



Seasonal changes and endocrine regulation of pejerrey (*Odontesthes bonariensis*) oogenesis in the wild



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ABSTRACT

The goal of this study was to evaluate the essential components controlling the brain–pituitary–gonad axis during pejerrey (*Odontesthes bonariensis*) oogenesis in the wild. Ovarian developmental stages from vitellogenesis up to ovulation were associated with increasing day length and water temperatures below 21 °C (winter and beginning of spring). Gonadal regression was observed when water temperature exceeded this value or when photoperiod decreased. Most females were arrested at primary growth stage during summer (high temperature) or at cortical alveoli stage between autumn and beginning of winter (short photoperiod). Plasma E₂ and transcript levels of *fshr*, *cyp19a1b* and *cyp19a1a* increased during vitellogenesis, while *fshb* remained high at all vitellogenic stages. A significant correlation between plasma sex steroids (T and E₂) and *cyp19a1b* as well as *lhcr* transcript levels was observed during vitellogenesis, suggesting a steroid positive feedback. *Gnrh-I*, Gth subunits and *lhcr* transcript levels increased significantly during late vitellogenesis and final maturation. Present results suggest that pejerrey vitellogenesis is controlled by Fsh/Fshr, stimulating gonadal aromatase and estradiol synthesis. Moreover, the increase of testosterone and estradiol during final vitellogenesis could induce coordinately the functioning of the Gnrh/Lh system (perhaps through brain P450 aromatase stimulation and brain estradiol increase) and the gonadal Lhcr synthesis to promote the final maturation of oocytes. All these stimulation mechanisms of gonadal development would be possible only under permissive environmental conditions.

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1. Introduction

In all vertebrates, female fish reproduction is regulated by different hormones and enzymes that jointly control the functioning of the brain–pituitary–gonad axis in order to stimulate gametogenesis and spawning (Lubzens et al., 2010). In temperate regions, photoperiod and temperature are the most important environmental cues that regulate fish reproductive axis and thereby promote the spawning seasonality (Pankhurst and Porter, 2003).

Gonadotropin releasing hormone (Gnrh) is an important neurohormone and neuromodulator implicated in fish reproductive control, which regulates the synthesis and release of the pituitary gonadotropins (Gths, Zohar et al., 2010). The Gths, follicle stimulating hormone (Fsh) and luteinizing hormone (Lh), and their respective receptors (Fshr and Lhcr) are critical in the endocrine control of gametogenesis, promoting mainly the synthesis of sex steroids through the stimulation of specific enzymes (Nagahama, 1994; Kumar et al., 2000). In females,

estradiol (E₂) is the main steroid that induces oocyte development, and the pathway that mediates the conversion of testosterone (T) to E₂ by means of gonadal P450 aromatase activity, represents a key step in the regulation of oogenesis (Lubzens et al., 2010).

Current knowledge about endocrine control of oogenesis suggests that, at least in synchronous spawner, Fsh through Fshr stimulates the P450 aromatase activity and E₂ production that promotes the vitellogenesis (Montserrat et al., 2004), while Gnrh, Lh and Lhcr stimulate a shift in the steroidogenic pathway toward the synthesis of the maturing inductor steroid (MIS), which promotes the final maturation and spawning (Nagahama and Yamashita, 2008; Lubzens et al., 2010). However, these regulation mechanisms are not clear for multiple spawner fish, in which different results have been reported depending on the species, the methodologies used or because different oocyte stages coexist in the same ovary. For instance, different patterns of Gths at gene expression levels have been found during the reproductive cycle of different fish species, such as goldfish *Carassius auratus* (Sohn et al., 1999), red seabream *Pagrus major* (Gen et al., 2003), and stickleback *Gasterosteus aculeatus* (Hellqvist et al., 2006). At plasma level, two increments of both Fsh and Lh plasma levels were observed in *Oreochromis niloticus*, one during vitellogenesis and the other during spawning (Aizen et al., 2007).

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It is known that sex steroids play a pivotal role in the communication (steroid feedback) between the gonads, the brain and the pituitary in order to regulate gonad development, although this mechanism is not fully understood (Zohar et al., 2010). In relation to steroid feedback, it is important to note the presence of brain aromatase in the brain as well as in the pituitary of fish (Pasmanik and Callard, 1985; Strobl-Mazzulla et al., 2008). Although brain aromatase has mainly been implicated in neurogenesis process, its role has also been suggested on fish reproductive control (Diotel et al., 2010; Strobl-Mazzulla et al., 2010). It must be noted that the activity and/or gene expression of brain aromatase fluctuate during the reproductive cycle in some teleost fish (Kazeto et al., 2003; Ezagouri et al., 2008; Rasheeda et al., 2010; Geraudie et al., 2011; Trubiroha et al., 2012).

Our fish model, the pejerrey (*Odontesthes bonariensis*), is a multiple spawner fish that inhabits lagoons of the Pampas region of Argentina. This species has a marked seasonal reproductive cycle, possessing a major spawning period during spring and a minor one in autumn (Calvo and Morriconi, 1972; Strüssmann, 1989). It has also been reported in captivity that both photoperiod and water temperature conditions determine jointly the pejerrey spawning season, the optimal temperatures being between 18 and 20 °C and a photoperiod of 14 h of light (Strüssmann, 1989; Miranda et al., 2006; Miranda et al., 2009).

