

## Extrahypophyseal expression of gonadotropin subunits in pejerrey *Odontesthes bonariensis* and effects of high water temperatures on their expression

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### ABSTRACT

It has been traditionally accepted that the gonadotropins (GtHs), follicle stimulating hormone (FSH) and luteinizing hormone (LH), are synthesized and secreted only by the pituitary. However, the presence of these hormones in extrapituitary tissues has been demonstrated in mammals, and more recently also in fish. In this study, we cloned the cDNAs and characterized the expression of *FSH-β*, *LH-β*, and glycoprotein hormone  $\alpha$  (*GPH-α*) subunits from brain and gonads of male and female pejerrey *Odontesthes bonariensis* at different stages of gonadal maturation. *In situ* hybridization revealed that, in addition to their classical location in pituitary cells, the three GtH transcripts were also located in the gonads. *FSH-β* and *GPH-α* subunits were found in the cytoplasm of oogonia, previtellogenic and vitellogenic oocytes in ovaries. *LH-β* expression was detected in previtellogenic and vitellogenic oocytes but not in oogonia. In males, the three subunits were expressed in spermatogonia and to a lesser extent in spermatocytes. Exposure of fish to high water temperatures that impair pejerrey reproduction also induced a decrease of extrahypophyseal expression of GtH subunits.

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### 1. Introduction

The pituitary gonadotropins (GtHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are key factors regulating gonadal maturation and function. In teleost fish, as in all vertebrates, these hormones are heterodimeric glycoproteins composed of a hormone-specific  $\beta$  subunit and a common  $\alpha$  subunit [13,47].

Traditionally it has been thought that GtHs are synthesized only in adenohypophyseal cells. However, LH and FSH producing neurons and their receptors have been identified using different techniques in the rat central nervous system [5,10,11,31]. The expression of GtH subunits (both at mRNA and protein levels) has also been demonstrated in mouse and rat gonads suggesting a local action [15,16,34,48,49].

Recent studies using cDNA characterization or immunocytochemistry have shown the presence of *FSH-β* and *LH-β* in the brain of the teleosts *Oreochromis niloticus*, *Danio rerio* and *Cichlasoma dimerus* [26,27,30,36]. Moreover, the presence of GtH subunits was reported in the ovary of *Sparus aurata* and *Silurus meridionalis* [45,46] and in the ovary and testis of *D. rerio* [36]. Their expression seemed to be stage dependent and subject to the regulation by gonadotropin-releasing hormone (GnRH) [45].

The control of LH and FSH synthesis and secretion is complex and involves an interplay between the gonads, pituitary and the hypothalamus [50]. In salmonids, it was suggested that FSH plays a major regulatory role during early stages of oogenesis and spermatogenesis and LH is mainly involved in the final stages of maturation [8,38,39].

Nevertheless, in multiple spawning fish both *FSH-β* and *LH-β* mRNA levels fluctuate in parallel during gonadal maturation [42,47]. According to this observation, it has been recently found in tilapia (*O. niloticus*) females two clear peaks of plasmatic FSH and LH levels in a reproductive cycle. One during vitellogenesis phase after spawning and the other just before spawning [1], suggesting that LH may play a role during vitellogenesis and not only during final oocyte maturation [1]. Taking together these considerations, it is possible that each gonadotropin regulates in a differential way, distinct oocyte stages present in the ovary at the same time [13].

It is also known that environmental factors, like photoperiod and temperature, influence the reproductive system in fish and that changes in these parameters can affect gonadal development and function [3,28]. For example, elevated water temperature in female Atlantic salmon (*Salmo salar*) can induce reproductive alterations as evidenced by decreased plasmatic levels of 17 $\beta$ -estradiol ( $E_2$ ) and vitellogenin [21,22,29]. It was also reported that high water temperatures caused inhibition of brain and pituitary gene expression related to reproduction in red seabream, *Pagrus major*

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[24]. In accordance with these observations, in our experimental model, the pejerrey *Odontesthes bonariensis*, it was demonstrated that short periods at water temperatures higher than 23 °C with no photoperiod manipulations completely blocked spawning, altered pituitary expression of GtHs and gonadal steroids plasmatic levels, and provoked gonadal regression [37]. Taken together, these results demonstrated that the delicate balance in the brain–pituitary–gonadal axis can be easily disrupted by elevated water temperatures in fish.

In this context, the aim of this research was to determine whether pejerrey gonadotropin subunit genes are expressed also in brain and gonads and if their expression is affected by high water temperatures as is the case in the pituitary.

## 2. Material and methods

### 2.1. Characterization of extrapituitary GtH subunit cDNAs

Three mature and immature animals of each sex from the stock maintained at the aquatic facilities of the Instituto de Investigaciones Biotecnológicas–Instituto Tecnológico de Chascomús (IIB–INTECH) were used. Mature fish (female GSI > 6; male GSI > 1) were selected from a tank with artificial reproductive stimulatory condition (18–20 °C, 14L: 10D) and immature fish (female GSI < 1; male GSI < 0.3) from a tank with artificial regressive reproductive condition (14–16 °C, 14D: 10L) following the criteria outlined by Miranda et al. [18,19].

