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Research paper

Gonadotropin subunits of the characiform *Astyanax altiparanae:* Molecular characterization, spatiotemporal expression and their possible role on female reproductive dysfunction in captivity

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ABSTRACT

To better understand the endocrine control of reproduction in Characiformes and the reproductive dysfunctions that commonly occur in migratory fish of this order when kept in captivity, we chose *Astyanax altiparanae*, which has asynchronous ovarian development and multiple spawning events, as model species. From *A. altiparanae* pituitary total RNA, we cloned the full-length cDNAs coding for the follicle-stimulating hormone β subunit (*fshb*), the luteinizing hormone β subunit (*lhb*), and the common gonadotropin α subunit (*gpha*). All three sequences showed the highest degree of amino acid identity with other homologous sequences from Siluriformes and Cypriniformes. Real-time, quantitative PCR analysis showed that *gpha*, *fshb* and *lhb* mRNAs were restricted to the pituitary gland. *In situ* hybridization and immunofluorescence, using specific-developed and characterized polyclonal antibodies, revealed that both gonadotropin β subunits mRNAs/proteins are expressed by distinct populations of gonadotropic cells in the *proximal pars distalis*. No marked variations for *lhb* transcripts levels were detected during the reproductive cycle, and 17α,20β-dihydroxy-4-pregnen-3-one plasma levels were also constant, suggesting that the reproductive dysfunction seen in *A. altiparanae* females in captivity are probably due to a lack of increase of Lh synthesis during spawning season. In contrast, *fshb* transcripts changed significantly during the reproductive cycle, although estradiol-17β (E_2) levels remained constant during the experiment, possibly due to a differential regulation of E_2 synthesis. Taken together, these data demonstrate the putative involvement of gonadotropin signaling on the impairment of the reproductive function in a migratory species when kept in captivity. Future experimental studies must be carried to clarify this hypothesis. All these data open the possibility for further basic and applied studies related to reproduction in this fish model.

1. Introduction

Among the multiple hormones involved in vertebrate reproduction, gonadotropins play a crucial role in the regulation of the hypothalamus-pituitary-gonadal (HPG) axis (Levavi-Sivan et al., 2010). Similar to other vertebrates, teleosts have two gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) (Swanson et al., 2003). Along with thyrotropin (Tsh), gonadotropins are members of the vertebrate glycoprotein hormone family, and composed by subunits that interact non-covalently forming heterodimers: a glycoprotein hormone α subunit (Gpha) and a hormone-specific β subunit. Moreover, each subunit is encoded by a distinct gene and shows a series of conserved cysteine residues (Levavi-Sivan et al., 2010).

Despite some findings describing the extrahypophyseal expression of piscine gonadotropins (Elisio et al., 2012; Pandolfi et al., 2009; So et al., 2005), these proteins are synthesized and secreted predominantly by pituitary gonadotropic cells. Gonadotropin synthesis and secretion occur mainly in response to hypothalamic gonadotropin-releasing hormone (GnRH). After their release, gonadotropins are transported through the bloodstream, and -upon reaching the gonads- promote the activation of their receptors, triggering events that result in gonadal steroidogenesis and

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gametogenesis (Levavi-Sivan et al., 2010). In females, estradiol-17β (E_2) and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DHP) are the major sexual steroids controlling ovarian development (Lubzens et al., 2010).

In Salmoniformes, which display synchronous or group-synchronous ovarian development, studies using measurements of plasma levels have demonstrated that Fsh acts in the early phases of the reproductive cycle (i.e. during vitellogenesis), while Lh acts on the final phases (e.g. during final oocyte maturation and ovulation) (Breton et al., 1998; Gomez et al., 1999; Prat et al., 1996). However, in Cypriniformes, Perciformes, and Pleuronectiformes species, which have asynchronous ovarian development, an increase of transcripts levels for both gonadotropins was detected during the late phases (Gen et al., 2000; Hassin et al., 2000; Kajimura et al., 2001; Sohn et al., 1999). Further, Neotropical species present a range of reproductive strategies, as group-synchronous and asynchronous ovarian development, suggesting different roles for gonadotropins among different reproductive strategies.

Most South American freshwater species used in aquaculture are migratory fishes. The latter, when kept in captivity, present several reproductive dysfunctions, a limiting factor for the sustainability of the aquaculture industry. Reproductive dysfunctions are common in aquaculture. Although in males there are less severe consequences on the development of the germ cells, in females it is possible to observe failures that range from vitellogenesis to spawning (Mylonas et al., 2010). Knowledge about reproductive dysfunctions in South American migratory fishes is scarce (Honji et al., 2013, 2015; Moreira et al., 2015) and the physiological basis of these processes requires a complete investigation of the HPG axis in each species throughout the reproductive cycle.

Astyanax altiparanae is a small-sized characiform species that performs short migrations and is widely distributed at the Upper Paraná River Basin (Carolsfeld et al., 2003). It has been used for human consumption as well as food for piscivorous fish in aquaculture. In addition, it presents an omnivorous feeding habit and rapid growth, reaching sexual maturity around four months in captivity, and displays sexual dimorphism (Yasui et al., 2015). Due to these features, *A. altiparanae* has been increasingly used as model for studies on reproduction, physiology, and ecotoxicology (Adolfi et al., 2015; Chehade et al., 2015; Costa et al., 2014; Gomes et al., 2013; Siqueira-Silva et al., 2015; Yasui et al., 2015). This species displays multiple spawning events and an asynchronous ovarian development; however, when kept in captivity, it usually does not spawn without hormonal induction or other exogenous manipulation (Chehade et al., 2015). To date, there is no basic information about the reproductive physiology of *A. altiparanae* either in the wild or in captivity.

Hence, this study presents the molecular characterization of the full-length gonadotropin subunit cDNA sequences of a representative species from the order Characiformes, *A. altiparanae*. We analyzed which tissues expressed the gonadotropin subunit mRNAs and investigated which cell types displayed gonadotropin subunits mRNA and protein expression in the pituitary. Moreover, in an effort to understand the reproductive issues for this species in captivity, we characterized the expression profiles of pituitary genes during a one-year reproductive cycle in captivity, in parallel to their sexual steroid levels and the analysis of their ovarian histology.

2. Material and methods

2.1. Animals and sampling

The specimens used in this study were reared and collected at the Aquaculture Station of Companhia Energética of São Paulo, Paraibuna, SP, Brazil (23°24′51″S, 45°35′59″W). Twenty adult females of *A. altiparanae* – average body weight (BW) of 27 g and average total length (TL) of 11.7 cm – were collected in March 2012 and used for the cloning of gonadotropin subunits.

In August 2012, six months-old females were separated and maintained in 200 m^3 fresh water tanks under natural photoperiod and temperature, and fed with commercial food, in order to perform the analysis of the reproductive cycle. From September 2012 to July 2013, adult females (n = 10) were bimonthly sampled to collection of plasma, pituitary, liver, and ovaries. All specimens were anaesthetized with 0.1% benzocaine and the body weight and total length were recorded followed by blood collection. Next, the animals were decapitated and their pituitaries were immediately collected in tubes containing RNAholder (Bioagency, São Paulo, SP, Brazil) and stored at −80 °C until the RNA extraction. In parallel, the gonadal and hepatic tissues were dissected and weighted for the calculation of the gonadosomatic index (GSI = (gonad weight/BW) * 100) and hepatosomatic index (HSI = (liver weight/BW) * 100).

In addition, in December 2012, another sampling of five adult males (∼12 g of BW and 9 cm of TL) and five females (∼28 g of BW and ∼11 cm of TL) was performed to collect several organs (or organ fragments) in order to analyze the supposed tissue distribution of gonadotropin subunit mRNAs. Also at this sampling, pituitaries from female specimens (n = 10) were collected to investigate the cellular expression of gonadotropin mRNAs and proteins. Finally, for Western blot, pituitaries were collected in September 2016 from adult males (∼15 g of BW and ∼10 cm of TL) and females (∼33 g of BW and ∼12,5 cm of TL) and pooled (three pools for sex, with 10 pituitaries in each pool) in 0,1 mM PMSF protease inhibitor (Sigma, Saint Louis, MO, USA), being stored at −80 °C. All experimental procedures adopted were approved by the Ethics Committee on Animal Experimentation of the Institute of Biomedical Sciences of the University of São Paulo, Brazil, n.130/2011.

2.2. RNA isolation and cDNA synthesis

Total RNA from four pooled pituitaries (five pituitaries, randomly picked, per pool) was extracted by homogenization in TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's recommendations. To eliminate potential genomic DNA contamination, the RNA samples were treated with Turbo DNA-free Kit (Ambion, Carlsbad, CA, USA). For cDNA cloning, the reverse transcription was performed using 2 μg of total RNA from each pool of pituitaries in combination with Superscript III and random primers, according to the manufacturer's instructions (Invitrogen). However, for qPCR reactions, cDNA samples were synthesized using 0.7 μg of DNAse-treated RNA of individual pituitaries. In addition, due to the small size of each pituitary, the RNA samples were extracted using the RNAaqueous Micro Kit (Ambion) while for the other organs, RNA was extracted by TRIzol.