As already mentioned, and notably for multiple spawner fish, the mechanisms associated with the coordinated functioning of the entire reproductive axis, and its interaction with the environmental cues, are far to be completely understood. In this context, the aim of the present study was to evaluate the essential components controlling the brain–pituitary–gonad axis during pejerrey oogenesis in order to obtain an overview of the natural functioning of the entire reproductive axis at each gonadal stage. Specifically, the expression of brain aromatase (*cyp19a1b*), brain GnRH variants (*gnrh-I*, *gnrh-II* and *gnrh-III*), pituitary Gth subunits (*gpha*, *fshb* and *lhb*), gonadal Gth receptors (*fshr* and *lhcr*), gonadal aromatase (*cyp19a1a*), and E₂ and T plasma levels were studied in the different gonad stages of pejerrey females from Chascomús Lagoon (Buenos Aires, Argentina). In addition, the seasonal occurrence of each gonadal stage was assessed throughout a whole year (May 2010–April 2011) in relation to the natural variations of photoperiod and water temperature recorded in the lagoon.

2. Materials and methods

2.1. Animal sampling

Adult pejerrey females were sampled monthly in Chascomús Lagoon (35°36'S 58°02'W) from May, 2010 to April, 2011 using a towing net, at 100 m far from the lagoon coast and approximately 1.2 m in depth. Fish caught were immediately taken to the Instituto de Investigaciones Biotecnológicas/Instituto Tecnológico de Chascomús laboratory. Every month 5 females were chosen (standard length, SL: 16.00 ± 0.29 cm; total weight, TW: 44.61 ± 2.74 g), euthanized with 2-phenoxyethanol and dissected. The size of all selected females was above the length of the first maturation reported for this species in Chascomús Lagoon (Calvo and Morriconi, 1972). Previously, blood samples were taken from the caudal vessels using heparinized syringes, and plasma samples were obtained by centrifugation at 4 °C and stored at –80 °C. Brains, pituitaries, gonads and livers were excised immediately after bleeding. Gonads and livers were weighed (GW and LW ± 0.1 g, respectively) for gonadosomatic index (GSI = 100GW/TW) and hepatosomatic index (HSI = 100LW/TW) calculations. A portion of each gonad, brain and pituitary were stored in TRIzol Reagent (Invitrogen, Germany) at 4 °C for approximately 24 h until processed for RNA extraction. The brains were sectioned, and only telencephalon, diencephalons and mesencephalon were used. A section of each gonad was fixed in Bouin's fixative and processed by routine methods for embedding in Paraplast Plus and histological analysis. 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. Histological analysis

Ovarian sections of 6 µm thick were stained with hematoxylin and eosin for observation of histological characteristics and estimation of the reproductive status of each animal. The proportion of oocytes at different developmental stages was analyzed at random in an area of 7.5 mm² in three different histological sections (separated by 1 mm each other) in the middle of the right ovary (see Elisio et al., 2012). The quantifications were performed on micrographs taken with a light microscope Nikon Eclipse E600, equipped with a digital photomicrographic system (Nikon Digital Sight DS-Fi1). Gonad stages were defined according to the proportion of different oocyte developmental stages present in the ovary following the guidelines proposed by Strüssmann (1989). Primary growth (PG): ovaries with all oocytes in primary growth stage (oocyte without evidence of cortical alveoli, lipids or yolk droplets); cortical alveoli (CA): ovaries with a clutch of oocytes in cortical alveoli stage (oocytes filled with cortical alveoli and lipid droplets) and without vitellogenic or final maturation oocytes; initial vitellogenesis (VtgA): ovaries with a leading clutch of oocytes at initial vitellogenesis stage (oocytes with small yolk droplets, cortical alveoli and lipid droplets that appear in the periphery of oocyte) and without final maturation oocytes; advanced vitellogenesis (VtgB): ovaries with a leading clutch of oocytes at advanced vitellogenesis stage (oocytes filled with large yolk droplets) and without final maturation oocytes; final maturation (FM): ovaries with a leading clutch of oocytes at final maturation stage (oocytes with fused yolk droplets and the germinal vesicle migrating or ex-centric); atretic (AT): ovaries with more than 10% of atretic oocytes (oocytes with chorionic filaments and follicle investments irregular in shape and shriveled, and with signs of nuclear and cytoplasmic disintegration); ovulated (OV): ovaries of ovulated or recently spawned females (shrunken follicle with its follicular layer folded). The histological features of the different gonad stages of pejerrey females are shown in Fig. 1.

2.3. Gene expression measurements

The relative transcript levels of brain GnRH variants (*gnrh-I*, *gnrh-II* and *gnrh-III*), brain aromatase (*cyp19a1b*), pituitary Gth subunits (*fshb*, *lhb*, *gpha*), gonadal Gth receptors (*fshr*, *lhcr*) and gonadal aromatase (*cyp19a1a*) genes were determined in each female using real-time RT-PCR with the standard curve method following the procedure published by Applied Biosystems (1997). The gene expression data were normalized using β-actin mRNA levels. For this purpose, total RNA was extracted for each sample using TRIzol Reagent following the manufacturer's instructions. Briefly, RNA samples were treated with DNase I (Invitrogen) and reverse transcribed using SuperScript III RNase H (Invitrogen) and oligo(dT)_{12–18}. Gene-specific primers for real-time PCR analysis were designed to generate amplicons no longer than 155 bp (Table 1) using the Primer Express software (Applied Biosystems, Foster City, CA, USA). The PCR mix consisted of 1 µL of diluted cDNA (ca. 100 ng), 1 pmol of each primer and 7.5 µL of FastStart Universal SYBR Green Master (ROX, Roche Applied Science, Mannheim, Germany) in a final volume of 15 µL. The reactions were performed in an M×3005P® QPCR System (Stratagene, Agilent Technology Company, Santa Clara, CA, USA). Amplification of the target genes was done simultaneously with β-actin in separate tubes and the results were analyzed with the Stratagene M×3005P® QPCR System software version 4.01. The efficiencies of RT-qPCR ranged between 80% and 100%. Dissociation-curves analyses were run after each real-time experiment to ensure that there was only one product. A reverse-transcriptase negative control was run for each template and primer pair.