They were killed with an overdose of 2-phenoxyethanol, and the brain, the pituitary, and gonads were dissected out and kept in RNA-later (Sigma, St. Louis, USA) at –80 °C until used. Total RNA for each tissue sample was extracted using TRIzol Reagent (Invitrogen, Germany). RNA samples were then treated with DNase I (Invitrogen, Germany) and reverse transcribed using SuperScript III RNase H (Invitrogen, Germany) and oligo(dT)12–18 following the manufacturer instructions. The cDNAs obtained were used as templates for PCR amplification with gene-specific primers (Table 1) designed based on the full length sequences previously obtained for pituitary *FSH-β*, *LH-β* and *GPH-α* (GenBank: DQ382280, AY319832 AY319833 [18]). Thermal cycling was performed on an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the following program: 94 °C for 3 min; 30 cycles of 94 °C for 20 s, 54 °C for 20 s, 72 °C for

30 s and 72 °C for 2 min. The products obtained by PCR were separated on a 1% agarose gel electrophoresis, purified using an Ultra-clean DNA purification kit (MO BIO Laboratories, Carlsbad, CA, USA), and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). PCR products from several independent PCR amplifications were cloned to avoid PCR errors caused by mis-incorporation. Plasmidic DNA was then purified by alkaline lysis and sequenced with an ALF Express II DNA sequencer (Pharmacia Ltd., Sandwich, Kent, UK) using Thermo sequence fluorescent label primer cycle sequencing kit with 7-deaza-dGTPt (Amersham Bioscience, Uppsala, Sweden).

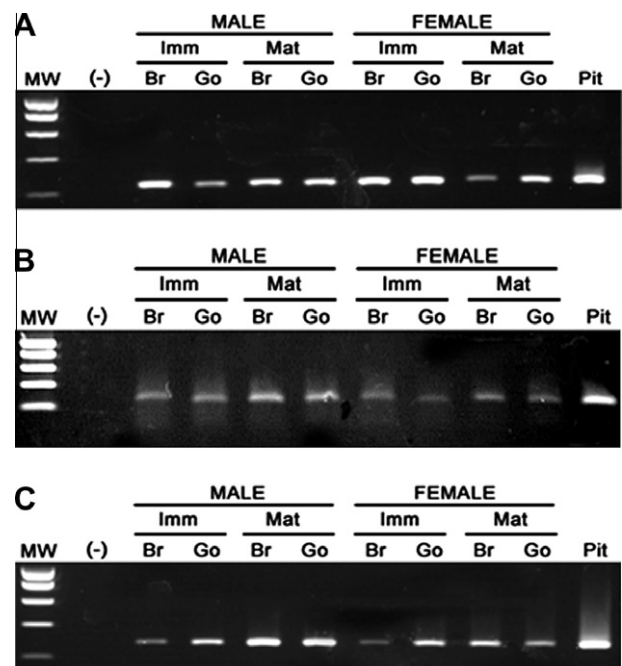
The nucleotide and predicted amino acid sequences for brain, ovary, testis, and pituitary forms were aligned by Clustal W multiple sequences alignment using DNASTAR 4.05 and their similarity was compared using the GeneDoc programme [20].

### 2.2. In situ hybridization (ISH)

Adult pejerrey of both sexes (3 mature males, IGS > 1 and 3 vitellogenic females, IGS > 6) were fixed by perfusion in 4% paraformaldehyde (PFA) and subsequently, brains with attached pituitaries and gonads were dissected out and processed as for routine histological preparations. *In situ* hybridizations were performed using probes based on the primer amplicons listed in Table 1. The riboprobe sequences were verified *in silico* and no hybridization with other unrelated transcripts were found. In order to obtain a specific signal the pituitary gland was used as a control. The different probes were labeled with a mix of rNTPs including Biotin-16-uridine-5'-triphosphate (Roche Applied Science) and UTPs, ATPs, CTPs and GTPs (Invitrogen). Six μm-thick sections were deparaffinized, dehydrated, and pretreated with proteinase K (5 μg/ml) for 7.5 min at room temperature. The reaction was stopped by washing in glycine-PBS buffer (2 mg/ml) for 10 min and the sections were then dipped in 100 mM triethylamine containing 0.25% anhydrous acetic acid for 10 min. For hybridization, sections were covered with 150 μl of Biotin-labeled sense or anti-

