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

Mariano Elisio, Tomás Chalde, Leandro A. Miranda *

Laboratorio de Ictiofisiología y Acuicultura, Instituto de Investigaciones Biotecnológicas-Instituto Tecnológico de Chascomús, (CONICET-UNSAM), Intendente Marino Km. 8.200 (B7130IWA), Chascomús, Buenos Aires, Argentina

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ABSTRACT

The endocrine mechanisms that regulate spermatogenesis and their interaction with environmental cues have been poorly studied compared with oogenesis in fish. The aim of this work was to study the spermatogenesis in pejerrey under the influence of photoperiod and water temperature fluctuation in the wild, evaluating the transcript levels of brain Gnrh variants and *cyp19a1b*, pituitary Gth subunits, gonadal Gth receptors, *11β-hsd*, and 11-KT plasma levels. Males at spermiogenic stage were observed during spring and autumn, under a photoperiod above 11 h of light and a water temperature below 23 °C. Most arrested males were observed in summer when water temperatures increased above 23 °C. Males at spermatogonial stage were mainly observed in autumn, while most males at spermatocytary stage were caught in winter. An increase of *gnrh-I*, *cyp19a1b*, *fshb*, *gpha* and *11β-hsd* transcripts and 11-KT plasma levels was observed during spermatogonial and/or spermatocytary stage (early spermatogenesis). The spermiogenic stage was associated to the maximum *gnrh-I* gene expression level and a significant increase of Gth receptors transcripts, being this fact more evident for *lhcgr*. During this last gonadal stage, *cyp19a1b* transcript level remained high, while *fshb* mRNA and 11-KT plasma levels showed a significant decreased compared to that occurred at the spermatocytary stage. Also, *gphα* and *11β-hsd* gene expression levels fell during spermiation up to similar values to those observed in arrested males. A significant correlation between 11-KT and *gnrh-I*, *cyp19a1b*, *gphα*, *fshb*, *11β-hsd* transcripts, and the number of spermatocytes was observed during spermatogenesis. All these findings suggested that in pejerrey, the spermatocyte proliferation occurs mainly during winter under the stimulation of 11-KT induced by FSH through the stimulation of specific enzymes, including the *11β-hsd* while spermiation occurs after photoperiod increase and with temperatures of the water below 23 °C, through the stimulation of *gnrh-I*, *cyp19a1b* and *lhcgr*.

1. Introduction

Spermatogenesis regulation in teleost fish is a very complex process involving the functioning of the brain–pituitary–gonad axis and influenced by environmental cues, being the photoperiod and temperature particularly important in temperate species (Nagahama, 1994; Pankhurst and Porter, 2003; Migaud et al., 2010). The full understanding of this process remains still unknown, and the studies developed for this purpose are less compared with those performed for oogenesis in fish.

As in other vertebrates the gonadotropin releasing hormone (Gnrh) jointly with the pituitary gonadotropins (Gths), follicle stimulating hormone (Fsh) and luteinizing hormone (Lh) represent a key control system for the regulation of spermatogenesis in fish (Zohar et al., 2010). The functioning of this system is influenced by environmental and hormonal factors, such as sexual steroids, that promote coordinately the seasonal cycle of gonadal maturation (Zohar et al., 2010). One of the major sexual steroids involved in the control of the Gnrh–Gths system in fish is the estradiol (E_2), which besides being synthesized in the gonads, can be synthesized in the brain (particularly in radial glia cells) and pituitary from both sexes by the P450 brain aromatase activity (Diotel et al., 2010; Zohar et al., 2010). This enzyme controls the brain–pituitary E_2 levels, and thus could play an important role in the synthesis and release of pituitary GtHs (Zohar et al., 2010). The Fsh and Lh regulate through their respective gonadal receptors (Fshr and Lhcgr) the gonadal steroidogenic pathways in order to promote the different stages of fish spermatogenesis (Levavi-Sivan et al., 2010; Schulz et al., 2010). The precise regulatory functions of Gths on spermatogenesis in teleost fish are not completely elucidated, and different mechanisms have been suggested depending on the

* Corresponding author. Fax: +54 2241 424048.
E-mail address: lmiranda@intech.gov.ar (L.A. Miranda).

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species or even the study methodology used (Levavi-Sivan et al., 2010; Schulz et al., 2010). Moreover, few studies were done demonstrating that *fshb* and *lhb* subunits increased during spermatogenesis and spermiation, and then decline sharply at post-spermiation stages (Kajimura et al., 2001; Mateos et al., 2003; Weltzein et al., 2003). At plasma level, only two species were evaluated (*Dicentrarchus labrax* and *Fundulus heteroclitus*), being reported an increase of Fsh levels from early stages of spermatogenesis (Molés et al., 2012; Shimizu et al., 2012), and a significant rise of Lh only during the spermiation (Rocha et al., 2009; Shimizu et al., 2012).