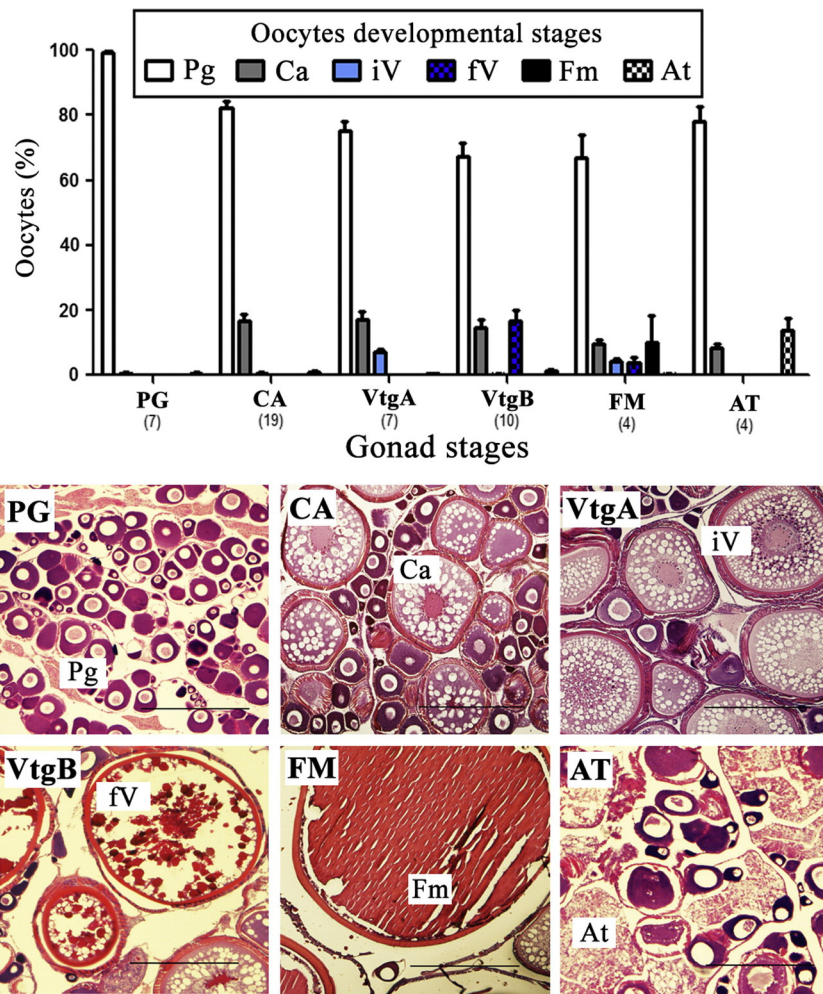


Fig. 1. Representative histological sections of pejerrey ovarian stages. Scale bars: 500 μ m. Proportion of oocytes at different developmental stages for each gonad stage is shown in the plot at the top of the figure. Values are mean \pm SEM. The numbers in parenthesis under each column indicate sample size. Pg: primary growth oocytes; Ca: cortical alveoli oocytes; iV: initial vitellogenic oocytes; fV: final vitellogenic oocytes; Fm: final maturation oocytes; At: atretic oocytes; PG: primary growth stage; CA: cortical alveoli stage; VtgA: initial vitellogenesis stage; VtgB: advanced vitellogenesis stage; FM: final maturation stage; AT: atretic stage.

2.4. Sex steroid measurements

Plasma levels of estradiol (E_2) and testosterone (T) were measured by an enzyme-linked immunosorbent assay (ELISA) using commercial kits, following the manufacturer's protocols (Cayman Chemicals, Ann Arbor, MI, USA). Serum samples were previously extracted with diethyl ether and resuspended in their initial volume of ELISA buffer. A standard curve was run for each ELISA plate. The lower limits of detection were 7.84 pg/mL for T and 15.8 pg/mL for E_2 . The intra-assay coefficients of variance were <10%.

2.5. Steroid feedback assessment

Linear regressions between sex steroids and gene transcript levels of the different components evaluated were analyzed during previtellogenesis (PG and AC) and during vitellogenesis (VtgA and VtgB).

2.6. Photoperiod and water temperature in Chascomús Lagoon

Water temperatures in Chascomús Lagoon were recorded every hour from May 2010 to April 2011, using waterproof electronic data loggers (Thermochron® iButton, Sunnyvale, CA, USA) at 1.5 m of depth. Daily maximum and minimum temperatures were calculated during

this period and plotted together with daily photoperiod (hours of light per day) recorded by the Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico Chascomús (IIB-INTECH) meteorological station in Chascomús County (35°34'S, 58°01'W).

2.7. Statistical analysis

Data are presented as the mean \pm standard error of the mean (SEM). The differences for all the parameters analyzed between different female reproductive stages were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Ordinary least squares algorithm and Fisher's test were used to evaluate the linear regression between steroids and gene expression levels. Results were considered statistically significant at $p < 0.05$. Logarithmic transformations were used when the data lack the assumptions of the statistical test. Statistical analyses were performed using SPSS 17.0 and GraphPad Prism 5.0 Software.