**Table 1**  
Oligonucleotide primers used for cDNA cloning, *in situ* hybridization (ISH) and RT-qPCR (RT).

Primer	Sequence (5'–3')	Amplification size (bp)
FSH-β F	TCAGCATCCCTgTggAgAgCTg	242
FSH-β R	TCCgCagTCAGTgTATACTgTTg	
LH-β F	TgTCATgAAgACgTggTTCAGc	253
LH-β R	TATgCgCTACgTTTgTTACTggA	
GPH-α F	gTCTCTTCTCTgTTgTCCTTCTTC	288
GPH-α R	gTgTggTTTTCTCAgCgTAATgTC	
β-Actin F	ggATgATATggAgAAgATCTgg	512
β-Actin R	ATggTgATgACCTgTCCgTC	
FSH-β F (ISH)	gTCAGCATCCCTgTggAgAgCTg	242
FSH-β R (ISH)	CCgCagTCAGTgTATACTgTTg	
LH-β F (ISH)	gTCATgAAgACgTggTT	252
LH-β R (ISH)	TATgCgCTACgTTTgTTACTggA	
GPH-α F (ISH)	gTCTCTTCTCTgTTgTCCTTCTTC	288
GPH-α R (ISH)	gTgTggTTTTCTCAgCgTAATgTC	
FSHβ F (RT)	ggCTgCCACCTgACTgTTAT	103
FSHβ R (RT)	TgAAgCACAgTCCTTCACATATgg	
LHβ F (RT)	CATCCAgTggAAgCAACCATCT	96
LHβ R (RT)	CgTgCACACACTTggTACATgT	
GPα F (RT)	gACATTACgCTgAgAAACCACACA	141
GPα R (RT)	CATAgAAgAgCgTCCACATgTTgA	
β-Actin F (RT)	CTCTgTgTACCACCTgTATCg	83
β-Actin R (RT)	gCAGAgCgTgACCTTCATAgATg	



**Fig. 1.** Expression of *FSH-β* (A), *LH-β* (B) and *GPH-α* (C) mRNAs in the brain and gonads of sexually immature and mature pejerrey of both sexes. Expression in the pituitary gland for comparison. MW: molecular weight marker; (-): negative control; Br: brain; Go: gonads; Pit: pituitary; Imm: immature; Mat: mature.

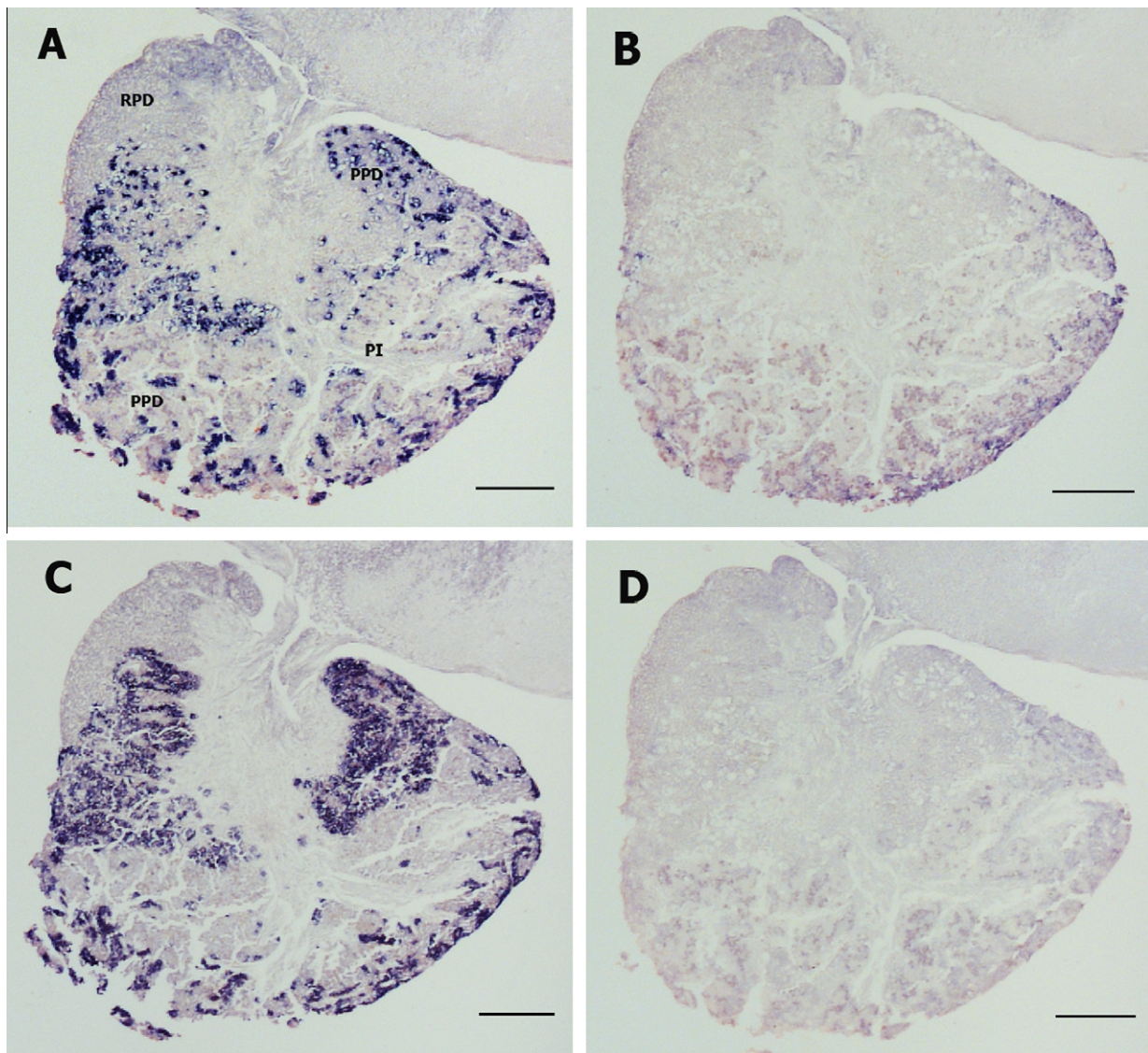
sense RNA probe solution (1 mg/ml) and incubated overnight in a moist chamber at 55 °C. After hybridization, sections were washed in 50% formamide/2X SSC and 0.1X SSC at 60 °C, and incubated 1 hour with Streptavidin-alkaline phosphatase-conjugate at 1/2000 dilution (Roche Applied Science, Penzberg, Germany). Final detection with NBT/BCIP was performed during 1.5 h at 25 °C following the manufacturer's (Roche Applied Science) protocols.