2.3. RT-PCR and cloning of partial sequences

We PCR amplified partial cDNA fragments using degenerate primer pairs (Table 1) designed based on conserved regions of *gpha*, *fshb* and *lhb* sequences. These regions were identified through Clustal X2 alignment software (http://www.clustal.org/clustal2/; Larkin et al., 2007) from some Cypriniformes (*Cyprinus carpio*, *Ctenopharyngodon idella, Danio rerio*) and Siluriformes (*Clarias gariepinus*, *Ictalurus punctatus, Silurus meridionalis*) species found in GenBank. These orders were used as a basis since no sequence information was available for the gonadotropin subunits in Characiformes at the beginning of this study. PCR amplifications were carried out in 25 μl reaction volumes using Platinum Taq

Table 1
Primers used for cDNA cloning and cRNA probe synthesis in this study.

Primer	Sequence 5′ → 3′	Use
gpha F1	TGGVTGTGARGARTGYRAACTCAA	Partial cDNA cloning
gpha R1	GTGCTRCARTGGCAGTCTGTG	Partial cDNA cloning
fshb F1	AGYGASGARTGTGGCAGCT	Partial cDNA cloning
fshb R1	CAYTCACAGCTSAGRGCCAC	Partial cDNA cloning
lhb F3[a]	TCTSTGGAGAAGGADGGCT	Partial cDNA cloning
lhb R3 [a]	CGAWGGTRCAGTCGGANGTG	Partial cDNA cloning
gpha5 outer	CTGTAGTTTTACATTGTTGACAAAAACCCTTTGGATT	RACE
gpha3 outer	CTCAAGGAGAACAGCATCTTCTCCAGACC	RACE
fshb5 outer	CTGCGCCGGCTTCTGCCACACGCAGGAA	RACE
fshb3 outer	GCCCGTCAGCTGGACAGTCTCGTATG	RACE
lhb5 outer	GACATTGCCTCACCAAGGATCCGGTGTA	RACE
lhb3 outer	GGTGCACAGGCTACAGTCACAACTGAGA	RACE
gpha sense [b]	T3Rpps-GTAAAACTACAGAACCACACAGACTGCCACTG	Probe synthesis
gpha anti[c]	T7Rpps-GGGTTGCAACTGAGGCTTTATTTTGAAAATTATTAG AA	Probe synthesis
fshb sense [b]	T3Rpps-GGCAGCTGCATCACCATCGACACCA	Probe synthesis
fshb anti [c]	T7Rpps-GATAGATAGTTTATTAACCATTTCCCAATA A	Probe synthesis
lhb sense [b]	T3Rpps-GGCTGCCCAAAATGCCTAGTGTTTCAGA	Probe synthesis
lhb anti [c]	T7Rpps-GGAAAAAGCAAAATGTTTTTATTGTATTAAGAGTGTACTT	Probe synthesis

[a] Derived from Parhar et al. (2003).
[b] Primers containing the T3 RNA polymerase promoter sequence at its 5′-end (T3Rpps).
[c] Primers containing the T7 RNA polymerase promoter sequence at its 5′-end (T7Rpps).

DNA Polymerase (Invitrogen) and 1 μl of cDNA obtained from the pooled pituitaries. After an initial denaturation step at 95 °C for 3 min, 35 cycles (denaturation at 95 °C for 30 s, annealing at 50 °C to 60 °C for 40 s and elongation at 72 °C for 2 min) of PCR amplifications were performed. PCR products of the expected size were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), cloned into pGEM-T vector (Promega), and transformed in *E. coli* cells. The *gpha*, *fshb* and *lhb* cDNAs from at least three independent clones were isolated with Wizard Plus SV Minipreps DNA Purification (Promega) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) and an ABI PRISM 3100 Genetic Analyzer. The *gpha*, *fshb* and *lhb* mRNA sequences obtained for *A. altiparanae* were confirmed by alignment with previously deposited gonadotropin subunit cDNA sequences at GenBank (http://www.ncbi.nlm.nih.gov/) using the BLASTN interface.

2.4. Rapid amplification of cDNA ends

To obtain the full-length gonadotropin subunit cDNAs, the rapid amplification of cDNA ends (RACE) technique was used (GeneRacer Kit with SuperScript III RT module, Invitrogen), according to the manufacturer's recommendations, with 2 μg of pituitary total RNA as input material. After the reverse transcription step with random primers, touchdown PCRs using primers provided by the kit in combination with gene-specific primers, based on the partial *A. altiparanae gpha*, *fshb* and *lhb* sequences (Table 2), were performed. These PCRs were performed in a 25 μl reaction volume using Platinum Taq DNA Polymerase High Fidelity (Invitrogen) with the following cycling conditions: an initial denaturation step at 94 °C for 2 min, five PCR cycles (denaturation step at 94 °C for 30 s and a combined annealing and elongation step at 72 °C for 5 min), followed by five other PCR cycles (denaturation step at 94 °C for 30 s, and a combined annealing and elongation step at 70 °C for 5 min), and finally 25 PCR cycles (denaturation step at 94 °C for 30 s, and a combined annealing and elongation step at 68 °C for 5 min). Next, PCR products were gel purified, cloned, and sequenced as described above.

2.5. Structural and phylogenetic analysis

The nomenclature guidelines proposed by the ZFIN zebrafish database (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines) were adopted for the cloned gene and deduced protein sequences. To deduce the amino acid sequences of the glycoprotein hormone subunit precursors from the full-length mRNA sequences, the Translate tool (http://web.expasy.org/translate/) was used, while the molecular weights were calculated with the Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The putative signal peptide cleavage sites were predicted using SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011). N- and O-linked glycosylation sites were predicted using NetNgLYC 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) and NetOGlyc (http://www.cbs.dtu.dk/services/NetOGlyc/), respectively.

Table 2
List of primers used to *agph*, *fshb* and *lhb* qPCR analyses in *Astyanax altiparanae.*

GenBank target accession	Primer	Sequence 5′ → 3′	Amplicon	R^2 (%)	Slope[a]
KJ544555	*gpha F_4417*	TGCTGTGTTGCCAAGGAAATC	70 bp	0,997	−2,32
	gpha R_4418	GGCAGTCTGTGTGGTTCTGTAGTTT			
KJ544557	*fshb F3_4525*	GTCCTGATGATTCTGCTGCT	105 bp	0,999	−2,39
	fshb R3_ 4526	GCATTCCTCGCTCTCCAC			
KJ544556	*lhb F_4419*	TGCCCAAAATGCCTAGTGTTTC	73 bp	0,999	−2,21
	lhb R_4420	TCTTGTACACCGGATCCTTGGT			
KM210283	*elfa1F_4501*	CACTGGTACCTCACAGGCTGACT	69 pb	0,998	−2,05
	elfa1_4503	CCAGCCTCAAACTCACCAACA			

[a] Slope obtained with 4-fold dilutions of pituitary cDNA.

Multiple alignment was performed with the full-length amino acid sequences of the three gonadotropins subunit sequences from several species (listed at Supplemental Table 1) available on GenBank using the Clustal X2 software. Phylogenetic trees were constructed by the Neighbor-Joining algorithm using Mega 6 software (Tamura et al., 2013). The robustness of the trees obtained was evaluated via 1000 bootstrapping replicates. The glycoprotein hormone α2 (ACI87878) sequence from *Petromyzon marinus* (sea lamprey) was used as the outgroup for the Gpha phylogenetic tree, whereas the gonadotropin β-like (AW56433) sequence from the same species was used as the outgroup for Fshb and Lhb phylogenetic trees.

2.6. Gene expression analysis by real-time quantitative PCR (qPCR)

The relative expression of *gpha, fshb* and *lhb* transcripts in individual pituitaries from females (n = 5) were analyzed with the Step One Plus Real-Time PCR System (Applied Biosystems) and calculated using the $2^{-(\Delta\Delta Ct)}$ method. The primers used were designed on the sequences previously obtained for *A. altiparanae*. The 20 μl reactions (10 μl SYBR Green Universal Master Mix [Applied Biosystems], 3 μl of each primer at 9 mM and 4 μl of 25-fold cDNA dilution) for each sample were run in duplicate in 96 well plates with the following cycling conditions: an initial denaturation and activation step at 50 °C for 2 min, and 95 °C for 10 min, followed by 40 cycles of denaturation step at 95 °C for 15 s and a combined annealing and elongation step at 60 °C for 1 min. Four-fold cDNA dilutions were used to calculate the amplification efficiency of each set of primers prior to their use, while melting curve analyses were applied to all reactions after 40 cycles. Furthermore, a tissue screen for the three transcripts was performed in brain, pituitary, gill, heart, liver, stomach, and ovary (n = 3) samples from females, and in testis (n = 3) samples from males. The transcript levels were normalized using the reference gene elongation factor 1 alpha (*ef1a*), based on a previously isolated partial 424 bp cDNA sequence from *A. altiparanae* (GenBank Accession No. KM210283) and its gene expression levels were stable in all organs analyzed (data not shown). To improve the presentation of the results, data from gene expression during the reproductive cycle were expressed as a ratio among the average data of July 13, which was set as 1. For tissue screen analyses, data were expressed as a ratio between pituitary and other organs.

