The effect of gonadal stages (vitellogenic and non-vitellogenic females) on the change of the model parameters was assessed by an analysis of variance (F-test). Results were considered statistically significant at $p < 0.05$. Statistical analyses were performed using GraphPad Prism ver. 5.0 (GraphPad Software, San Diego, USA), and R ver. 3.1.0 (R Foundation for Statistical Computing, Austria) software.

3. Results

3.1. Shbg transcript abundance in pejerrey hepatopancreas at different gonadal stages

Hepatopancreas from VtgB females presented significantly lower *shbg* transcript abundance when compared to this tissue from females at other gonadal stages: PG, CA, VtgA, FM and AT (Fig. 1). The stability of *b-actin*, as reference gene, was constant between all the gonadal stages and was not influenced by E_2 plasma levels (Supplementary Fig. 1).

3.2. Relationship between E_2 plasma levels and hepatopancreatic shbg transcript abundance

Hepatopancreatic *shbg* abundance was significantly associated with E_2 plasma levels, showing a negative relationship, and did not change significantly between vitellogenic and non-vitellogenic females, but became more consistent when only vitellogenic females were considered, with an increase of R^2 from 0.22 to 0.43 (Fig. 2).

3.3. Localization of ir-Shbg in pejerrey ovary at different reproductive stages

Previtellogenic ovaries showed ir-Shbg in the cytoplasm of some PG oocytes (Fig. 3; PG row). At the CA stage, diffuse ir-Shbg staining was present in some cortical alveoli and strong signals were observed in the chorionic filaments (Fig. 3; CA line). As the gonadal stage progressed, the ir-Shbg stain was observed towards the periphery of the oocyte, in accordance with a displacement of cortical alveoli during vitellogenesis

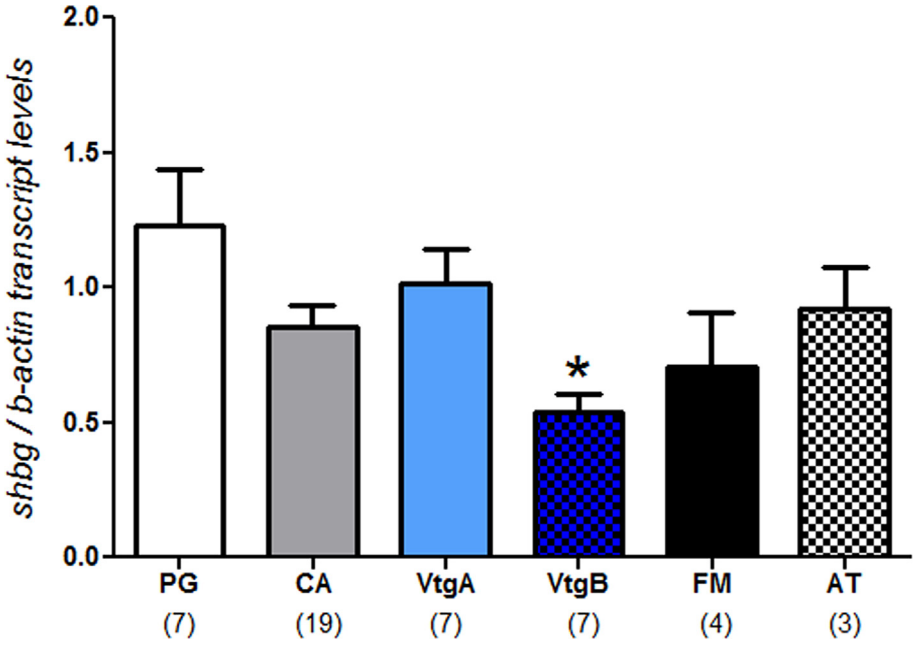

Fig. 1. Relative hepatopancreatic shbg transcript levels in pejerrey females during oogenesis. Values are mean ± SEM (numbers between brackets under each column indicate sample size). PG: Primary Growth; CA: Cortical Alveoli; VtgA: Initial vitellogenesis; VtgB: Final vitellogenesis; FM: Final Maturation; AT: Atretic stage. Asterisk means significant difference between gonadal stages ($p < 0.05$).

(Fig. 3; VtgA, VtgB and FM lines).