### 2.3. Gene expression by RT-qPCR

The brain, ovary and testis cDNAs used in this analysis were obtained from a previous experiment [37]. Briefly, adult pejerrey with external signs of active reproductive status (milting males and females with dilated abdominal region and genital pore) were randomly distributed in three groups of five males and five females and stocked in 3000-L indoor tanks kept under natural light conditions (14L:10D) and three different temperatures (19, 23, and 27 °C). After 8 days all fish were killed by an overdose of the anesthetic 2-phenoxyethanol and immediately dissected for extraction of the brain, and gonads. Tissues were processed as described in Section 2.1. Gene-specific primers for RT-qPCR analysis of FSH- $\beta$ , LH- $\beta$  and GPH- $\alpha$  were designed to generate amplicons not longer

than 155 bp [19] using the Primer Express software (Applied Biosystems, Foster City, CA). The PCR mix consisted of 1  $\mu$ l of diluted cDNA (approx. 100 ng), 5 pmol of each primer and 12.5  $\mu$ l of SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 25  $\mu$ l. The reactions were performed in a ABI7300 Real time PCR System (Applied Biosystems). Amplifications of the target genes were done simultaneously with  $\beta$ -actin (reference gene, NCBI Accession EF044319) to normalize the abundance of each particular transcript. The standard curve method was used for gene quantification and RT-qPCR efficiency ranged between 80–100%. Analyses of dissociation curves were run after each real time experiment to ensure that only one product was produced. A reverse transcriptase negative control was run from each template and primer pairs in order to control for false positives. The mRNA transcript abundance of GtH subunits were normalized to that of pejerrey  $\beta$ -actin and reported as fold changes relative to the initial condition levels.

The data are presented as the mean  $\pm$  standard error of the mean (SEM). The differences between temperature groups were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test. Differences were considered statistically significant at  $p < 0.05$ .



**Fig. 2.** Localization of FSH- $\beta$  (A), LH- $\beta$  (B), and GPH- $\alpha$  (C) transcripts in the pejerrey pituitary gland. Control sections were hybridized with GPH- $\alpha$  sense. Scale bars: 250  $\mu$ m; RPD: rostral pars distalis; PPD: proximalis pars distalis; PI: pars intermedia.

All fish were treated in an appropriate manner following the Institutional Animal Use and Care Committees.

### 3. Results

#### 3.1. GtH subunit gene expressions

Specific PCR products for *FSH-β*, *LH-β* and *GPH-α* were amplified from cDNAs obtained from brain and gonads of mature and immature pejerrey of both sexes (Fig. 1). The partial sequences obtained for *FSH-β*, *LH-β*, and *GPH-α* consisted of 242, 253, and 288 bp nucleotides, respectively, regardless of the source tissue. Sequence alignment and analyses revealed that the homology among brain, gonads and pituitary *FSH-β*, *LH-β*, and *GPH-α* PCR products were 100% at both the nucleotidic and the aminoacidic levels respectively. PCR products were not observed when the samples were not treated with reverse transcriptase, indicating that there was no genomic DNA contamination in the samples (data not shown).

#### 3.2. Localization of the GtH subunits

The tissue distribution of the expression of different GtH subunits was analyzed by *in situ* hybridization. As expected, the three GtH subunit mRNAs were detected in cells of pituitary *proximalis pars distalis* (PPD) and *pars intermedia* (PI, Fig. 2). *FSH-β* mRNA was found throughout the PPD with stronger signals at the border and the central region of the PPD and PI (Fig. 2A) while *LH-β* cells were identified only at the border of these regions (Fig. 2B). Positive signals for *GPH-α* subunit were also found overlapping the *FSH-β* and *LH-β* subunit patterns (Fig. 2C). No significant signals were observed in the control sections using *GPH-α* sense probe (Fig. 2D).