2.7. In situ hybridization (ISH)

Individual pituitaries from adult females (n = 5) were collected and immediately fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.4) at 4 °C for 24 h, dehydrated in increasing concentrations of ethanol, diaphanized in xylene, and embedded in Paraplast. Parasagittal and coronal serial sections (5 μm) were obtained, affixed on positively-charged slides (Superfrost Plus Microscope slides, Fisher Scientific, Pittsburgh, PA, USA), and dried at 37 °C for 1 day. For probe synthesis, PCR products for *gpha* (370 bp), *fshb* (411 bp), and *lhb* (400 bp) were amplified using primers designed with T3 or T7 promoters at their 5′-ends (Table 1) and used to generate sense and antisense digoxigenin (DIG)-labeled cRNA probes by *in vitro* transcription with T3/T7 RNA polymerase in combination with a DIG RNA Labeling Kit (Roche, Mannheim, Germany). Next, pituitary sections were used for *in situ* hybridizations following the protocol described by Cerdà et al. (2008), using 1 μg of each probe (sense or antisense). The RNA hybrids were detected using the alkaline phosphatase activity by addition of NBT/BCIP (Roche) substrate. The sections were mounted with Aquamount (Dako, Carpinteria, CA, USA), analyzed by light microcopy, and photographed.

2.8. Production of polyclonal antibody from A. altiparanae gonadotropin beta subunits

Affinity purified-Fshb antisera were produced in rabbit using as epitope a peptide corresponding to amino acid residues 58–69 from *A. altiparanae* Fshb, with an additional cysteine to its C terminus and conjugated to the keyhole limpet hemocyanin (KLH) carrier protein. Protein G-Lhb antisera were raised in rat using as epitope a peptide corresponding to amino acid residues 64–75, using the same conditions as described above. All steps of production were conducted by GenScript, Piscataway, NJ, USA. Antibody specificity was confirmed by Western blot and immunohistochemistry, as described below.

2.9. Western blot

To test the specificity of anti-*A. altiparanae* Fshb and Lhb polyclonal antibodies, an analysis on 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot was performed. Six pools containing-pituitaries were homogenized in 40 μl of cytoplasmic extraction buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, and 0.3% NP-40) supplemented with protease inhibitor cocktail (Sigma, Saint Louis, MO, USA). After denaturation at 95 °C for 5 min, 30 μg from the different lysates (pools) were submitted to SDS-PAGE, and blotted into nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in TBS-T (20 mM Tris, 140 mM NaCl, 0.1% Tween [pH 7.6]) at room temperature for 1 h, then incubated with anti-*A. altiparanae* Fshb or Lhb antibodies (1:1000) overnight, at 4 °C. Control lines were performed by incubating pituitary homogenates with the preadsorbed primary antibodies in their working dilution with an excess of their respective peptides (1:10). After incubation with respective primary antibodies, membranes were incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (1:2500) at room temperature for 1 h with gentle rocking, and visualized on G:BOX Chemi XX6 (Syngene) using an enhanced chemiluminescence (ECL) detection system.

2.10. Immunohistochemistry and immunofluorescence

Whole heads from adult females (n = 5) were collected and fixed in Bouin Solution at room temperature for 24 h, after brain exposure and jaw removal. Next, the samples were washed in running water, decalcified in RDO Gold (Apex, Aurora, IL, USA), dehydrated, and embedded in Paraplast. Serial, parasagittal (5 μm) sections were obtained and affixed in poly-L-lysine coated slides.

For single immunohistochemistry, sections were rehydrated in descending alcohol series until phosphate buffered saline (PBS, pH 7.4). Endogenous peroxidase was inactivated using 3% hydrogen peroxide in PBS. Thereafter, sections were blocked in 5% goat serum and 0.1% bovine serum albumin (BSA) in PBS with 0.1% Tween (PBS-T). Incubation with rabbit anti-*A. altiparanae* Fshb (1:1500) or rat anti-*A. altiparanae* Lhb (1:20), diluted in 1% goat serum 0.1% BSA in 0.1% PBS-T, was performed overnight at 4 °C. Next, sections were washed in PBS and incubated with Super Picture Polymer (Invitrogen) conjugated to HRP at room temperature for 10 min, washed again, and the immunostaining was revealed with 3,3′-diaminobenzidine (DAB, Invitrogen). Counterstain was performed with Mayer's hematoxylin, and the sections were dehydrated and mounted.

For double-label immunofluorescence, after rehydrated, sections were immersed in 1% glycine and blocked as described. As secondary antibodies, anti-rabbit IgG-Alexa 488 (Fshb) and anti-rat IgG-Alexa 594 (Lhb) from Invitrogen were used, both diluted 1:100 in PBS and incubated at room temperature for 1 h. Next, the sections were washed and mounted with Prolong Gold

mounting media (Invitrogen), and analyzed by conventional fluorescence microscope.

The specificity of *A. altiparanae* Fshb and Lhb antibodies were confirmed by the replacement of each primary antibody with different solutions: (a) primary antibody preadsorbed with its respective epitope in excess (1:5), (b) with their respective pre immune serum, and (c) by PBS. All specificity controls were not immunoreactive.

2.11. Sex steroid quantification

Plasma levels of E_2 and 17α,20β-DHP were quantified by an enzyme-linked immunosorbent assay (ELISA) using commercial kits (Cayman, Cayman Chemical Company, Michigan, USA). These kits were previously validated for other two Characiformes, *Hoplias malabaricus* (Gomes et al., 2015) and *Salminus hilarii* (Moreira et al., 2015). For this purpose, blood samples from caudal vasculature were collected using heparinized syringes, centrifuged, and the plasma obtained was aliquoted and stored at −80 °C until analysis. All samples were analyzed in duplicate following the manufacturer's instructions. The absorbance measurements were conducted at 405 nm in a microplate reader (Spectra Max 250 Molecular Devices) and the results were expressed in pg/ml. The detection limit for E_2 assays was 19 pg/ml, and 15 pg/ml for 17α,20β-DHP.

2.12. Ovarian histology

For histological analysis, samples from the middle region of the ovary were dissected and fixed in Karnovsky solution (2% glutaraldehyde and 4% paraformaldehyde in Sorensen buffer [0.1 M, pH 7.2]) at 4 °C for 24 h. Then, samples were washed in water, dehydrated in increasing concentrations of ethanol, and embedded in Historesin (Leica HistoResin, Weltzlar, Germany). Semi-serial sections (3 μm) were obtained and subjected to Hematoxylin-Eosin staining, followed by light microscopy analysis, and photographed. The reproductive phases and germinative cells were classified according to the nomenclature proposed by Brown-Peterson et al. (2011).

2.13. Statistical analysis

The data obtained bimonthly for GSI, HSI, qPCR and ELISA were expressed as mean ± standard error of the mean (SEM) and then subjected to one-way ANOVA, followed by Tukey's test. The tests were performed in the GraphPad Prism 4 software (San Diego, CA, USA), and the significance value was determined at $p < 0.05$.

3. Results

3.1. Cloning and in silico analysis of A. altiparanae gpha, fshb and lhb cDNAs

PCR amplification using degenerate primers yielded partial cDNA sequences for *A. altiparanae gpha*, *fshb* and *lhb* of 240, 212, and 260 base pairs (bp), respectively. Thereafter, their full-length cDNAs were obtained by RACE PCR. The complete *gpha* cDNA (GenBank Accession No. KJ544555; Supplemental Fig. 1A) was 744 nucleotides in length and consisted of a 5′-untranslated region (5′-UTR) of 40 nucleotides, an open reading frame (ORF) of 357 nucleotides (nucleotides 41–397), followed by a 3′-untranslated region (3′-UTR) of 347 nucleotides (nucleotides 398–744). A polyadenylation signal (AATAAA; nucleotides 686–691) was recognized 21 nucleotides upstream of the 37 nucleotides-long poly (A) tail (nucleotides 708–744). The deduced amino acid sequence comprised of 118 amino acids, consisting of a putative 24 amino acids-long signal peptide followed by the 94 amino acids-long mature Gpha peptide, with a calculated molecular weight of 10.78 kDa. The Gpha peptide contains two putative N-linked glycosylation sites at positions 79–81 (Asn-Ile-Thr) and 104–106 (Asn-His-Thr), taking the signal peptide into account in the amino acid numbering. The alignment of the *A. altiparanae* Gpha sequence with other teleost species and human shows that it contains 10 highly conserved cysteines residues (Fig. 1A).