A major focus of current discussion seems to be the differential involvement of GtHs and their gonadal receptors on the Leydig cells to stimulate the synthesis of 11-ketotestosterone (11-KT), which is the major androgen that promote fish spermatogenesis (Schulz et al., 2010). Since Leydig cells in fish express both the Lhgcr and Fshr, being the latter sensitive to both pituitary GtHs (García-López et al., 2009; Chauvigné et al., 2012), the mechanism of 11-KT synthesis during spermatogenesis is very complex and depends largely on the prevailing endocrine environment at each gonadal stage and even for each species.

One of the major mechanisms by which Gth/Gth receptors regulate the testicular synthesis of 11-KT in fish is the stimulation of specific steroidogenic enzymes (Kazeto et al., 2008; Marín-Juez et al., 2011). In this sense, the conversion of T into 11-KT represents an important regulatory step in spermatogenesis control, similarly to that occurring with aromatase in the ovary. The last pathway in the conversion of T into 11-KT is catalyzed by the 11β-hydroxysteroid dehydrogenase (11β-hsd). It is worth to note that the gene expression of this enzyme increases significantly during mid and late spermatogenesis in rainbow trout showing their maximum levels coincidently with the highest 11-KT plasma values (Kusakabe et al., 2006).

Our study model, the pejerrey (*Odontesthes bonariensis*), is a multiple spawner fish that inhabits lagoons from the Pampas region of Argentina. This specie has a marked seasonal reproductive cycle (Calvo and Morriconi, 1972; Strüssmann, 1989; Elisio et al., 2014), largely influenced by photoperiod and water temperature conditions (Miranda et al., 2006; Miranda et al., 2007, 2009; Soria et al., 2008; Elisio et al., 2012). It has recently been reported that pejerrey spawning season on its typical habitat (Pampas shallow lakes) begins when the photoperiod increases during late winter, and extends until water temperature rises above 21 °C, event that commonly occurs during late spring, early summer (Elisio et al., 2014).

In this context, the aim of the present study was to evaluate the essential components controlling the brain–pituitary–gonad axis during the pejerrey spermatogenesis under natural environmental conditions. Specifically, the expression of brain Gnrh variants (*gnrh-I*, *gnrh-II*, *gnrh-III*), brain aromatase (*cyp19a1b*), pituitary Gths subunits (*fshb*, *lhb*, *gphα*), gonadal Gth receptors (*fshr*, *lhcgr*), 11β-Hydroxysteroid dehydrogenase (*11β-hsd*), and 11-KT plasma levels were studied during the different spermatogenic stages of pejerrey from *Chascomús* shallow lake (*Buenos Aires* Province, *Argentina*).

In addition, the seasonal occurrence of testicular stages was assessed throughout a whole year (May 2010 – April 2011) in relation to the natural variations of photoperiod and water temperatures recorded in the shallow lake.

2. Materials and methods

2.1. Animal sampling

Adult pejerrey males were sampled monthly in *Chascomús* Lake (35°36′S 58°02′W) from May 2010 to April 2011, using a towing net, and immediately taken to the *Instituto de Investigaciones Biotecnológicas/Instituto Tecnológico de Chascomús* laboratory. Every month 5 males were randomly chosen from the total animals caught (standard length: 15.96 ± 0.24 cm; total weight: 43.09 ± 2.23 g), euthanized with 2-phenoxyethanol and dissected. The size of all selected males was above of the length of first maturation reported for this specie in *Chascomús* Lake (Calvo and Morriconi, 1972). Previously, blood samples were taken from the caudal peduncle 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, brains and pituitaries were stored in TRIzol Reagent (Invitrogen, Germany) at 4 °C for approximately 24 h until processed for RNA extraction. The brains were sectioned in anterior brain (telencephalon and diencephalons), and mid brain (mesencephalon without *Optic Tectum*) and these parts were processed together. 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

Testicular sections of 6 μm thickness were stained with hematoxylin and eosin for the observation of histological characteristics and estimation of the reproductive status of each animal. The number of spermatogonia and spermatocytes was counted in five different spermatogenic tubules in five different histological sections per fish. In the same spermatogenic tubules the presence of spermatids and spermatozoa was evaluated (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). Spermatogenic stages were defined according to the number of the different types of germinal cells in the spermatogenic tubules, following the guidelines proposed by Strüssmann (1989) and Nóbrega et al. (2009). Arrested (**A**): spermatogenic tubules with the scarcest number of all types of germinal cells; spermatogonial stage (**SG**): spermatogenic tubules with the highest number of spermatogonias. The number of spermatocytes is relatively scarce, and the spermatids and spermatozoa begin to appear at the final side of spermatogenic tubules; spermatocytary stage (**SC**): spermatogenic tubules with the highest number of spermatocytes and the highest spermatocyte/spermatogonia rate. Most of spermatogenic tubules possess spermatids, and a minor proportion of them also possess spermatozoa; spermiogenic stage (**SP**): The number of spermatogonia and spermatocytes is relatively scarce, even though higher than in arrested males. Most of spermatogenic tubules possess spermatozoa. The testicular lumen is full of spermatozoa. The histological features of the different spermatogenic stages of pejerrey are shown in Fig. 1.