In the case of ovaries, *FSH-β* transcripts were clearly found in the cytoplasm of oogonia (Fig. 3A), previtelogenic and vitelogenic oocytes (Fig. 3B). Similar findings were observed for *GPH-α* (Fig. 3C and D) and *LH-β* but signals of the latter could not be detected in oogonia (Fig. 3E and F). A *FSH-β* sense probe was used as control (Fig. 3G and H).

In the case of testis, the three GtH subunits were identified in spermatogonia and slight signals were also detected in spermatocytes (Fig. 4A–C). No signals were observed in control testis sections using a *LH-β* sense probe (Fig. 4D).

In spite of numerous attempts, it was not possible to localize GtH subunit mRNAs in the brain (data not shown).

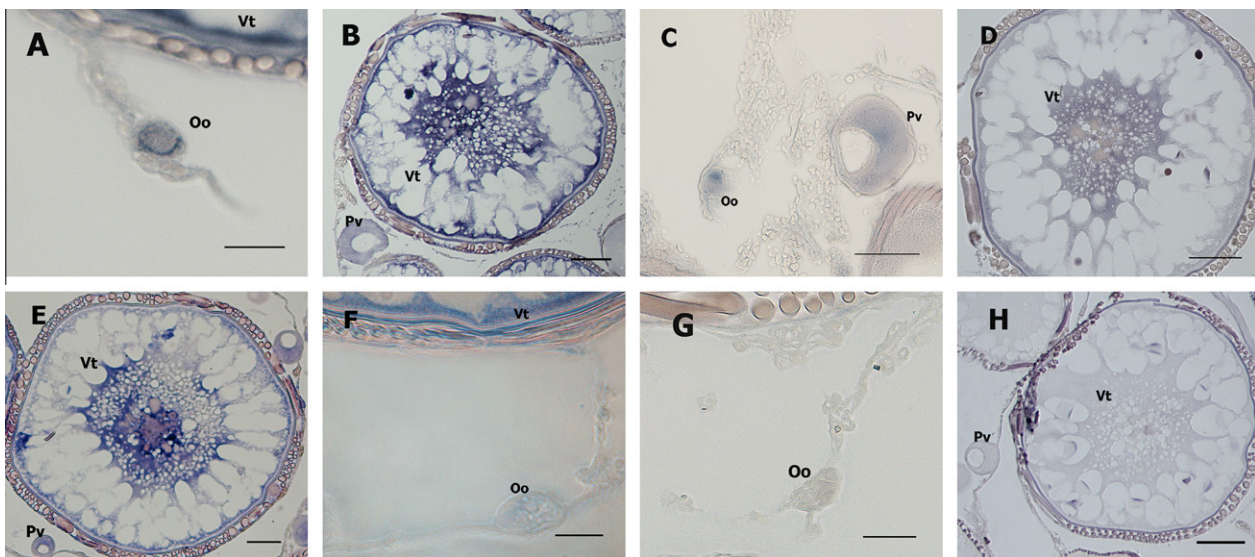
#### 3.3. Effects of high water temperatures on the extrahypophyseal expression of GtH subunit genes

The extrahypophyseal expression of GtH subunits in fish exposed to high water temperature was analyzed by RT-qPCR. In the brain, mRNA levels significantly decreased in relation to the increase in temperature for both sexes (Fig. 5A and B), with the exception of *FSH-β* in male brains (Fig. 5A). In the gonad, all GtH subunits evidenced a significant down-expression at increased temperatures regardless of sex (Fig. 6A and B). This effect was more evident for all GtH subunits in female ovaries (Fig. 6A).