The *fshb* cDNA (GenBank Accession No. KJ544557) was 609 nucleotides long and contains an ORF of 399 nucleotides, with the first nucleotide of the ATG start codon located at nucleotide 33 and the last nucleotide of its stop codon at nucleotide 431 (Supplementary Fig. 1B). Consequently, the ORF was preceded by a 5′-UTR of 32 nucleotides (positions 1–32), and followed by a 3′-UTR of 133 nucleotides (nucleotides 432–564). The *fshb* 3′-UTR contains a polyadenylation signal (AATAAA; nucleotides 545–550) located 20 nucleotides upstream of the 45-nucleotides-long poly (A) tail (nucleotides 565–609). The deduced amino acid sequence yielded a peptide of 132 amino acids, starting with a putative signal peptide of 17 amino acids and followed by the mature Fshb peptide of 115 amino acids, with a calculated molecular weight of 12.44 kDa. The *A. altiparanae* Fshb sequence contains only a single putative N-linked glycosylation site at positions 29–31 (Asn-Ile-Ser) and a total of 13 cysteine residues (Supplemental Fig. 1B). An additional cysteine residue (Cys^{21}) is located in the N-terminal region (marked as 3′ in Fig. 1B), before the first conserved cysteine residue seen in the tetrapod Fshb amino acid sequences.

The complete *lhb* cDNA (GenBank Accession No. KJ544556) consists of 638 nucleotides, with an ORF of 429 nucleotides that starts with nucleotide 53 (*i.e.* the first nucleotide of the ATG start codon) and runs to nucleotide 481 (*i.e.* the last nucleotide of the stop codon; Supplementary Fig. 1C). The ORF of this cDNA is preceded by a 52 nucleotides-long 5′-UTR (nucleotides 1–52), while the ORF extends into a 103 nucleotides-long 3′-UTR (nucleotides 482–584). A typical polyadenylation signal (AATAAA; nucleotides 562–567) is located 23 nucleotides upstream of the poly (A) tail (nucleotides 585–638). The deduced Lhb peptide consists of 142 amino acids, with a putative signal peptide of 25 amino acids and a mature Lhb peptide of 117 amino acids. The calculated molecular weight of the mature Lhb peptide was 13.26 kDa. The mature Lhb peptide also contains a single putative N-linked glycosylation site at positions 35–37 (Asn-Glu-Thr), as well as a single putative O-linked glycosylation site at position 126 (Ser). Moreover, the *A. altiparanae* Lhb peptide contains 12 highly conserved cysteine residues (Fig. 1C).

In silico analysis showed that the *A. altiparanae* Fshb and Lhb subunits shared 31% amino acids identity, while the *A. altiparanae* Gpha subunit shared 8% and 10% of identity with the *A. altiparanae* Fshb and Lhb subunits, respectively. The *A. altiparanae* Gpha sequence showed the highest amino acid identity with other Gpha sequences: from *A. mexicanus* (92%), *Clarias gariepinus* (82%), and other Siluriformes species (80–82%), followed by Cypriniformes (55–75%), Osteoglossiformes (67%), Anguiliformes (63%), and Salmoniformes (58–67%). The *A. altiparanae* Fshb sequence showed the highest amino acid identity with other Fshb sequences: from *A. mexicanus* (92%), followed by *Clarias gariepinus* and *Ictalurus punctatus* (68%), from other Siluriformes species (65–68%), and then from Cypriniformes (52–63%), Anguiliformes (51%), and Salmoniformes (40%). The Lhb sequence of *A. altiparanae* displayed the highest amino acid identity with two Lhb variant sequences from *A. mexicanus* (96%), other Lhb sequences from *Silurus meridionalis* (77%), and from other Siluriformes species (74–77%), followed by 66–76%, 64–65%, and 63% of amino acid identity with Lhb sequences from Cypriniformes, Anguiliformes, and Salmoniformes, respectively.

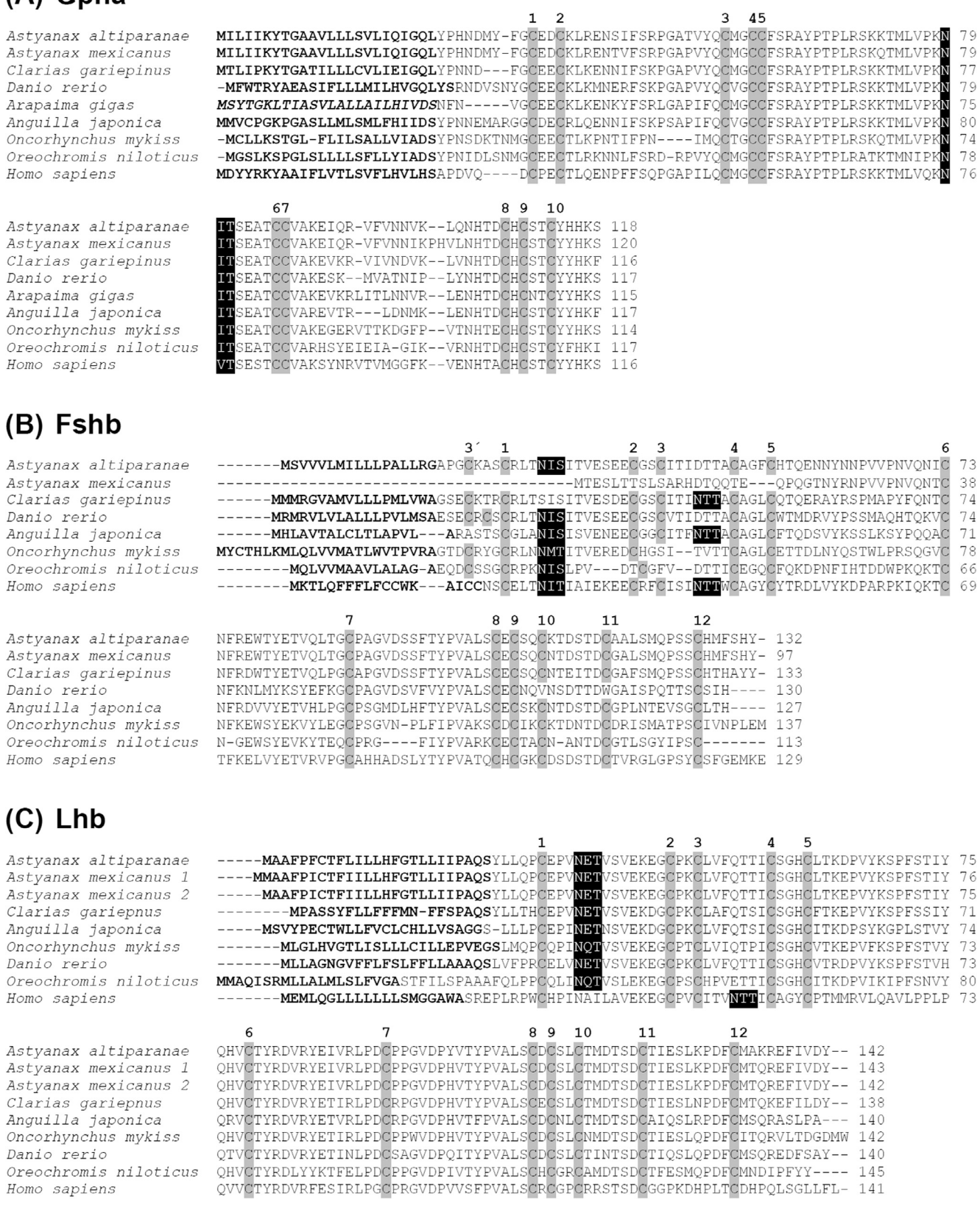

Fig. 1. Alignment of deduced amino acid sequences of Gpha (A), Fshb (B), and Lhb (C) gonadotropin subunits of *A. altiparanae* with human and other representative teleost species from the orders Characiformes, Siluriformes, Cypriniformes, Osteoglossiformes, Anguiliformes, Salmoniformes, and Perciformes. Gaps (marked by a dash) were introduced to maximize the alignments of the cysteine residues. The putative signal peptide is indicated in bold. The conserved cysteine residues are marked by gray boxes and numbered as in tetrapods. The putative N-linked glycosylation sites are marked by black boxes.

Phylogenetic analysis revealed that all three *A. altiparanae* gonadotropin subunits form a monophyletic group with gonadotropin subunit peptides from Siluriformes species (Fig. 2). In general, the topology of the trees for all gonadotropin subunits is similar to each other, which suggests that all gonadotropin subunits in *A. altiparanae* (Characiformes) are closest to Siluriformes, Cypriniformes, Osteoglossiformes, Anguiliformes, and Salmoniformes species, in this order (Fig. 2).

3.2. Tissue distribution and cellular expression in the pituitary gland

The gonadotropin subunit (*gpha*, *lhb* and *fshb)* mRNA expression was investigated in several tissues and organs of *A. altiparanae*. The expression of these transcripts was restricted to the pituitary gland (data not shown). Using *in situ* hybridization, we also investigated which cell types expressed the gonadotropin subunit mRNAs in pituitary. A strong signal for *gpha* mRNA was detected in the *proximal pars distalis* (PPD, Fig. 3A). This signal was distributed extensively through the PPD indicating that the mRNA for this subunit is expressed by Fsh, Lh, and Tsh cells. As expected, the signal for *fshb* was also detected in the PPD, distributed in small clusters (Fig. 3B). In contrast, the signal for *lhb* had a broader distribution and occupied the most part of the PPD (Fig. 3C), indicating a higher number of *lhb*-expressing cells. No signal was detected when sense probes were used (Fig. 3D), indicating specific hybridization for each of the anti-sense probes.