3. Results

3.1. Influence of photoperiod and water temperature on pejerrey ovarian development in the wild

The analysis of the percentage of pejerrey ovarian stages during a complete year in Chascomús Lagoon was shown in Fig. 2. The highest

Table 1
Oligonucleotide primers used for real time RT-PCR.

Primer and amplification size (bp)	Sequence (5'-3')
<i>GnRH-I</i> (103)	
Forward	TgCACCTTgCCTgTTgTgg
Reverse	gCgTCCATTTCCCTgTCggT
<i>GnRH-II</i> (104)	
Forward	CTACTTgAgACCCCAgAggCagAA
Reverse	AgCAgCgAAAgATggAAAgCagTC
<i>GnRH-III</i> (154)	
Forward	gAggCAAgCagCagAgTTATggTg
Reverse	CTCCTCTgTgCCCATCATCT
<i>Cyp19a1b</i> (71)	
Forward	CCATCTTgATTACTCTgTTgTCTCgTT
Reverse	CTTgATgCTgTTgAggTTgCA
<i>Fsh-β</i> (103)	
Forward	ggCTgCCACCTCgACTgTTAT
Reverse	TgAAgCACAgTCCTTCACATATgg
<i>Lh-β</i> (96)	
Forward	CATCCAgTggAAgCAACCATCT
Reverse	CgTgCACACACTTTggTACATgT
<i>Gph-α</i> (141)	
Forward	gACATTACgCTgAgAAACCACACA
Reverse	CATAgAAgAgCgTCCACATgTTgA
<i>Fsh-R</i> (83)	
Forward	TggCAAACTAACgTACCCTTCA
Reverse	gTCgCCACAAAACAAGTTCCA
<i>Lh-R</i> (98)	
Forward	gCCATgCCAACACTgACTTCTATg
Reverse	gggTTTCTgTTggCCACTTgT
<i>cyp19a1a</i> (100)	
Forward	gCgAgCTgTCTgCTgAgAA
Reverse	AggAgCagCagCATgAAgAAgA
<i>β-Actin</i> (83)	
Forward	CTCTggTCgTACCCTgTATCg
Reverse	gCAgAgCgTAgCCTTCATAgATg

proportion of vitellogenic females was found in winter (July–August) when photoperiod began to increase (above 10 h of light) and water temperatures were minimal. Ovulated females were caught only during August–September–October period, coincidently with an increase of photoperiod from 11 to 13 h of light and with an increase of daily maximum water temperatures from 10 to 19 °C. Also, the highest and variable values of GSI and HSI were observed during this last period. Females characterized as atretic were found mainly in November, after the water temperature rose above 21 °C (up to 22 °C). Also, in this month some VtgB and FM females were caught. During summer (December–January–February), most of the females were in PG stage (arresting period) with daily maximum temperatures generally above 25 °C. During this season, but in minor proportion, some vitellogenic and CA females were found. The highest proportion of CA females was found in autumn (March–April–May–June) when photoperiod and water temperature decreased. While some vitellogenic and AT females were found during March–April–May period, in June, when photoperiod was minimum, 100% of the females were in CA stage (Fig. 2).

3.2. The reproductive endocrine axis during pejerrey oogenesis

Gonadosomatic index and HSI increased proportionally with gonad development, reaching their maximum values at FM stage. GSI increased significantly from VtgA to FM, while HSI increased from VtgB to FM. In AT females both indexes showed similar values to those observed in CA females (Fig. 3).

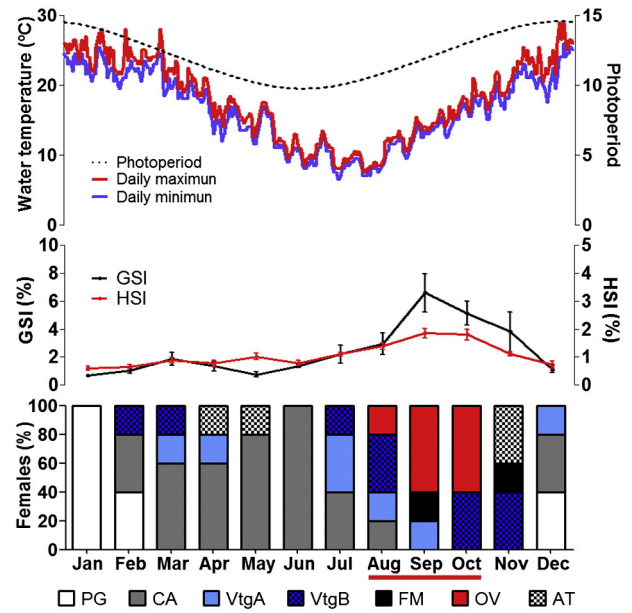


Fig. 2. Monthly percentage of pejerrey females at different gonad stages caught in Chascomús Lagoon in one year. Photoperiod and daily minimum and maximum water temperature are shown at the top of the figure. Monthly mean \pm SEM of pejerrey females gonadosomatic index (GSI) and hepatosomatic index (HSI) is shown in the middle part of the figure. Monthly sample size = 5. The red line under the X axis indicates the presumptive spawning period. PG: primary growth stage; CA: cortical alveoli stage; VtgA: initial vitellogenesis stage; VtgB: advanced vitellogenesis stage; FM: final maturation stage; OV: ovulated females; AT: atretic stage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

At brain level, *gnrh-I* increased with the advancement of gonad development, showing the highest values at FM stage. A similar pattern of variation was observed for *cyp19a1b*, but this gene showed its maximum expression values during VtgB. In AT females, both *gnrh-I* and *cyp19a1b* showed low expression levels similar to those observed in CA females. While *gnrh-III* gene expression increased significantly only in VtgA stage compared with PG females, *gnrh-II* showed no statistically significant difference between females at all gonad stages (Fig. 4).