4. Discussion

Shbg is a plasma protein that specifically carries sex steroid from it source to target tissue, determine the free and bioavailable plasma steroid concentration and controls its metabolic clearance (Mendel (1989); Laurent et al. (2016); Hammond, 2011; 2016). Shbg is present in the blood of all vertebrates (Bobe et al., 2010; Hammond, 2011), except birds (Malisch and Breuner, 2010). In all species, the liver is the main site of its expression (Hammond, 2011, 2016), and while ir-Shbg is absent in pejerrey hepatocytes, a strong signal has been observed in the pancreas, which together with the liver forms the hepatopancreas (González et al., 2017). Moreover, in female pejerrey, hepatopancreatic *shbg* transcript abundance varies between reproductive and non-reproductive seasons (González et al., 2017). In order to better understand the participation of Shbg in reproduction in pejerrey, we therefore analyzed hepatopancreatic *shbg* transcript levels in females at different gonadal stages. As Shbg was also detected in their oocytes (González et al., 2017), its ovarian localization was followed during oocyte maturation.

Notably, the lowest hepatopancreatic *shbg* transcript levels coincided with the highest levels of plasma E_2 and T in this species (Elisio et al., 2014), and a negative relationship between E_2 plasma levels and the expression of hepatopancreatic *shbg* was observed, being highest during vitellogenic stages. Similar results were observed in sea bass, where Shbg plasma levels presented the lowest value during the reproductive season (Miguel-Queralt et al., 2007). In this work the authors proposed that in sea bass, changes in feeding and the nutritional/metabolic status between reproductive and non-reproductive seasons could be important factors involved in the regulation of Shbg plasma levels. Nevertheless, in pejerrey, regardless of whether *shbg* is regulated by sex steroids and other factors, its decrease at the final stage of vitellogenesis would serve to maintain reduced plasma E_2 levels, which is consistent with the hypothesis of a protective role of Shbg for biologically active plasma steroids (Bobe et al., 2010; Mendel (1989)). This

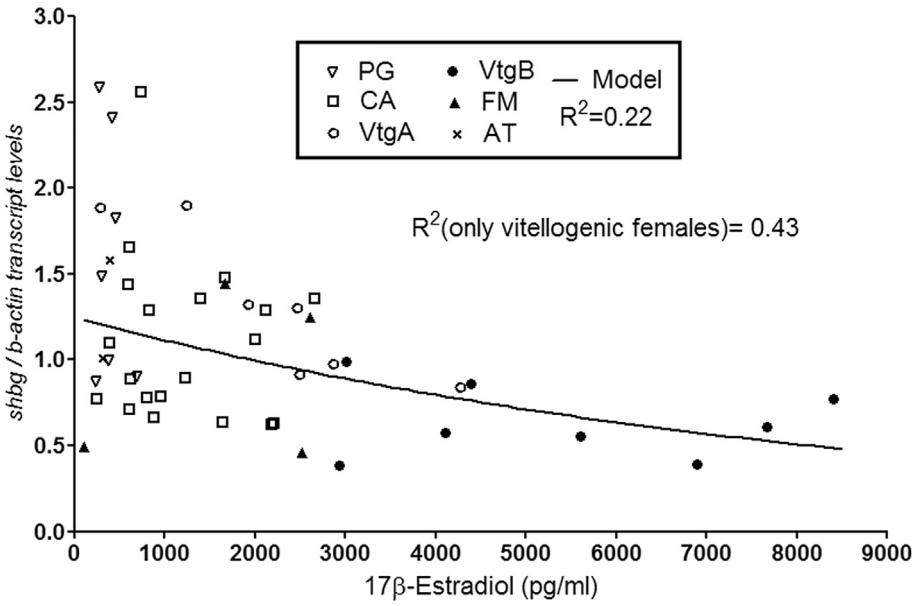

Fig. 2. Corelation between E_2 plasma levels and hepatopancreatic *shbg* transcript levels in pejerrey females. The linear regressions are statistically significant (Fisher's test, $p < 0.05$). The value of coefficient of determination (R^2) for each case is shown on top of each plot. R^2 considering all gonadal stages = 0.22 and only considering VtgA and VtgB stages = 0.43.