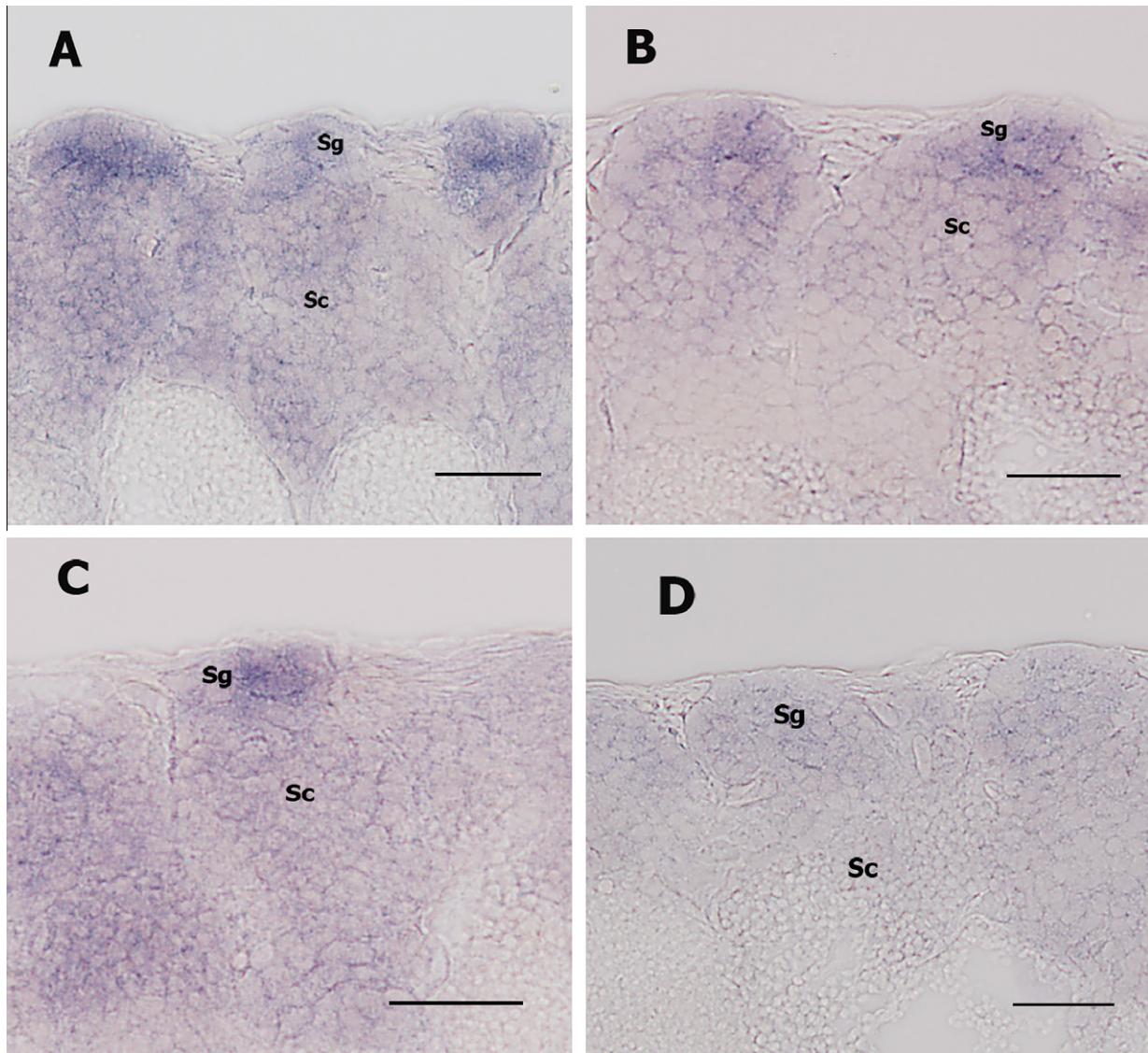
### 4. Discussion

As mentioned above, it has been traditionally accepted that FSH and LH are synthesized only in the pituitary gland and they are indispensable for the regulation of gonadal sex steroid production and gametogenesis in vertebrates. Nevertheless, since the discovery of GtHs in extrapituitary sites such as brain and gonads, new studies are being performed in order to elucidate the physiological implications of extrahypophyseal gonadotropin production [25].

As already observed in other animal models, *FSH-β*, *LH-β* and *GPH-α* subunits cDNAs were detected in the brain and gonads of adult pejerrey of both sexes. Similar findings have been reported in mammals [6,7,11,14,34] and more recently in teleosts [27,30,36,45,46]. The homology among pejerrey brain, gonads and pituitary *FSH-β*, *LH-β* and *GPH-α* PCR products suggests that the extrapituitary pejerrey GtHs are encoded by the same genes as those in the pituitary. This is the same situation as for the brain GtHs in *O. niloticus* [30]. Nevertheless, in *S. aurata*, it has been shown that the ovarian *LH-β* is driven by a different promoter than pituitary *LH-β*, and generates a longer transcript due to a longer 5'-UTR [45]. A similar observation has been made also in the rat ovary by Schirman-Hildesheim et al. [34]. Further studies are needed to see if this is the case in our model species.



**Fig. 3.** Localization of *FSH-β*, *LH-β*, and *GPH-α* transcripts in the pejerrey ovary. Signals for *FSH-β* in Oo (A), Pv and Vt oocyte (B) and for *GPH-α* in Oo, Pv (C) and Vt oocyte (D) were detected. For *LH-β*, positive signals were observed in Pv and Vt oocyte (E) meanwhile no signals were observed in Oo (F). Control sections were hybridized with *FSH-β* sense (G and H). Scale bars: 25 μm (A, F, and G), 50 μm (C), and 100 μm (B, D, E, and H); Oo: oogonia; Pv: previtelogenic oocytes; Vt: vitelogenic oocytes.