At pituitary level, *fshb* and *gphα* transcript levels increased significantly from CA stage, remained at elevated levels during vitellogenesis, and increased again during FM stage. On the other hand, *lhb* showed a significant increase only at FM stage. The lower gene expression for the three Gth subunits was found in AT females (Fig. 5).

Both *fshr* and *cyp19a1a* transcript levels in the ovary showed similar patterns of variation along oogenesis, increasing significantly during gonad development up to VtgB stage, and decreasing during FM and

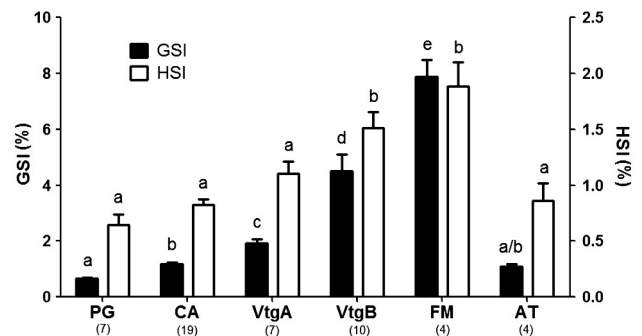


Fig. 3. Gonadosomatic index (GSI) and hepatosomatic index (HSI) of pejerrey females at different gonad stages. Values are mean \pm SEM. Different letters represent significant difference between gonad stages (Tukey's multiple comparison test, $p < 0.05$). The numbers in parenthesis under each column indicate sample size. PG: primary growth stage; CA: cortical alveoli stage; VtgA: initial vitellogenesis stage; VtgB: final vitellogenesis stage; FM: final maturation stage; AT: atretic stage.

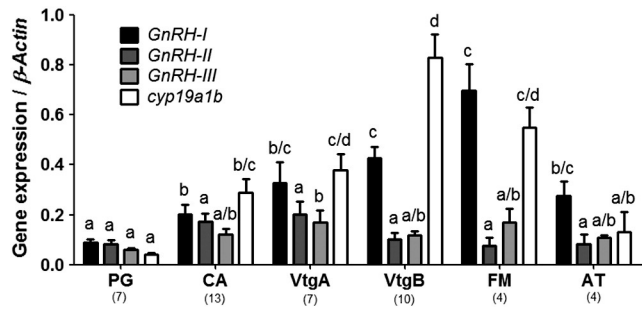


Fig. 4. Gene transcript levels of *gnrh-I*, *gnrh-II*, *gnrh-III*, and *cyp19a1b* in the brain of pejerrey females at different gonad stages. Values are mean \pm SEM. Different letters represent significant difference between gonad stages (Tukey's multiple comparison test, $p < 0.05$). The numbers in parenthesis under each column indicate sample size. PG: primary growth stage; CA: cortical alveoli stage; VtgA: initial vitellogenesis stage; VtgB: advanced vitellogenesis stage; FM: final maturation stage; AT: atretic stage.

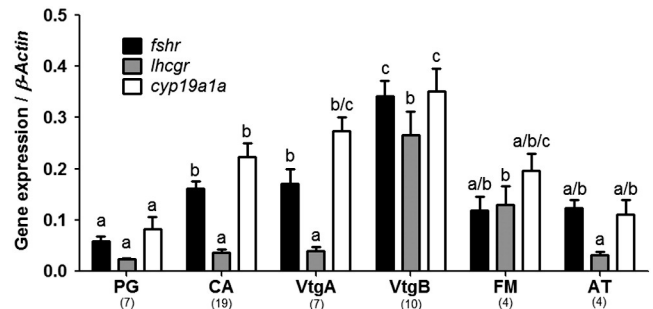


Fig. 6. Gene transcript levels of *fshr*, *lhcr*, and *cyp19a1a* in the ovary of pejerrey females at different gonad stages. Values are mean \pm SEM. Different letters represent significant difference between gonad stages (Tukey's multiple comparison test, $p < 0.05$). The numbers in parenthesis under each column indicate sample size. PG: primary growth stage; CA: cortical alveoli stage; VtgA: initial vitellogenesis stage; VtgB: advanced vitellogenesis stage; FM: final maturation stage; AT: atretic stage.

AT stages with values similar to those observed in CA females. For *lhcr* gene expression, a significant increase was observed only in VtgB and FM stages (Fig. 6).

Estradiol plasma levels increased in proportion with gonad development up to VtgB, and decreased significantly in FM stage. On the other hand, two clear peaks were observed for T plasma levels during gonad development, a smaller one during CA stage, and the most elevated and statistically different from other stages at VtgB stage. In AT females, both T and E_2 decreased significantly up to plasma levels similar to those observed in PG females (Fig. 7).

3.3. Steroid feedback assessment

Linear regression assessment between sex steroid plasma levels and gene expression levels during vitellogenesis period (VtgA and VtgB stages), showed significant regressions between both T and E_2 , and *cyp19a1b* in the brain and *lhcr* in the ovary (Fig. 8). In contrast, no significant regressions were observed for previtellogenesis stages (data not shown).