2.3. Gene expression measurements

The relative levels of brain Gnrh variants (*gnrh-I*, *gnrh-II*, *gnrh-III*), brain aromatase (*cyp19a1b*), pituitary Gth subunits (*fshb*, *lhb*, *gphα*), gonadal Gth receptors (*fshr*, *lhcgr*) and 11β-Hydroxysteroid dehydrogenase (*11β-hsd*) were determined in each male using real-time RT-PCR with the standard curve method following the procedure published by Applied Biosystem (1997). The gene expression data were normalized using β-actin mRNA levels. For

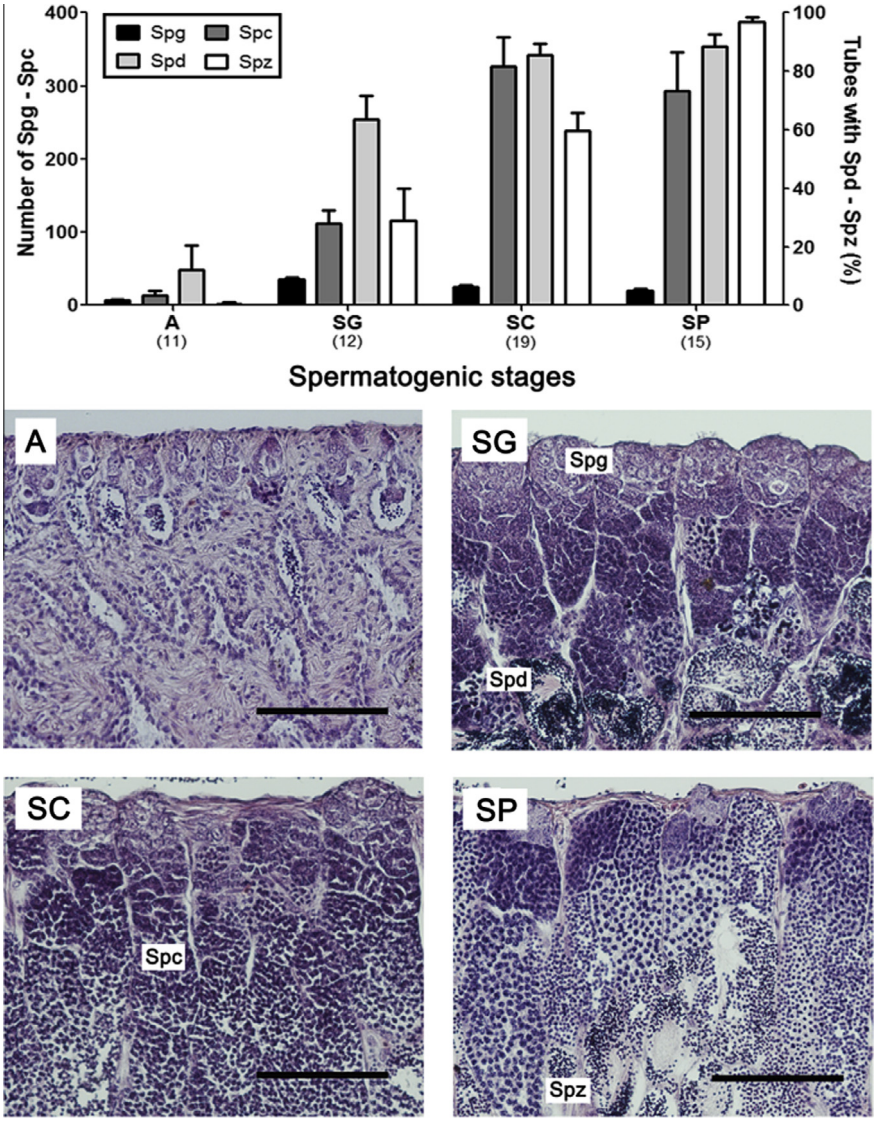

Fig. 1. Representative histological sections of pejerrey *O. bonariensis* spermatogenic stages. The number of spermatogonia (**Spg**) and spermatocytes (**Spc**); and the percentages of tubules with spermatids (**Spd**) and spermatozoa (**Spz**) in testis at different spermatogenesis stages are shown in the plot at the top of the figure. Values are mean ± SEM. The numbers in brackets under each column indicate sample size. **A**: arrested; **SG**: spermatogonial stage; **SC**: spermatocytary stage; **SP**: spermiogenic stage. Scale bars: 100 μm.

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 Mx3005P® QPCR System (Stratagene, Agilent Technology Company, Santa Clara, CA, USA). Amplification of the target genes were done simultaneously with β-actin in separates tubes and the results were analyzed with the Stratagene Mx3005P® QPCR System software version 4.01. The standard curve method was used for gene quantification and RT-qPCR efficiency 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.