The antibodies developed against synthetic peptides from *A. altiparanae* gonadotropin beta subunits provided specific immunostaining of each gonadotropic cell population (Fig. 3E, F, I, J). All controls performed during antibodies characterization (see Material & Methods) did not result in immunostaining (Fig. 3G, H, K, L). Confirming our results from the *in situ* hybridization, both gonadotropic cells were detected at PPD (Fig. 3E, I). Fshb-expressing cells were round to polygonal shaped and distributed in clusters, as well as bordering the neurohypophyseal projections (Fig. 3E, F). Lhb-expressing cells were abundant, round-shaped cells randomly distributed at PPD (Fig. I, J). The employment of double-label immunofluorescence revealed that Fshb and Lhb proteins are expressed by distinct gonadotropic cell populations (Fig. 4A–F).

The presence of Fshb and Lhb polypeptides in *A. Altiparanae* pituitaries from males and females was confirmed by Western blot analysis. The anti-Fshb antibody identified two bands, a stronger band of ~15 kDa, and a second weaker smeared band of ~21 kDa (Fig. 3M). With the anti-Lhb antibody, a single band of ~20 kDa was detected (Fig. 3M). All reactive bands related to Fshb and Lhb subunits were no longer detected when using the preadsorbed antibodies (Fig. 3M).

3.3. Temporal pituitary expression of gonadotropin mRNAs and plasma level of sexual steroids

During the period investigated, no statistically significant changes in *gpha* transcripts levels were observed ($p > 0.05$) (Fig. 5A). In contrast, the highest levels of *fshb* (Fig. 5B) were observed in samples from November 2012 ($p < 0.05$), after which levels decreased dramatically in Jan 2013, and remained constant until March 2013. Then, *fshb* transcripts levels started to gradually increase again until the last point of sampling, July 2013 ($p < 0.05$). The levels for *lhb* (Fig. 5C) did not present marked changes during the annual cycle. They were constant during most of the year, with an exception of a decrease observed in November 2012 and January 2013 ($p < 0.05$). No increase or peak in its levels was seen during the reproductive season (November 2012 to March 2013). Concerning the sexual steroids determination, E_2 and 17α,20β-DHP plasma levels were detected in all animals during the entire period of the study (Fig. 6). However, no variations in E_2 and 17α,20β-DHP levels were observed ($p > 0.05$).

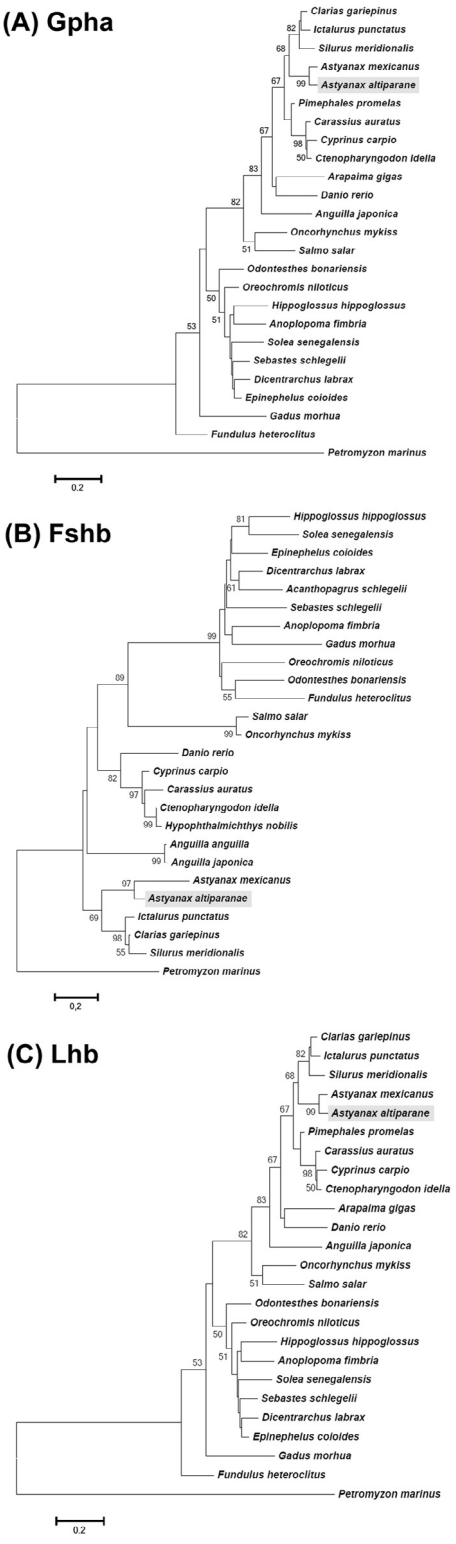

Fig. 2. Phylogenetic relationships of Gpha (A), Fshb (B), and Lhb (C) gonadotropin subunits of *A. altiparanae* with other teleost species. The tree was reconstructed using the Neigbor-Joining algorithm (1000 replications). The number indicates the bootstrap and only nodes with support >50% bootstrap are indicated. The glycoprotein hormone α2 (ACI87878) sequence from *Petromyzon marinus* was used as outgroup for Gpha analysis, while its gonadotropin β-like (AW56433) sequence was used as outgroup for Fshb and Lhb analysis. The marker length corresponds to 20% sequence difference.

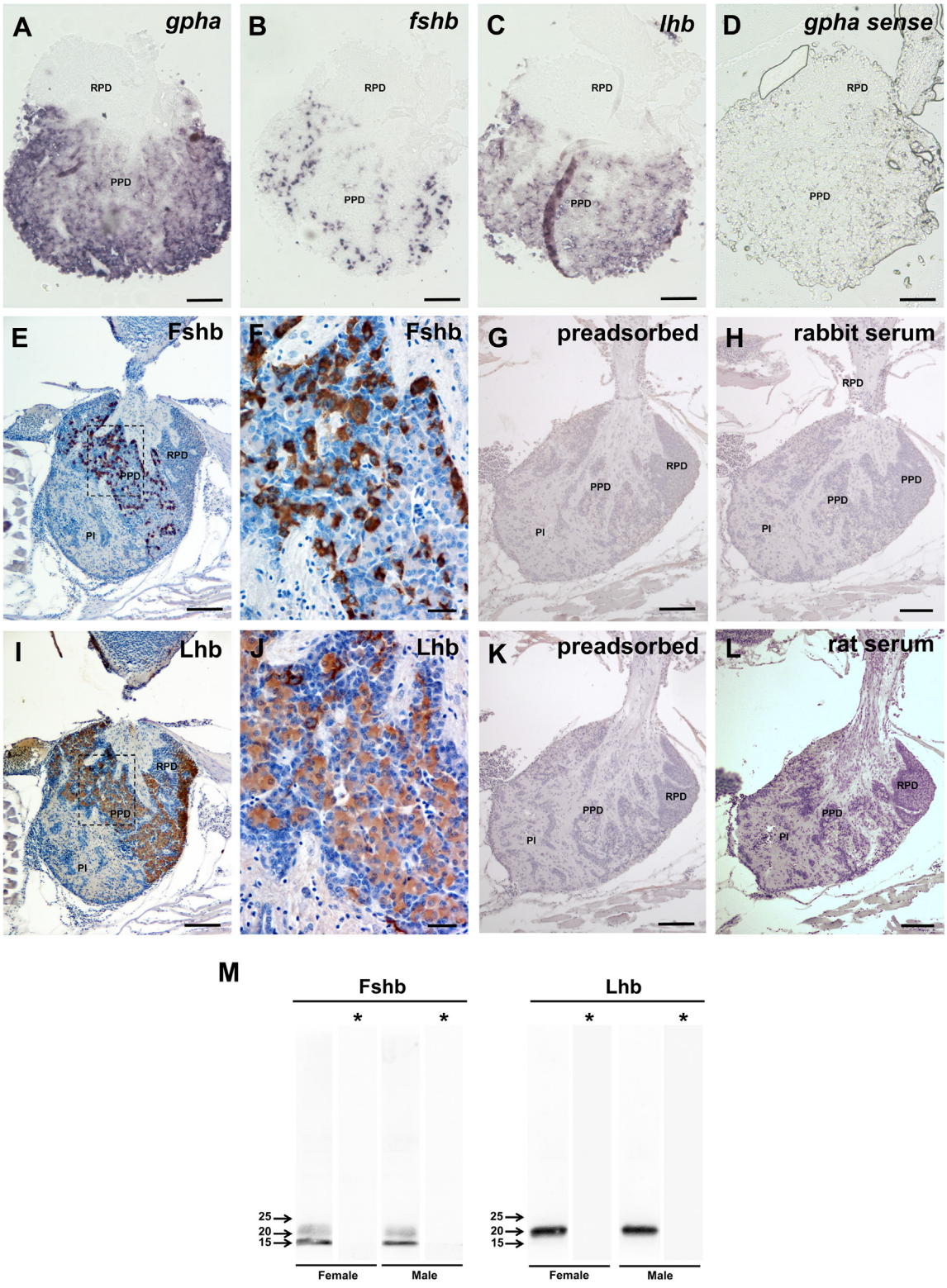

Fig. 3. *In situ* hybridization for detection of *gpha, fshb*, and *lhb* mRNAs and characterization of Fshb and Lhb proteins in *A. altiparanae* pituitaries using specific polyclonal antibodies. A) anti-sense probe for *gpha*, (B) anti-sense probe for *fshb*, (C) anti-sense probe for *lhb*, (D) hybridization with sense probe for *gpha*. Hybridizations with *fshb* and *lhb* sense probes gave negative results (data not shown). (E) Immunostaining with anti-Fshb, (F) high magnification from the dashed field in E, (I) immunostaining with anti-Lhb, (J) high magnification from the dashed field in I. Control reactions using preadsorbed anti-Fshb (G), anti-Lhb (K), and their respective peptides. Control reactions using normal rabbit serum (H) and normal rat serum as primary antibody (L). Note that the sections A–D are coronal sections and do not show the *pars intermedia* (PI), while the others are parasagittal sections. (M) Western blot analyses of pituitary homogenates carried out using anti-*A. altiparanae* Fshb and Lhb antibodies. Asterisks (*) indicate the lines where pituitary homogenates were incubated with the antibodies preadsorbed with their respective synthetic peptides used for immunization. *Rostral pars distalis* (RPD), *proximal pars distalis* (PPD), *pars intermedia* (PI). *Scale bar:* 200 μm (A–E, G–I, K, L), 20 μm (F, J).