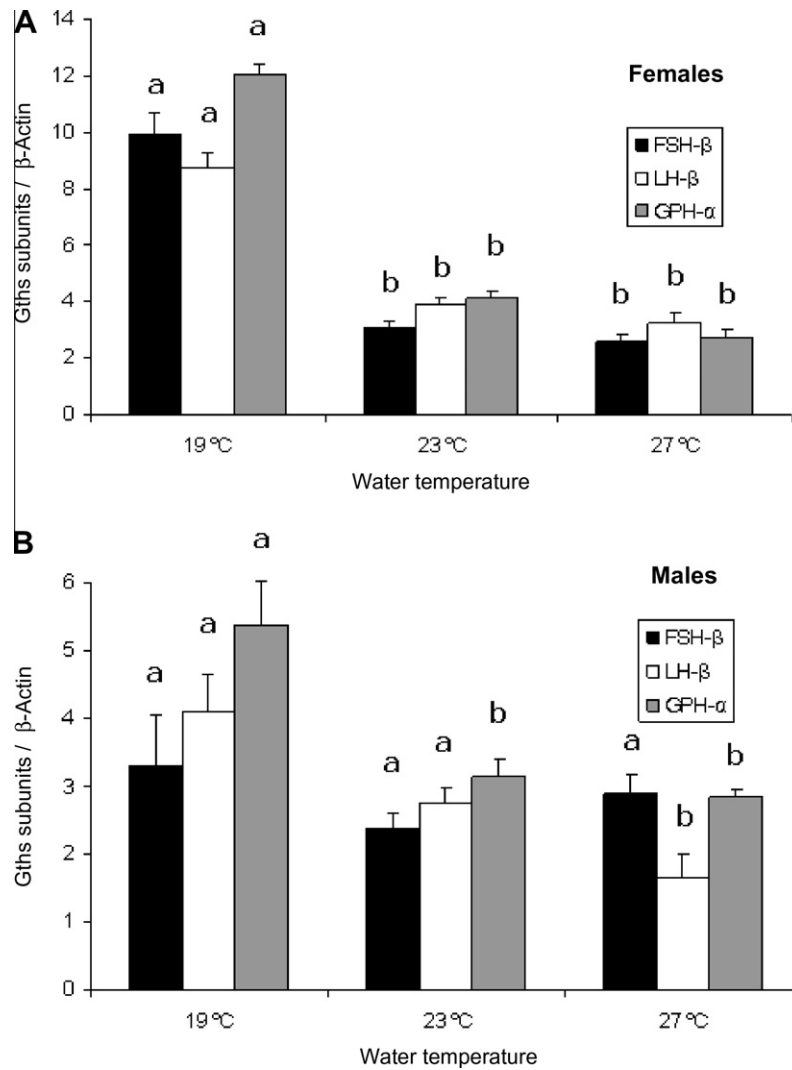
**Fig. 4.** Localization of *FSH-β* (A), *LH-β* (B), and *GPH-α* (C) in the pejerrey testis. Control sections were hybridized with *LH-β* sense (D). Scale bars: 25  $\mu$ m; Sg: spermatogonia; Sc: spermatocytes.

The distribution of GtH subunits in the preoptic and hypothalamic areas of mammalian [11] and fish [27,30] brains has been demonstrated by immunocytochemistry (ICC). In spite of repeated attempts during this study, it was not possible to find them in pejerrey brains by means of ISH. The same limitation was reported by Parhar et al. [30] in *O. niloticus*, who attributed the failure of ISH to detect GtH subunits to their low brain expression levels. Despite of the lack of information about the function of GtHs in fish brain, information available from mammals suggests they may be related with brain development and sexual behavior [2,12].

The specificity of the different ISH probes was first tested in the pituitary gland. The distribution patterns for pituitary *FSH-β* transcripts mainly throughout the PPD and in the PI periphery and for *LH-β* transcripts only at the border of these regions confirmed previous observations in the same species obtained by ICC [17,41] as well as in other teleosts [4,35,36,44]. As expected, pejerrey *GPH-α* subunit cells were found following approximately the same distribution pattern of that of *FSH-β* and *LH-β* but were more concentrated at the dorsal part of PPD where thyroid stimulating hormone producing cells were identified by ICC in the same species [41].

Using the same probes in pejerrey vitellogenic ovaries, the presence of the three GtH subunits was demonstrated in oocytes of

various developmental stages. An exception was *LH-β*, which could not be found in oogonia. Similar findings have been reported in *S. aurata* ovary, where *LH-β* was observed only in vitellogenic oocytes [45]. It is known that ovarian folliculogenesis is a complex process integrating both systemic endocrine hormones and intra ovarian factors. In teleosts, the reproductive rhythms of multiple spawners, like pejerrey, differ from annual spawners by involving asynchronous development of multiple batches of oocytes, which are ovulated and spawned at regular intervals [32,40]. Hence, the regulation of oocytes at different developmental stages might be supported by differential intrafollicular behavior. It has been hypothesized that both pituitary FSH and LH are required to stimulate the heterogeneous batches of follicles in multiple spawning females. Their differential responsiveness to FSH and LH might be regulated by modulating the temporal synthesis of the respective receptors in the developing follicles [23], and this could be a possible role for intraovarian gonadotropins. Moreover, it has been found in *in vitro* experiments with *S. aurata* that ovarian GtH expression can respond to GnRH [45], suggesting the existence of a local GnRH-GtH axis in the gonads. This axis might occur also in pejerrey since the expression of GnRH and one of its receptors was recently demonstrated in the ovary of this species [9].



**Fig. 5.** Pejerrey FSH- $\beta$ , LH- $\beta$ , and GPH- $\alpha$  gene expression in the brain of females (A;  $n = 6$ ) and males (B;  $n = 4$ ) reared at 19, 23 and 27 °C for 8 days. Values are mean  $\pm$  SEM. Different letters represent significant differences between temperatures for the same gene ( $p < 0.05$ ).