2.4. Sex steroid measurements

Plasma levels of 11-ketotestosterone (11-KT) 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 re-suspended in their initial volume of ELISA buffer. A standard curve was run for each ELISA plate. The lower limit of detection was 1.56 pg/mL and the intra-assay coefficients of variance was <10%.

2.5. Photoperiod and water temperature in Chascomús Lake

Water temperatures in *Chascomús* Lake were recorded every hour from May 2010 to April 2011, using waterproof electronic data loggers (Thermochron® iButton, Sunnyvale, CA, USA) at

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

Primer and amplification size (bp)	Sequence (5′–3′)
Gnrh-I (103):	
Forward	TgCACCTTgCCTgTTgTgg
Reverse	gCgTCCATTTTCCCTgTCggT
Gnrh-II (104):	
Forward	CTACTTgAgACCCCAgAggCAgAA
Reverse	AgCAgCgAAAgATggAAAgCAgTC
Gnrh-III (154):	
Forward	gAggCAAgCAgCAgAgTTATggTg
Reverse	CTCCTCCTgTgCCCATCATCCT
Cyp19a1b (71):	
Forward	CCATCTTgATTACTCTgTTgTCTCgTT
Reverse	CTTgATgCTgTTgAggTTgCA
Fshb (103):	
Forward	ggCTgCCACCTCgACTgTTAT
Reverse	TgAAgCACAgTCCTTCACATATgg
Lhb (96):	
Forward	CATCCAgTggAAgCAACCATCT
Reverse	CgTgCACACACTTTggTACATgT
Gphα (141):	
Forward	gACATTACgCTgAgAAACCACACA
Reverse	CATAgAAgAgCgTCCACATgTTgA
Fshr (83):	
Forward	TggCAAAACTAACgTACCCTTCA
Reverse	gTCgCCACAAAACAAgTTCCA
Lhcgr (98):	
Forward	gCCATgCCAACACTgACTTCTATAg
Reverse	gggTTTCTgTTggCCACTTgT
11β-hsd (64):	
Forward	CgAgCTgTCTCTgATgTCCAAC
Reverse	colTgCTCAgAgTgCCgAAgAAgT
β-Actin (83):	
Forward	CTCTggTCgTACCACTggTATCg
Reverse	gCAgAgCgTAgCCTTCATAgATg

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).

2.6. Steroid feedback assessment

Linear regressions between the levels of 11-KT and the expression of the different genes considered, as well as the number of spermatogonia and spermatocytes observed in the spermatogenic tubules were analyzed. Since the reproductive function in **A** males was quiescent, this spermatogenic stage was avoided in the analysis.

2.7. Statistic analysis

Data are presented as the mean ± standard error of the mean (SEM). The differences between different spermatogenic stages were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The differences between temperature and photoperiod conditions were assessed using the Student's *t* test. Ordinary Least Squares and Fisher's Test were used to evaluate the statistical significance of the linear regressions. Results were considered statistically significant at $p < 0.05$. Logarithmic transformations were used if the data lacked 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 spermatogenic cycle in the wild

Analysis of pejerrey spermatogenic cycle during a complete year in *Chascomús* Lake is shown in Fig. 2. The highest number of pejerrey at **SP** stage was found from August to December (end of winter, spring), coinciding with the highest values of IGS. Moreover, some **SP** males were caught during March and April (early autumn), coinciding again with a small increase in the IGS values. During periods when **SP** males were found, the photoperiod was above 11 h of light, while daily maximum water temperatures were generally below 23 °C. Most males were at **A** stage when water temperature increased during summer (December, January and February), with daily maximum values above 23 °C. In addition, some males at **SG** and **SC** stages were found during December and February, respectively. Most males at **SG** stage were found from April to June (late autumn, early winter), after the decrease of photoperiod and water temperature. On the other hand, **SC** males were found almost throughout the year, although the highest number of them was caught between July and August, when photoperiod began to increase (above 10 h of light) and water temperature was the minimal (around 8 °C). Also, some **A** males were caught during May and June, with low values of both photoperiod and water temperature (Fig. 2).

3.2. The reproductive endocrine axis during pejerrey spermatogenesis

In general, GSI and HSI increased proportionally with gonadal maturation. GSI increased significantly from **SG** to **SP** stage, while HSI increased from **SG** to **SC** stage, and then decreased during **SP** stage up to intermediate levels between those observed at **A** and **SG** stages (Fig. 3).