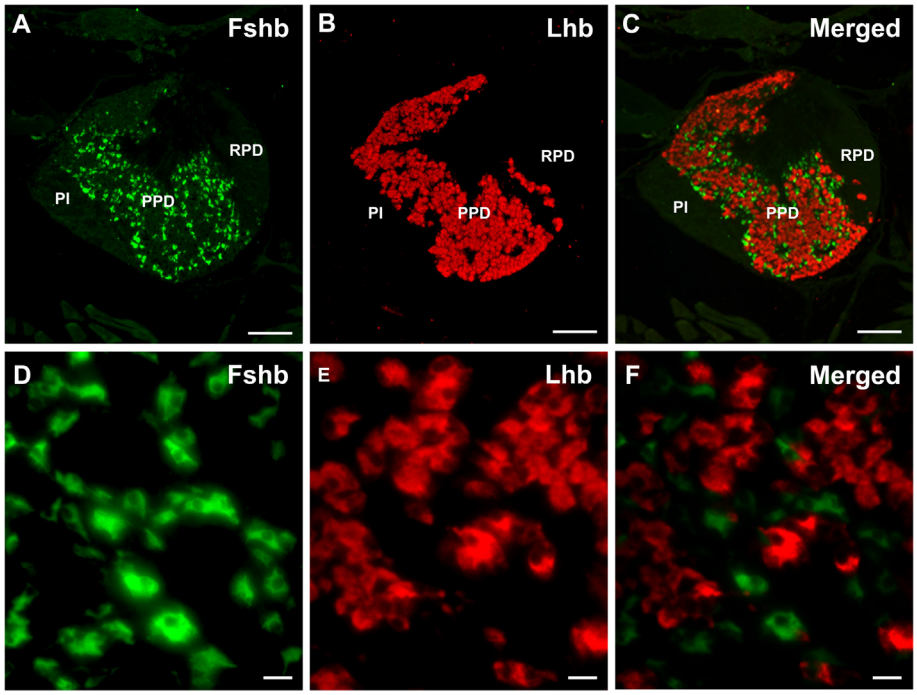

Fig. 4. Double-immunofluorescence investigation of gonadotropic cell duality in *A. altiparanae* using specific polyclonal antibodies. (A, D) Immunostaining with anti-Fshb labeled with Alexa 488 (green), (B, E) immunostaining with anti-Lhb labeled with Alexa 594 (red). (D, E) High magnification from the *proximal pars distalis* (PPD). Note that each signal (Fshb or Lhb) is present in distinct gonadotropic cells and the signals do not co-localize. The fluorescence of each fluorochrome and the merged images (C, F) were derived from the same section. *Rostral pars distalis* (RPD) and *pars intermedia* (PI). *Scale bar:* 100 μm (A–C), 10 μm (D–F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Reproductive parameters

Female *A. altiparanae* kept in captivity were sampled during their first reproductive cycle, from September 2012 to July 2013. Their GSI (Fig. 7A) varied significantly during the year ($p < 0.05$) and peaked on January 2013, indicating that the reproductive season in this species occurs during the summer, in Brazil. Although these females were not induced to reproduce, their GSI decreased after the spawning season and returned to the same values as found at the beginning of the sampling (September 2012). In contrast, no clear variation in HSI was detected during the whole period of study (Fig. 7B). Regarding the dynamic in the ovaries (Fig. 7C–H), vitellogenic oocytes were detected throughout the sampling period. Due this feature, all females analyzed were at spawning capable phase throughout the reproductive cycle, according to the nomenclature proposed by Brown-Peterson et al. (2011). Cortical alveolar oocytes were seen from September 2012 until March 2013 (Fig. 7C–F), suggesting that different clusters of oocytes developed during the reproductive season. Perinuclear oocytes were detected from May 2013 (Fig. 7G), marking the beginning of a new reproductive cycle. A few atretic oocytes were found in all analyzed specimen. No post-ovulatory complexes were detected, indicating that females were unable to reach final oocyte maturation, ovulation, and spawning during the reproductive season when in captivity.

4. Discussion

There are recent reports on the isolation of partial gonadotropin β subunit cDNAs from two Characiformes species, *S. hillari* (Moreira et al., 2015) and *Piaractus mesopotamicus* (Kuradomi et al., 2016). However, to our knowledge, this is the first study describing the cloning and molecular characterization of the full-length *gpha*, *fshb*, and *lhb* glycoprotein hormone subunit cDNAs in a representative species from this order, *Astyanax altiparanae,* which is one of the most representatives in diversity of species, and has ecological and economic value.

In the primary sequences of the *A. altiparanae* gonadotropin subunits, a series of conserved cysteine residues were recognized, with 10, 13, and 12 residues in Gpha, Fshb, and Lhb, respectively. Cysteine residues establish disulfide bridges and loop formation, essential for determining the tertiary structure of different proteins (Vitt et al., 2001). The presence of 10 cysteine residues in Gpha and 12 in Lhb are highly conserved features in teleosts and mammals. These cysteines are also located in evolutionary conserved positions in the proteins and form five and six intramolecular disulfide bridges in Gpha and Lh, respectively (Levavi-Sivan et al., 2010; Swanson et al., 2003). In contrast, the number of cysteines residues for Fshb is less conserved. Fshb sequences in ancestral lineages of fish as elasmobranchs (Querat et al., 2001), chondrosteans (Querat et al., 2000), and eels (Degani et al., 2003) contain 12 conserved cysteines, similar as found in tetrapods. However, Siluriformes (*I. punctatus* and *C. gariepinus*) and Cypriniformes (*Cyprinus carpio* and *Carassius auratus*) species show an additional cysteine residue in the N-terminal region of this hormone subunit (Chang et al., 1988; Liu et al., 2001; Vischer et al., 2003; Yoshiura et al., 1997), thus making the total number of cysteines 13; only *Danio rerio* is an exception in this respect (So et al., 2005). This data corroborates accumulated evidence that 13 cysteine residues is an exclusive feature in Fshb subunits in the superorder Ostariophysi (Levavi-Sivan et al., 2010).

The attachment of oligosaccharides is relevant to peptide folding, sorting, intra-cellular storage, receptor binding, and activation, as well as physiological clearance rates of glycoprotein hormones (Hearn and Gomme, 2000). Similar as in other fish and mammalian species, *A. altiparanae* Gpha and Lhb display two and one fully conserved potential N-linked glycosylation sites, respectively (Levavi-Sivan et al., 2010; Swanson et al., 2003). In contrast, the deduced amino acid sequence of *A. altiparanae* Fshb displays only a single