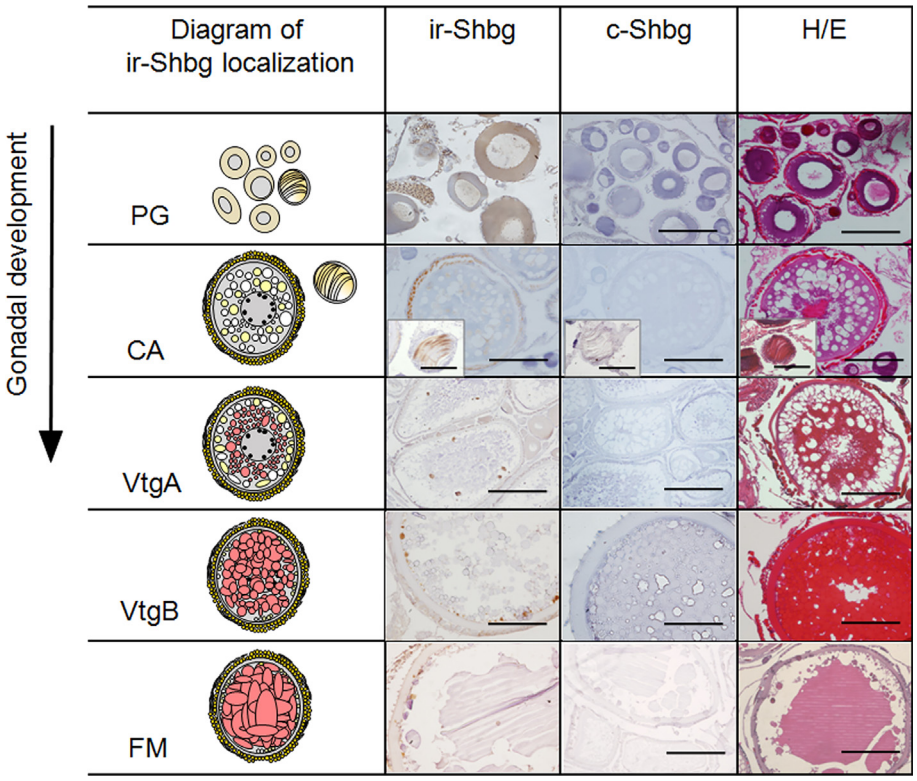

Fig. 3. Immunoreactive Shbg localization in pejerrey ovary at different gonadal stages. PG, CA, VtgA, VtgA and FM. From left to right the first column shows a diagram of the ir-Shbg localization in the oocyte as gonadal development progresses (from top to bottom). The ir-Shbg signal is indicated in yellow and yolk in red. The second column represents ir-Shbg in pejerrey ovary and the third negative controls. The fourth column shows the hematoxylin-eosin colored sections. The length of the scale bar is 100 µm.

happens when the steroidogenic shift occurs during oocyte maturation and ovarian follicle layers start to produce MIH (17α, 20β-dihydroxy-4-pregnen-3-one, in most teleosts), as reviewed by Nagahama and Yamashita (2008), and it is known that Shbg does not bind progestagens in teleosts (Bobe et al., 2010).

On the other hand, very low *shbg* transcript levels have been observed in the pejerrey ovary and ir-Shbg was reported in the oocytes (Bobe et al., 2010). These low *shbg* transcript levels may account for ir-Shbg in the ovary, but ir-Shbg was not found in other tissues/organs (brain, muscle and kidney) with similarly low *shbg* transcript levels, and is possible that the Shbg in oocytes originate through the sequestration of plasma Shbg. On this respect, plasma serum SHBG is sequestered within the endometrial stroma of mice by fibulin-like extracellular matrix associated proteins (Ng et al., 2006). If a similar pathway is present in pejerrey gonads where Shbg is mainly outside the oocyte plasma membrane, it is possible than Shbg is trapped in the chorionic filaments and then internalized in the oocyte, explaining the diffuse staining in cortical alveoli. Although the immunodetection of Shbg was previously reported in the ovary of sea bass (Miguel-Queralt et al., 2007), and Shbg binding activity was observed in the ovarian interstitial fluid of the spotted weakfish *Cynoscion nebulosus* (Laidley and Thomas, 1997), in pejerrey ovaries, ir-Shbg was mainly restricted to the cortical alveoli. In this species, as vitellogenesis progressed, the ir-Shbg staining was localized towards the periphery. This is consistent with the migration of the cortical alveoli to the egg plasma membrane, in the so-called cortical reaction at fertilization in fish (Lubzens et al., 2010), leading to the restructuring of the egg envelop proteins to form the chorion (Selman et al., 1993).

Although there is little information about the chemical composition of the cortical alveoli, it is mainly composed by glycoproteins (Gallo and Costantini, 2012) like the fibulins (Segade, 2010), the mechanism of Shbg accumulation in the cortical alveoli is intriguing. Additional studies are therefore warranted to determine if Shbg serves to transport E_2 from the follicular layers into the oocyte, and if Shbg is produced within the oocyte itself to play a role during oocyte development. There are reports that aromatase is expressed inside oocytes of the frog, *Xenopus laevis* (Gohin et al., 2011a) and rainbow trout (Gohin et al., 2011b), and it is possible that Shbg produced within the oocytes may regulate E_2 actions during oocyte development.

In summary, our study showed a negative relationship between hepatopancreatic *shbg* expression and E2 plasma levels in pejerrey females studied during an annual reproductive cycle. Beside, showed a new immunolocalization of ir-Shbg inside the cortical alveoli on the ovary, which could be suggests a novel role for Shbg in the regulation of E_2 action during oocyte development and reproduction.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.11.010.

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