At brain level, *gnrh-I* transcript levels increased in proportion with gonadal maturation, showing the highest values at **SP** stage. *Gnrh-III* gene expression only showed a significant increase at **SG**

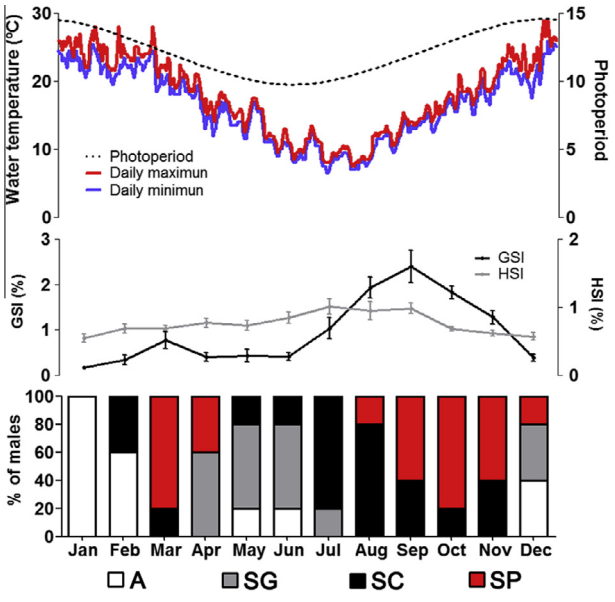

Fig. 2. Monthly percentage of pejerrey males at different gonad stages caught in *Chascomús* Lake during one year. Photoperiod and daily minimum and maximum water temperature are shown at the top of the figure. Monthly mean (±SEM) of pejerrey males gonadosomatic index (GSI) and hepatosomatic index (HSI) are shown in the middle part of the figure. Monthly sample size = 5. **A**: arrested; **SG**: spermatogonial stage; **SC**: spermatocytary stage; **SP**: spermiogenic stage.

stage compared with males at **A** stage. *Gnrh-II* transcript levels showed no significant variations between different spermatogenic stages (Fig. 4). On the other hand, *cyp19a1b* gene expression showed a significant increase during **SC** and **SP** stages.

At pituitary level, *fshb* gene expression increased in proportion with gonadal maturation up to **SC** stage, and then decreased during **SP** stage up to values similar to those obtained in males at **A** and **SG** stages. A similar pattern of variation was observed for *gphα* transcript levels. No significant changes between spermatogenic stages were observed for *lhb* gene expression; however its level tended to increase in males at **SG**, **SC** and **SP** stages compared with **A** males (Fig. 5).

At gonadal level, *fshr* gene expression remained invariable in most spermatogenesis stages, and showed a significant increase only in males at **SP** stage compared with those at **SG** stage. The maximum *lhcgr* gene expression levels were obtained in males at **SP** stage, with an average value statistically different to those observed at **A** and **SG** stages. On the other hand, *11β-hsd* transcript levels increased significantly from **SC** stage and then decreased during **SP** stage up to intermediate expression levels between those observed at **SC** and **SG** stages (Fig. 6).

The 11-KT plasma levels increased in proportion to the advance of spermatogenesis up to **SC** stage, in which values were up to 4 times higher than those observed during **A** and **SG** stages. Males at **SP** stage had 11-KT levels similar to those observed in males at **SG** stage (Fig. 7).

The linear regressions between 11-KT plasma levels and the different components of the reproductive axis analyzed in males at **SG**, **SC** and **SP** stages were statistically significant for *gnrh-I*, *cyp19a1b*, *gphα*, *fshb*, *11β-hsd* gene expression levels and also for the spermatocytes number in the spermatogenic tubules. The highest correlation coefficient values ($r > 0.6$) were obtained for *fshb* transcript levels and for spermatocytes number (Table 2).

4. Discussion

The present study showed that pejerrey had a clear seasonal spermatogenesis cycle during the assessed year in *Chascomús* Lake. Males at SP stage (spawning season) were found coincidently with a photoperiod above 11 h of light and water temperature conditions below 23 °C. Similar findings were observed in experimental conditions, where fish were able to mature only at a long photoperiod condition (Miranda et al., 2009), and under a water temperature below 23 °C (Soria et al., 2008; Elisio et al., 2012). Considering these last evidences, spring was the main season in which photoperiod and water temperature conditions were favorable for pejerrey gonadal maturation and spawning in *Chascomús* Lake. In fact, the highest number of SP males was caught during this period, coinciding further with the presence of ovulated females

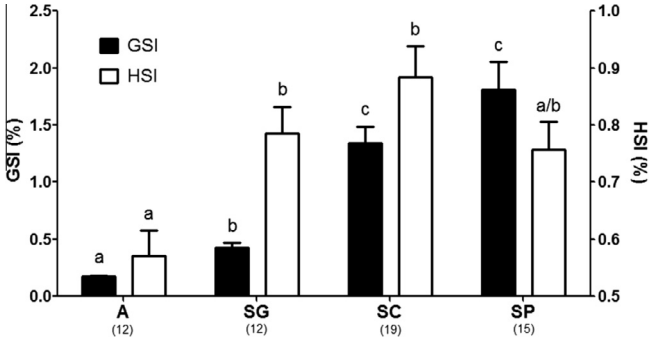

Fig. 3. Gonadosomatic index (GSI) and hepatosomatic index (HSI) of pejerrey males at different gonad stages. Values are mean ± SEM. Different letters represent significant difference between gonad stages (Tukey's multiple comparison test, $p < 0.05$). Logarithmic transformation was used in the GSI data for the statistical test. The numbers in brackets under each column indicate sample size. **A**: arrested; **SG**: spermatogonial stage; **SC**: spermatocytary stage; **SP**: spermiogenic stage.