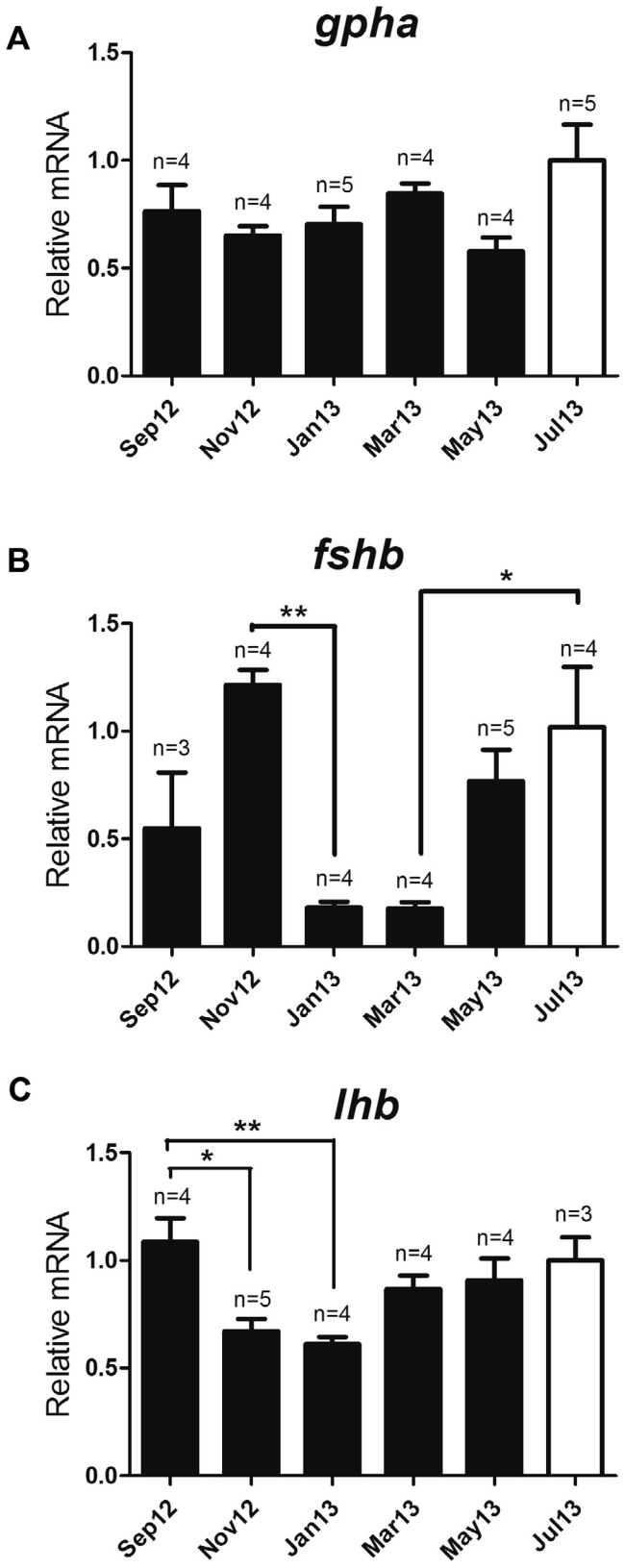

Fig. 5. Relative pituitary expression of *gpha* (A), *fshb* (B), and *lhb* (C) mRNAs of adult females of *A. altiparanae* during the annual reproductive cycle in captive, from September 2012 to July 2013. Data are expressed as mean ± SEM and presented as a ratio among the data obtained in July 2013 (white column, reference group). Asterisks denotes different levels of significance after the Tukey's test, *($p < 0.05$ and >0.01), **($p < 0.01$ and >0.001).

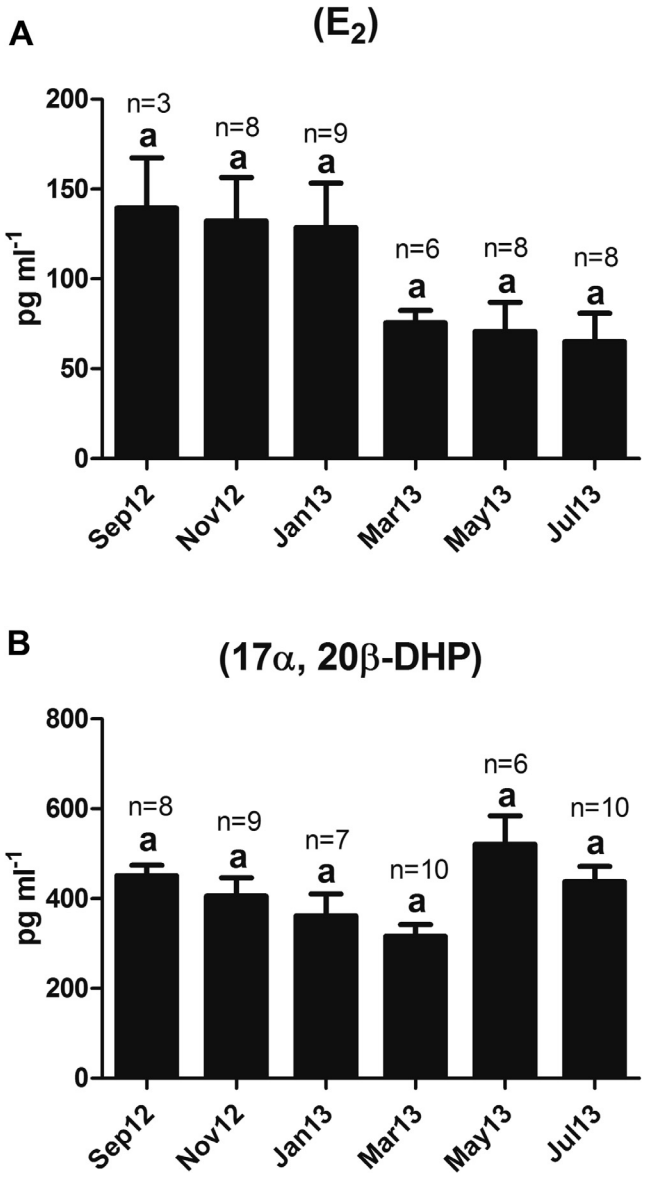

Fig. 6. Plasma levels of estradiol-17β (E_2) and 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-DHP) of adult females of *A. altiparanae* during the annual reproductive cycle in captive, from September 2012 to July 2013. Data are expressed as mean ± SEM and months sharing the same letter were not significantly different according to Tukey's test ($p > 0.05$).

potential N-linked glycosylation site, located in the N-terminal region. In general, Fshb sequences present more variations in relation to N-linked glycosylation sites (Swanson et al., 2003). Fshb subunits of primitive fish contain two potential N-linked glycosylation sites, while only one glycosylation site was detected in the Fshb sequences of Cypriniformes (So et al., 2005; Yoshiura et al., 1997), Perciformes (Mateos et al., 2003), Salmoniformes (Swanson et al., 2003), Siluriformes (Vischer et al., 2003), and now also Characiformes, as shown in this study.

In comparison with the *A. altiparanae* Gpha and Lhb sequences, the Fshb sequence displays a lower amino acid identity with Fshb sequences of species from several other orders. This was expected as it is well established that teleost Fshb primary sequences are less conserved between species (Levavi-Sivan et al., 2010), even in regions interacting with their cognate receptor. Accordingly,

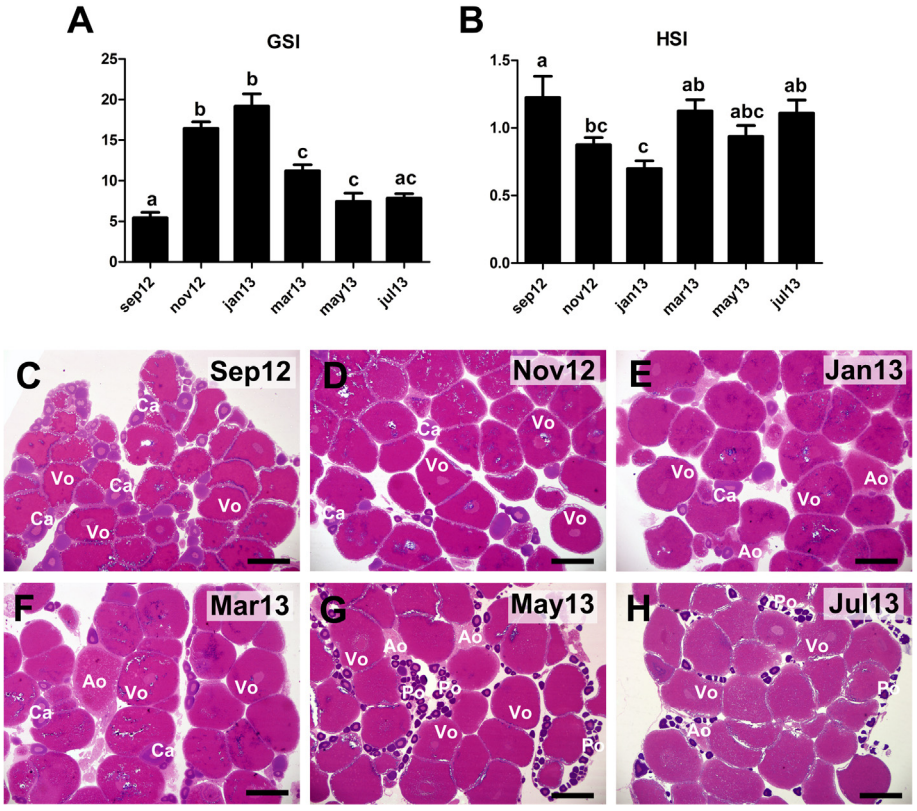

Fig. 7. Gonadosomatic index (A), hepatosomatic index (B), and representative histological sections from ovaries (C–H) of adult females of *A. altiparanae* during the annual reproductive cycle in captive, from September 2012 to July 2013. Months sharing the same letter were not significantly different according to Tukey's test ($p > 0.05$). The GSI varied significantly during the year and peaked on January 13. Note that vitellogenic oocytes (Vo) were found in ovaries during all months assessed while no post-ovulatory complexes were detected in any time. Atretic oocytes (Ao), cortical alveolar oocytes (Ca), and perinucleolar oocytes (Po). *Scale bar:* 500 μm.