4. Discussion

Based on the present results and considering ovulated females as evidence of spawning, it is possible to suggest that pejerrey population from Chascomús Lagoon begins to spawn at the end of winter (mid-August) with a peak during September–October, and ends in November. It has been suggested in different lagoons from the Pampas region, that pejerrey presents a major spawning period during spring and a smaller one in autumn (Calvo and Morriconi, 1972; Freyre et al., 2009). However, no evidence of ovulated females was observed during autumn in the

present study. Taking into account the demonstrated influence of photoperiod and water temperature on pejerrey reproductive control (Soria et al., 2008; Miranda et al., 2009; Elisio et al., 2012), the occurrence of its autumn spawning period could depend largely on prevailing environmental conditions. In the present study, short photoperiod during winter (below to 10 h of light) or water temperatures above 21 °C during summer were associated with gonad regression and arresting. Hence, if water temperature after summer decreases down to proper conditions for gametogenesis, and photoperiod is still long, females would begin its gonad development. In fact, some vitellogenic females were observed during autumn in the present study. Nonetheless, those females would spawn only if the gonad maturation occurs before the photoperiod fall. Similarly, the duration of spring spawning season would also depend on the moment of year in which water temperature increases. For instance, in Lake Titicaca (Bolivia–Perú), where water temperature is never excessively elevated during summer, pejerrey appears to have an uninterrupted spawning season from spring to autumn (Strüssmann et al., 2010).

Since the high water temperature in January and the short photoperiod in June were associated with a 100% of PG and CA pejerrey females, it appears that high water temperatures arrest gonad development from CA stage, while short photoperiod conditions seem to exert such arrest as from the vitellogenic stage.

The detailed characterization of reproductive endocrine axis during pejerrey oogenesis shows a differential regulation for each gonadal stage. Pejerrey females at PG stage were associated with low expression levels of all *Gnrh* variants, *cyp19a1b*, and *Gth* subunits. These last findings suggest that PG stage is not regulated by brain–pituitary–gonad axis. In accordance with this suggestion, it has been demonstrated in hypophysectomized fish that oogenesis can progress only up to the

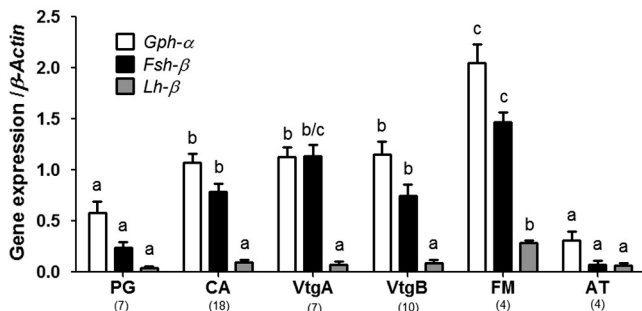


Fig. 5. Gene transcript levels of *fshb*, *lhb* and *gha* in the pituitary of pejerrey females at different gonad stages. Values are mean \pm SEM. Different letters represent significant difference between gonad stages (Tukey's multiple comparison test, $p < 0.05$). The numbers in parenthesis under each column indicate sample size. PG: primary growth stage; CA: cortical alveoli stage; VtgA: initial vitellogenesis stage; VtgB: advanced vitellogenesis stage; FM: final maturation stage; AT: atretic stage.

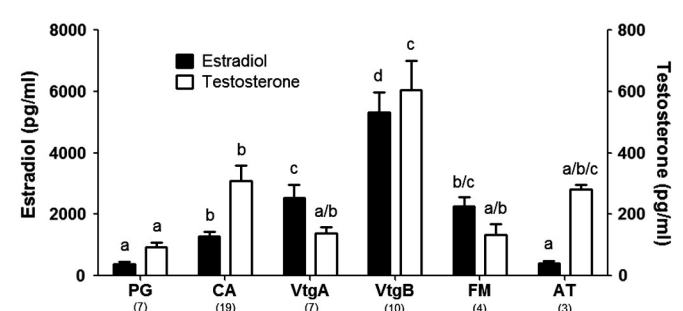


Fig. 7. Estradiol and testosterone plasma levels of pejerrey females at different gonad stages. Values are mean \pm SEM. Different letters represent significant difference between gonad stages (Tukey's multiple comparison test, $p < 0.05$). The numbers in parenthesis under each column indicate sample size. PG: primary growth stage; CA: cortical alveoli stage; VtgA: initial vitellogenesis stage; VtgB: advanced vitellogenesis stage; FM: final maturation stage; AT: atretic stage.