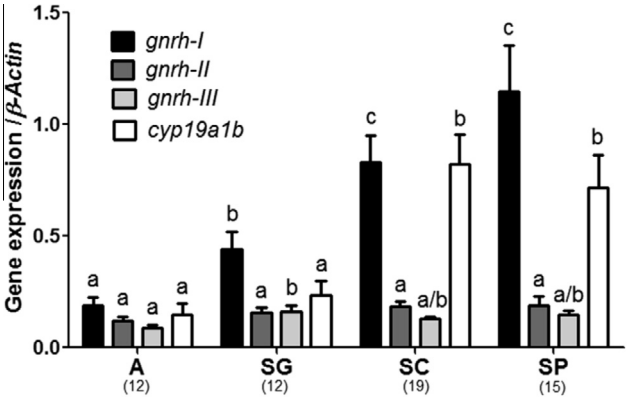

Fig. 4. Gene transcript levels of *gnrh-I*, *gnrh-II*, *gnrh-III*, and *cyp19a1b* in the brain of pejerrey males at different gonad stages. Values are mean ± SEM. Different letters represent significant difference between gonad stages (Tukey's multiple comparison test, $p < 0.05$). Logarithmic transformation was used in the *gnrh-I* and *cyp19a1b* data for the statistical test. The numbers in brackets under each column indicate sample size. **A**: arrested; **SG**: spermatogonial stage; **SC**: spermatocytary stage; **SP**: spermiogenic stage.

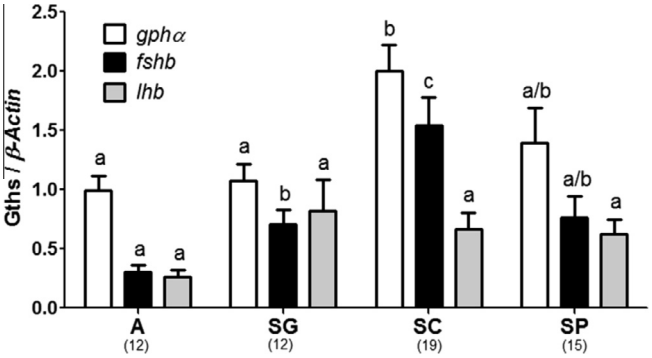

Fig. 5. Gene transcript levels of *gphα*, *fshb*, and *lhb* in the pituitary of pejerrey males at different gonad stages. Values are mean ± SEM. Different letters represent significant difference between gonad stages (Tukey's multiple comparison test, $p < 0.05$). Logarithmic transformation was used in the *gphα* and *fshb* data for the statistical test. The numbers in brackets under each column indicate sample size. **A**: arrested; **SG**: spermatogonial stage; **SC**: spermatocytary stage; **SP**: spermiogenic stage.

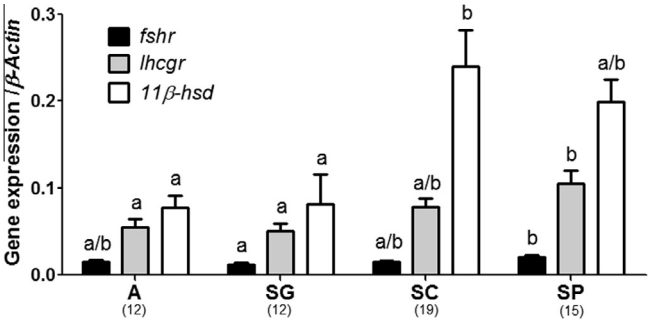

Fig. 6. Gene transcript levels of *fshr*, *lhcgr*, and *11β-hsd* in the testis of pejerrey males at different gonad stages. Values are mean ± SEM. Different letters represent significant difference between gonad stages (Tukey's multiple comparison test, $p < 0.05$). Logarithmic transformation was used in the *11β-hsd* data for the statistical test. The numbers in brackets under each column indicate sample size. **A**: arrested; **SG**: spermatogonial stage; **SC**: spermatocytary stage; **SP**: spermiogenic stage.

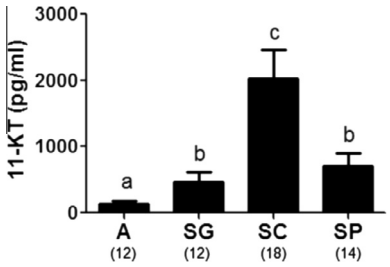

Fig. 7. 11-Ketotestosterone plasma levels of pejerrey males at different gonad stages. Values are mean ± SEM. Different letters represent significant difference between gonad stages (Tukey's multiple comparison test, $p < 0.05$). Logarithmic transformation was used in the data for the statistical test. The numbers in brackets under each column indicate sample size. **A**: arrested; **SG**: spermatogonial stage; **SC**: spermatocytary stage; **SP**: spermiogenic stage.