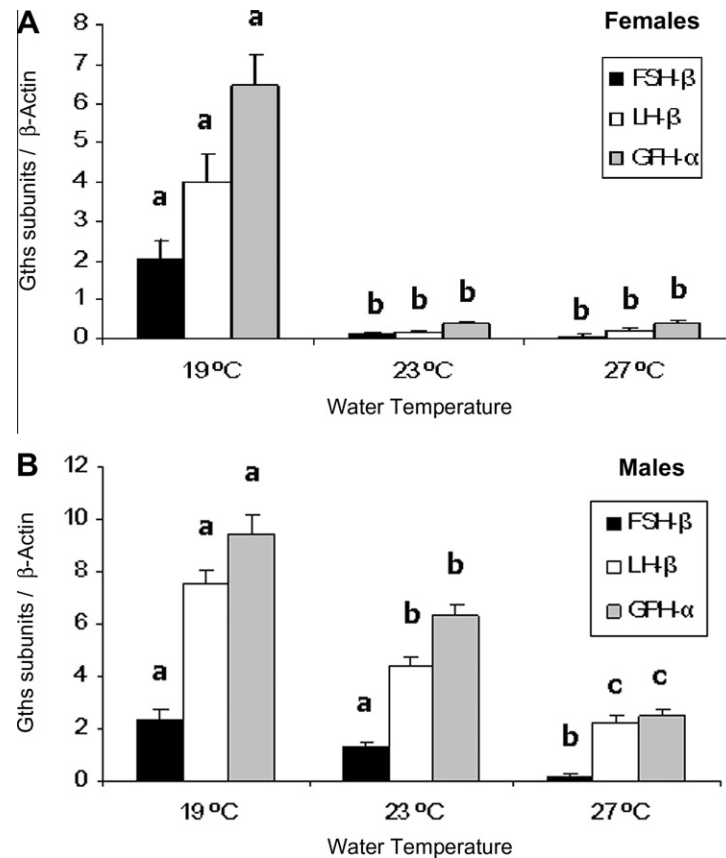
GtH transcripts have been detected by PCR in testicular tissue of some fish species [33,36] but, to the best of our knowledge, the present results are the first demonstration by *ISH* of their anatomical localization in fish testes. In pejerrey testis, the three GtH subunits were clearly expressed in spermatogonia and to a lesser extent in spermatocytes, which is similar to what has been observed by ICC in mouse testis [15].

In a previous study we tried to identify gonadotropin cells in pejerrey brain and gonads by immunocytochemistry using antibodies against chum salmon FSH- $\beta$  and LH- $\beta$ . However, it was not possible to find positive signals in the studied tissues in juveniles or adults fish of both sexes [17]. Moreover, in the present study western blot analysis was performed with the mentioned antibodies, using pituitary, brain, testis and ovary homogenates from mature pejerrey. Immunoreactive proteins were only detected in the pituitary gland and had an estimated molecular weights in the range of those published for GtH- $\beta$  subunits of other teleost species [(data not shown) [43]. The fact, that it was not possible to detect GtH bands in brain and gonads could be due to its low level of protein expression, and the low sensibility of the heterologous antibodies used.

Recent studies have demonstrated that elevated water temperatures can provoke reproductive impairment caused by alterations

on the brain-pituitary-gonad axis [21,22,24,29]. In our experimental model it has been recently demonstrated that high water temperatures impaired spawning and induced gonadal regression [37]. It is important then to note that in that study high water temperature clearly decreased the expression of pituitary GtH subunits. Analysis of the expression of brain and gonadal GtH subunits in samples from the same fish used by Soria et al. [37] showed a clear decline in all of them. This was particularly noticeable at the gonadal level and more evident in females than in males. Taken together, it is evident that high temperatures can induce a decrease of GtH expression at the brain, pituitary and gonadal levels, and this concerted effect might be behind the reproductive impairment observed in the form of gonadal regression and failure to spawn.

The discovery of the extrapituitary GtHs and its possible role in the local regulation of different aspects of vertebrate reproduction open a broad range of academic issues and applications for this knowledge in aquaculture production. However, new approaches would be necessary to find out whether the observed extrahypophysal expression actually leads to local production of hormones and if it does, to fully elucidate the function of locally produced hormones and distinguish their actions from those of their pituitary homologues. According to this, specific antibodies against pejerrey GtH subunits are being developing in our laboratory in or-



**Fig. 6.** Pejerrey FSH- $\beta$ , LH- $\beta$ , and GPH- $\alpha$  gene expression in the gonads of females (A;  $n = 6$ ) and males (B;  $n = 4$ ) reared at 19, 23 and 27 °C for 8 days. Values are mean  $\pm$  SEM. Different letters represent significant differences between temperatures of the same gene ( $p < 0.05$ ).

der to map them in the brain, perform ontogenical studies, and find out the beginning of GtH extrapituitary expression and its relation with sex differentiation and reproduction.

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