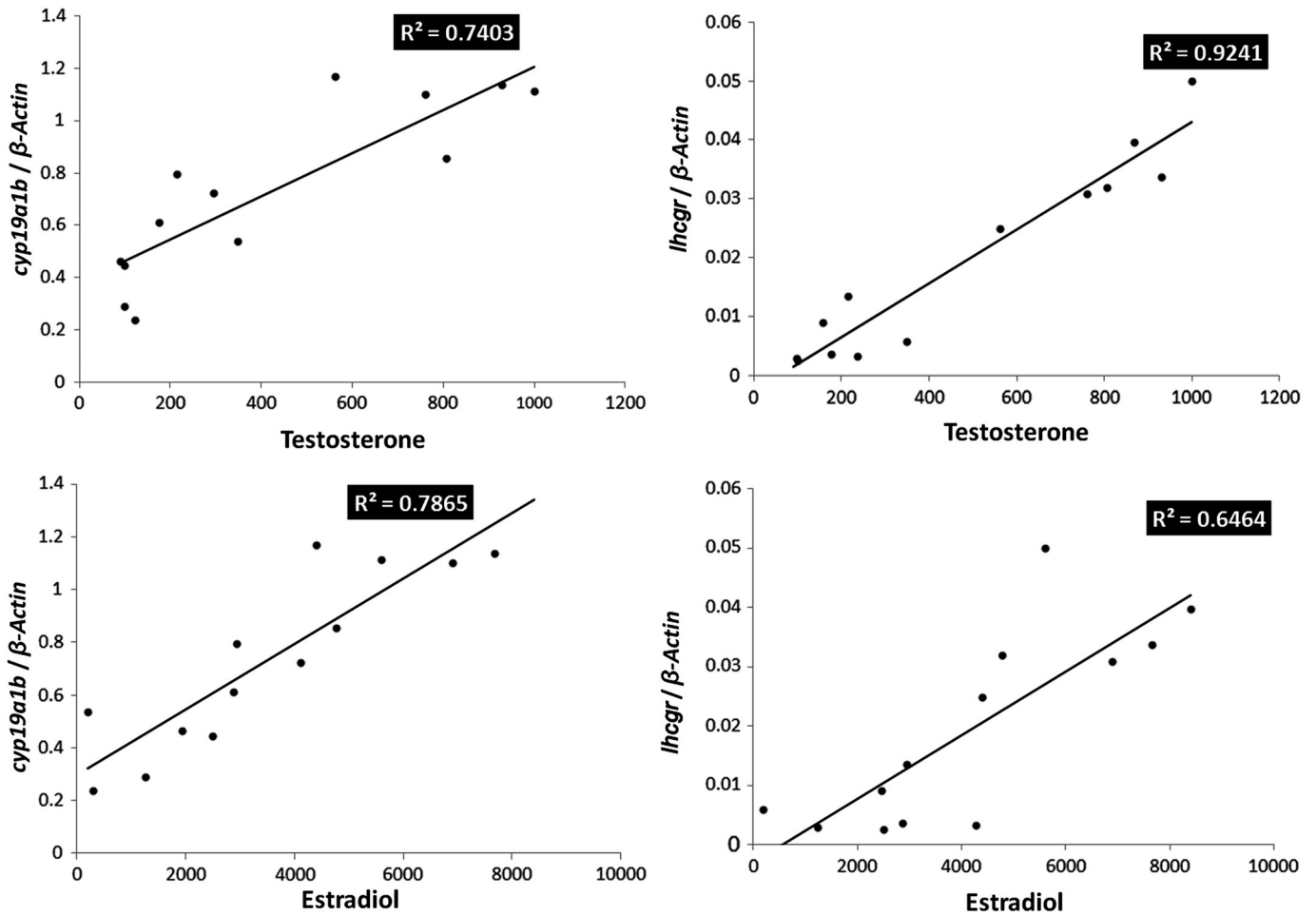


Fig. 8. Linear regressions between sex steroid plasma levels and gene transcript levels of brain *cyp19a1b* and ovarian *lhcr* in vitellogenic pejerrey females. The linear regressions are statistically significant (Fisher's test, $p < 0.05$). The value of coefficient of determination (R^2) for each case is shown on top of each plot.

earliest stage of cortical alveoli (Billard, 1992). Moreover, PG females showed low transcript levels of ovarian Gth receptors and *cyp19a1a*, which could explain the low plasmatic levels of sex steroids found in this gonad stage.

In contrast to PG stage, CA females showed elevated gene expression of *gnrh-I*, *cyp19a1b*, *fshb*, *fshr* and *cyp19a1a*, jointly with an increase of T and E_2 plasma levels. According to these results, it has been demonstrated in coho salmon (*Oncorhynchus kisutch*) that the progression from PG to CA stage was associated with an increase of *fshr* expression, and Fsh and E_2 plasma levels (Campbell et al., 2006; Luckenbach et al., 2008). Nonetheless, the precise regulation of the cortical alveoli oocytes is not completely understood (Luckenbach et al., 2008; Lubzens et al., 2010).

Gnrh-I and *cyp19a1b* expressions increased during the progress of pejerrey vitellogenesis, being this fact more evident for the last one, which reached the maximum value at VtgB stage. Similar results for *gnrh-I* were found in females of *Morone saxatilis* (Holland et al., 2001), while an increase of brain aromatase activity and/or gene expression were also observed during the reproductive cycle of several teleost fish (*Danio rerio*, *Ictalurus punctatus*, Kazeto et al., 2003; *Trichogaster trichopterus*, Ezagouri et al., 2008; *Clarias gariepinus*, Rasheeda et al., 2010; *Rutilus rutilus*, Geraudie et al., 2011). On the other hand, *gnrh-III* only showed a significant increase during the initial vitellogenesis (VtgA). Since pejerrey *Gnrh-III* is present in axon terminals that colocalize with *Gnrh-I* neurons (Guilgur, 2008), this neurohormone could be implicated in the stimulation of *Gnrh-I*/Gths system in order to promote the beginning of vitellogenic phase. Pituitary *fshb* transcript levels increased during pejerrey vitellogenesis, while *lhb* levels remained as low as in the PG stage. Similar pattern of pituitary Gths