Table 2
Regression analysis between 11-ketotestosterone plasma levels and gene transcript levels of *gnrh-I*, *gnrh-II*, *gnrh-III*, *cyp19a1b*, *gphα*, *fshb*, *lhb*, *fshr*, *lhcgr*, *11β-hsd*, and number of spermatogonia (**Spg**) and spermatocyte (**Spc**) in pejerrey males at **SG**, **SC** and **SP** stages from *Chascomús* Lake. The values of the correlation coefficient, *r* and the uncorrelation probability, *p* are shown for each regression. $n = 48$.

11-KT with	*r*	*p* (uncorrelation)
gnrh-I	0.37	0.0079*
gnrh-II	0.02	0.8799
gnrh-III	0.00	0.9986
cyp19a1b	0.39	0.0068*
gphα	0.58	<0.0001*
fshb	0.64	<0.0001*
lhb	0.19	0.2241
fshr	−0.23	0.1082
lhcgr	−0.02	0.9045
11β-hsd	0.38	0.0076*
Spg	0.10	0.4916
Spc	0.64	<0.0001*

* $p < 0.05$.

(Elisio et al., 2014). Moreover, some SP males were caught during a short autumn period after water temperature decreased while photoperiod was still long. These last findings explain the marked reproductive seasonality previously characterized in different pejerrey wild populations (Calvo and Morriconi, 1972; Strüssmann, 1989; Freyre et al., 2009; Elisio et al., 2014). It is interesting to note that while short photoperiod appears to inhibit only the last stage of spermatogenesis, the high water temperature appears to cause a complete gonadal regression, suggesting that these environmental cues are differentially decoded by the reproductive axis.

The endocrine analysis performed in this study showed a clear differential pattern of pejerrey spermatogenesis regulation depending on the gonadal stage. Arrested males had the lowest values of all endocrine components assessed, suggesting a lack of spermatogenesis stimulation, similarly to that observed for this stage in captivity condition (Miranda et al., 2007). Compared with A males, the SG stage was associated with a significant increase of *gnrh-I*, *gnrh-III* and *fshb* gene expression, being the only stage in which *gnrh-III* showed a significant change. A similar result for *gnrh-III* transcript levels was also observed in pejerrey females during the beginning of vitellogenesis (Elisio et al., 2014). Since it was demonstrated that Gnrh can stimulate Fsh in fish (Zohar et al., 2010) and Gnrh-III in pejerrey is present in axons terminals near Gnrh-I neurons (Guilgur, 2008), an interaction between these neurohormones to stimulate the pituitary Fsh and promote the beginning of spermatogenesis could exist in pejerrey. It must be noted that the effect of Fsh during early spermatogenesis has been associated with the stimulation of testicular androgens synthesis (Ohta et al., 2007). In accordance with this, the 11-KT plasma levels in the present study increased at SG stage, suggesting that this steroid could be implicated in the stimulation of this testicular stage in pejerrey, as it was already demonstrated in other teleost fish (Miura et al., 1991; Campbell et al., 2003; Amer et al., 2001; Ohta et al., 2007).

During pejerrey SC stage, *gnrh-I* and *fshb* transcript levels increased compared with that found in SG males. Associated with this fact, a significant increase of the *11β-hsd* gene expression and 11-KT plasma levels was also observed. Similar findings were reported for *11β-hsd* in *Oncorhynchus mykiss* (Kusakabe et al., 2003, 2006) and for *fshb* in teleost fish (Hassin et al., 2000; Rahman et al., 2003; Vischer et al., 2003; Hellqvist et al., 2006; Maugars and Schmitz, 2008; Almeida et al., 2011). It is interesting to note that also Fsh, which was demonstrated to stimulate steroidogenic enzymes that promote 11-KT synthesis in fish (Ohta et al., 2007; Kazeto et al., 2008; Chauvigné et al., 2012; Sambroni et al., 2013), increase in plasma during this spermatogenic stage (Prat et al., 1996; Gomez et al., 1999; Campbell et al., 2003; Molés et al., 2012; Shimizu et al., 2012). According to the known action of 11-KT as inductor of spermatogenesis in fish (Schulz et al., 2010), the plasma levels of this androgen correlated significantly and positively with the number of spermatocytes counted in pejerrey testis. Taken together these findings, it is possible to suggest that pejerrey spermatocyte proliferation is promoted by 11-KT induced by Fsh through the stimulation of specific steroidogenic enzymes such as 11β-hsd. Moreover, the significant and positive correlation observed between *fshb* and 11-KT plasma levels during the progress of pejerrey spermatogenesis, suggests a positive androgen feedback on pituitary FSH, such as it was demonstrated in fish males of other species (Borg et al., 1998; Hellqvist et al., 2008). On the other hand, the significant correlation between *11β-hsd* gene expression and 11-KT plasma levels, suggest that this enzyme could play a key regulatory role during the synthesis of this steroid.

Males at SP stage in this study showed the highest *gnrh-I* gene expression levels, similarly to that previously reported by Miranda et al. (2007) in captive-reared pejerrey fish. This result is in agreement with the increase of Gnrh and Lh pituitary content observed during the spawning season in males of other teleost fish such as *Spaurus aurata* (Holland et al., 1998) and *Morone saxatilis* (Holland et al., 2001). It must be noted that Gnrh is widely related to the Lh synthesis and release for inducing the spermiation in fish (Zohar et al., 2010). In accordance with this, Lh plasma levels increase during the later stages of spermatogenesis in all species evaluated so far (Prat et al., 1996; Gomez et al., 1999; Campbell et al., 2003; Rocha et al., 2009; Shimizu et al., 2012). However, only a tendency to increase was observed in *lhb* gene expression during pejerrey spermatogenesis, with no significant changes during SP stage. A similar result was also registered by Rahman et al. (2003) in *Seriola quinqueradiata*. It would be interesting to assess if an increase in pituitary and/or plasma Lh content is observed during spermiation in these species.

The *gnrh-I* mRNA increase in pejerrey at SP stage was also associated with a significant decrease in pituitary *fshb* gene expression, and a significant increase in the transcript levels of both Gth receptors. A similar finding was also observed for *fshb* expression in *M. saxatilis* (Hassin et al., 2000), *S. quinqueradiata* (Rahman et al., 2003), *Gasterosteus aculeatus* (Hellqvist et al., 2006), *Salmo salar* (Maugars and Schmitz, 2008), and *Gadus morhua* (Almeida et al., 2011), being in line with the decrease in Fsh plasma levels registered during the last stage of spermatogenesis in several teleost fish (Prat et al., 1996; Gomez et al., 1999; Molés et al., 2012). On the other hand, the increase in Gth receptors gene expression levels observed in pejerrey at SP stage was also reported during the same spermatogenic stage in all fish species evaluated so far

(Kusakabe et al., 2006; Maugars and Schmitz, 2008; Rocha et al., 2009; Almeida et al., 2011). This gene expression pattern observed during pejerrey spermiation was associated with a significant fall of 11-KT plasma levels. In agreement with this, a change in the steroidogenic pathway from 11-KT to 17,20β-dihydroxypregn-4-en-3-one (the major maturing inductor steroid in fish) synthesis has been observed during the last step of spermatogenesis in fish (Scott et al., 2010).

Interestingly, a significant increase of brain *cyp19a1b* transcript levels was observed in pejerrey males at SC and SP stages. Although this enzyme has been widely implicated in neurogenesis process (Diotel et al., 2010; Strobl-Mazzulla et al., 2010), its relation to the reproductive cycle was suggested in several teleost fish (Kazeto et al., 2003; Ezagouri et al., 2008; Rasheeda et al., 2010; Geraudie et al., 2011). Since it was demonstrated that the aromatization of T modify the pituitary Lh content and secretion in fish males (Antonopoulou et al., 1999, 2009; Khan et al., 1999), it is possible that the high levels of *cyp19a1b* observed during these gonadal stages is related to an endocrine mechanism that increase the endogenous E_2 production to control the Lh-induced spermiation and spawning.

It must be noted that the Gnrh-Lh system, which is related to the stimuli of the last step of spermatogenesis, is largely affected by photoperiod condition (Bromage et al., 2001; Migaud et al., 2010). This fact could explain why pejerrey males at SP stage were only caught during periods in which photoperiod was long. However, independently of photoperiod condition, **SP** males were not found during summer. It must be noted that during this season water temperature showed the maximum values (in general above 23 °C), and most pejerrey males were at A stage. This finding indicates that a thermal disruption of pejerrey spermatogenesis occurs under high water temperatures, decreasing plasma levels of T and 11-KT as it was already demonstrated under experimental conditions (Soria et al., 2008; Elisio et al., 2012). It would be interesting to study if the thermal sensitivity of gonadal steroidogenic enzymes, similarly to that demonstrated in pejerrey females (Miranda et al., 2013), triggers this thermal disruption of spermatogenesis.

In summary, this study showed that the early stages of spermatogenesis in pejerrey fish were associated with 11-KT control, probably induced by Fsh through the stimulation of specific steroidogenic enzymes, including the 11β-hsd. The spermiation and spawning, probably is triggered by the Gnrh-Lh system (stimulated by the increase of photoperiod) through the induction of a change in the steroidogenic pathway from 11-KT to the maturing inductor steroid. All these endocrine mechanisms that promote spermatogenesis would be inhibited if water temperatures surpass critical values, such as commonly occurs during summer in the pejerrey natural habitat.

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