expression during fish vitellogenesis was found in several multiple spawners such as *I. punctatus* (Kumar and Trant, 2004), *G. aculeatus* (Hellqvist et al., 2006), *Conger myriaster*, *M. saxatilis*, *O. niloticus* (reviewed in Levavi-Sivan et al., 2010), and *Scomber japonicus* (Nyuji et al., 2011). According to this Gths expression patterns, an increase of Fsh plasma levels was observed during vitellogenesis (*O. niloticus*, Aizen et al., 2007; *Dicentrarchus labrax*, Molés et al., 2012). The elevated transcript levels of pituitary *fshb* during pejerrey vitellogenesis were associated with an increase of ovarian *fshr* and *cyp19a1a* expression. Similar findings were published in other teleost fish for both *fshr* (*D. rerio*, Kwok et al., 2005; *Hippoglossus hippoglossus*, Kobayashi et al., 2008; *D. labrax*, Rocha et al., 2009; *Pseudolabrus sieboldi*, Kitano et al., 2011) and P450 aromatase, gene expression and activity (*P. major*, Gen et al., 2001; *Anguilla japonica*, Ijiri et al., 2003; *C. gariepinus*, Rasheeda et al., 2010). The increase of *fshr* and *cyp19a1a* transcript levels was also coincident with a rise of E_2 plasma levels, which has already been observed in teleost species (Kumar et al., 2000; García-López et al., 2007; Muncaster et al., 2010; Pham et al., 2011), and seems to be a common event during fish vitellogenesis (Nagahama, 1994; Lubzens et al., 2010). In agreement with the known E_2 stimulatory effects on hepatic vitellogenin synthesis and releasing (Nagahama, 1994), pejerrey HSI increased significantly during vitellogenesis. Taken together, these results suggest that pejerrey Fsh and *Fshr* stimulate gonadal P450 aromatase activity and E_2 synthesis to induce the ovarian development, such as it was demonstrated in other teleosts (Montserrat et al., 2004; Molés et al., 2008; Luckenbach et al., 2011).

It must be noted that pejerrey *lhcr* transcript levels remained low during the first stages of gametogenesis, increasing significantly in

VtgB and FM stages. This event has also been observed in other teleost fish such as *D. rerio* (Kwok et al., 2005), *A. japonica* (Jeng et al., 2007), *H. hippoglossus* (Kobayashi et al., 2008), *Salmo salar* (Andersson et al., 2009), and *P. sieboldi* (Kitano et al., 2011). Regulation of Gth receptors synthesis has little been studied in fish, and the physiological significance of this last finding is still unknown (Jeng et al., 2007). The significant linear regression between sex steroids and *lhcr* transcript levels found in the present study might suggest that, E₂ and/or T can stimulate the synthesis of this receptor during vitellogenic phase. Similar conclusions were obtained in *Sparus aurata* by means of in vitro stimulation of ovarian tissue with different sex steroids (Wong and Zohar, 2003), as well as in mammals, where E₂ induces *lhcr* transcript levels in granulosa cells (Shimizu et al., 2005). Considering the stimulatory effect of this Gth receptor on the final maturation (Lubzens et al., 2010), these last results could be interpreted as a mechanism by which the increase of sex steroid during vitellogenesis induces the synthesis of *lhcr*, preparing the ovary for the Lh stimulation during the oocyte final maturation. Furthermore, the results obtained in this study also suggested that E₂ and/or T exert a positive feedback on *cyp19a1b* during pejerrey vitellogenesis, as it was demonstrated in other teleosts (Dietel et al., 2010; Rasheeda et al., 2010). The elevated levels of brain aromatase transcript levels and plasmatic T during pejerrey final vitellogenesis, suggest also a high production of brain E₂. As it was observed in some teleost fish, E₂ can stimulate the GnRH system, although this effect would be caused indirectly, probably by means of Kiss (Zohar et al., 2010). Taking together these findings, it may be possible that a mechanism of feedback by which the rise of plasma sex steroids during final vitellogenesis increases brain E₂ production to stimulate the GnRH/Lh system and thus the final maturation. In line with this suggestion, both *gnrh-1* and *lhb* transcript levels showed their maximum levels at FM stage, similarly to those reported in other teleosts (Holland et al., 2001; Kumar and Trant, 2004; Hellqvist et al., 2006). It is well known that the effect of GnRH in the stimulation of Lh synthesis and releasing (Zohar et al., 2010), as well as the role of this pituitary hormone on the steroidogenic pathway changes toward the synthesis of the MIS to induce the gonad final maturation in fish (Nagahama and Yamashita, 2008). In accordance with this, a decrease of *cyp19a1a* transcript levels, T and E₂ plasma levels was observed in pejerrey females at FM stage.

It must be noted that a significant increase of *fshb* was also observed at FM stage. Similarly, a rise of Fsh plasma levels was observed during the spawning period in *O. niloticus* (Aizen et al., 2007), probably due to recruitment of a new generation of vitellogenic oocytes. This finding could be associated with the stimulation of growth of a new oocyte clutch, such as it occurs in multiple spawner fish during the same reproductive season.

Gonadal regression in pejerrey females (AT stage) was associated with a decrease in all the parameters of the reproductive axis analyzed. However, the endocrine mechanism that triggered atresia may be different depending on the environmental conditions in which those fish lived (short photoperiod or high water temperature). For instance, it was recently demonstrated in pejerrey that high temperatures disrupt directly the *cyp19a1a* transcript levels and the E₂ synthesis in in vitro ovarian cultures, suggesting this event as the mechanism causing ovarian atresia under high water temperature conditions (Miranda et al., 2013). Furthermore, it was also demonstrated that short photoperiod or high water temperature induced atresia inhibiting mainly E₂ plasma levels (Miranda et al., 2009; 2013; Elisio et al., 2012).

5. Conclusions

The present findings jointly with the information existent so far suggest that GnRH, Fsh, Fshr, gonadal P450 aromatase and E₂ are important components to stimulate vitellogenesis in fish. Moreover, the increase of T and E₂ during final vitellogenesis (when oocytes finish their growth) could induce coordinately the functioning of the GnRH/Lh system (perhaps through brain P450 aromatase stimulation and brain E₂

increase) and the gonadal *Lhcgr* synthesis, in order to promote a change in the steroidogenic pathway toward the maturing inductor steroid and thus the gonadal final maturation. It must be highlighted that all these stimulation mechanisms of gonadal development would be possible only if the GnRH/GtHs system is under an adequate photoperiod condition, and if water temperature is permissive for gonadal steroidogenesis.

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