So et al. (2005) have proposed that the lower conservation in Fshb sequences implies a functional divergence, suggesting that further investigations into the roles and physiological relevance of Fsh in different groups of teleosts are necessary.

In an attempt to detect extrahypophyseal expression sites for the *A. altiparanae* gonadotropins subunit mRNAs, we performed a tissue screen on samples of different organs of mature males and females using real-time quantitative PCR. Our data confirm that their presence is restricted to the pituitary gland, as seen in other species as *Ctenopharyngodon idella* (Zhou et al., 2010) and *Solea senegalensis* (Cerdà et al., 2008). In contrast, the expression of gonadotropin subunit mRNAs has been detected in the brain, ovaries, and testis in some species (Elisio et al., 2012; Huang et al., 2009; Pandolfi et al., 2006; Parhar et al., 2003; So et al., 2005; Wu et al., 2009). In addition, an unexpected expression of these subunits was found in non-HPG tissues, as in kidney, muscle, gill, and liver of *D. rerio* (So et al., 2005) and in spleen, kidney, and gill of *Anguilla marmorata* (Huang et al., 2009). Extrahypophyseal gonadotropins could function as autocrine/paracrine factors, allowing fine adjustment of the HPG axis, although their exact roles are still unknown (Levavi-Sivan et al., 2010). In view of these previous reports, our data suggest that the occurrence of extrahypophyseal gonadotropins can be species-specific.

The use of homologous antibodies or specific probes has unequivocally identified individual Fsh- and Lh-expressing cells (Naito et al., 1993; Nozaki et al., 1990; Shimizu et al., 2003; So et al., 2005; Weltzien et al., 2003) and, additionally, a third population of pituitary cells that express both hormone subunits (Cerdà et al., 2008; García-Hernández et al., 2002; Vischer et al., 2003). Until our work, antibodies against gonadotropin subunits from Neotropical fish were not yet available. Thus, cellular expression of gonadotropins in the pituitary has been investigated only in a few Characiformes species using heterologous antibodies (Borella et al., 1997; Honji et al., 2013; Jesus et al., 2014). Our specific antibodies developed against Fshb and Lhb synthetic peptides from *A. altiparanae* identified the distribution of these cells at pituitary, and support the data reviewed by Agulleiro et al. (2006). Moreover, they give evidence to the duality of gonadotropic cells also in a species from the order Characiformes, increasing the data available in this field.

In addition, Western blot analysis revealed that the polyclonal antibodies against *A. altiparanae* gonadotropin beta subunits were specific. They recognized bands close to their predicted molecular mass, and do not detect these bands after preincubation of the antisera with higher amounts of their respective immunizing peptides. Two specific bands closer to each other were detected by the anti-*A. altiparanae* Fshb, a result similar to that seen in *C. dimerus* (Pandolfi et al., 2006), probably due to a differential content of glycosylation in Fshb chains.

In our study, the activity of the pituitary-gonad axis in captive females of *A. altiparanae* was assessed by gene expression profiles of the gonadotropin subunit mRNAs in relation to sex steroid serum levels and ovarian histology, analyzed bimonthly during the reproductive cycle, from September 2012 to July 2013. The levels of *fshb* transcripts showed significant changes throughout the year. The highest levels were detected on November 2012, concomitant to the gradual increase in GSI and its peak on January 2013. It is well known that Fsh plays two major roles during ovarian development, stimulating oogonial proliferation as well as the vitellogenesis processes (Lubzens et al., 2010). Increase of *fshb*/Fsh levels occurs during vitellogenesis in most teleost species, which promotes oocyte growth in parallel with an increase in GSI

(Levavi-Sivan et al., 2010), physiological features also observed in *A. altiparanae*. In addition, the second increase in *fshb* transcript levels observed as from May 2013 may be associated with oogonial proliferation, once a large population of perinuclear oocytes was detected at this time.

However, evidence is accumulating that Fsh signaling may have many other roles during ovarian development (Guzmán et al., 2014; Luckenbach et al., 2013; So et al., 2005). Based on the high levels of *fshb*/Fsh detected during or just after ovulation, it has been suggested that Fsh also could act during late events of the reproductive cycle (So et al., 2005). Fsh would signal to the ovaries the beginning of a new reproductive cycle in species with single spawning (Breton et al., 1998; Gomez et al., 1999), and further stimulates the development of new oocyte batches during the same reproductive season in species with multiple spawning (Aizen et al., 2007; Elisio et al., 2014). In addition, a suite of Fsh-regulated genes during *Oncorhynchus kisutch* oogenesis was recently identified, by *in vitro* and *in vivo* studies. These genes are involved in cell proliferation, differentiation, survival, and extracellular matrix remodeling (Guzmán et al., 2014; Luckenbach et al., 2013). Such data show that Fsh may have different roles at specific stages of oocyte development. In *A. altiparanae*, our data show continuous vitellogenesis and their accentuation during the spawning season. Moreover, Fsh also could function in recruiting new batches of oocytes to the secondary growth, as already assumed for other species with multiple spawning (Kajimura et al., 2001; Sohn et al., 1999). In this way, it would be interesting to investigate these putative new roles related to Fsh and their downstream signaling that drives an asynchronous ovarian development, as occur in *A. altiparanae*.

The changes detected in the *fshb* mRNA levels were not reflected in the plasma levels of E_2, which remained constant throughout the cycle, differing from many studies that associated the vitellogenesis progression to an increase in E_2 plasma levels (Elisio et al., 2014; Tacon et al., 2000). This profile of E_2 levels was also detected in *H. malabaricus*, a characiform that displays group-synchronous ovarian development and has multiple spawning events. In this species, vitellogenic oocytes were detected only in spring and summer (Gomes et al., 2015), which differs from our findings. Sex steroids act by signaling the status of gonadal development to the brain and pituitary by positive and negative feedback (Levavi-Sivan et al., 2010). The lack of relation between pituitary *fshb* transcripts and E_2 serum levels that we observed may be due to various factors, such as post-transcriptional regulation, differential regulation of Fsh secretion, and their dynamics in the serum, as observed in *S. senegalensis* males (Chauvigné et al., 2016), or even by a differential expression and activation of Fshr in the ovaries. All of these hypotheses must be explored in futures studies.

In fish, Lh promotes the initiation of germ cell meiosis and final oocyte maturation, ovulation, and spawning via the progestogen 17α,20β-DHP, which is considered as the maturation-inducing hormone in most fish species (Lubzens et al., 2010). In species with synchronous ovarian development, high levels of *lhb*/Lh or their increase occur during final oocyte maturation and ovulation (Gomez et al., 1999; Swanson et al., 2003). But in species with asynchronous ovarian development and multiple spawning, both *fshb* and *lhb* increase in parallel with the progression of gonadal development (Mateos et al., 2003; Weltzien et al., 2003). The pituitary levels of *lhb* mRNA found in *A. altiparanae* females were constant during most part of reproductive cycle, differing from other studies in species with multiple spawning. In addition, *lhb* levels did not increase even during the spawning season, in contrast to many studies that have detected an increase and peak in *lhb* transcript levels/Lhb pituitary content and their serum levels during final oocyte maturation, ovulation, and spawning in wild animals (Elisio et al., 2014; Gomez et al., 1999; Kim et al., 2005) or animals kept in captivity submitted to induced reproduction (Honji et al., 2015). Therefore, our data suggest that a disturbance in Lh synthesis may be the first cause of the reproductive impairment in this species when kept in captivity. Accordingly, we highlight that new experimental approaches *in vivo* must be performed to clarify our hypothesis, as for example, to test whether females respond to exogenous Lh or human chorionic gonadotropin (hCG) intraperitoneally injected. Once the synthesis of recombinant Fsh and Lh from *A. altiparanae* are in progress, we believe that in a near future this question must be clarified.

As expected, considering the *lhb* mRNA profile in *A. altiparanae*, no changes in 17α,20β-DHP serum levels were detected during the first reproductive cycle. The same constant profile of 17α,20β-DHP was detected in cultured female *S. senegalensis* (García-López et al., 2007). The authors suggested that reproductive dysfunction in *S. senegalensis* occurred because the oocytes were unable to respond to this steroid in captive environmental conditions. In contrast, in *S. hilarii* no differences in 17α,20β-DHP levels were detected between wild and captive animals, even when *lhb* transcripts were low (Moreira et al., 2015). This highlights that the lack of ovulation in captivity, in the case of *S. hilarii*, is probably not caused by problems related to *lhb* synthesis and/or modulation of progestogen production by gonadotropins. Thereby, it was speculated that many other genetic disturbances can affect the HPG axis in captivity (Moreira et al., 2015).

Our histological analyses revealed that *A. altiparanae* females completed the ovarian development, presenting oocytes in secondary growth. In contrast, these females were not able to naturally reach the oocyte final maturation, ovulate, and spawn spontaneously, as no mature oocytes and post-ovulatory complexes were detected in any specimen, pointing to a reproductive dysfunction (Mylonas et al., 2010). We assumed that the impairment of migration and all the other physical and chemical features and social cues of the captive environment do not stimulate the neuroendocrine network to increase Lh synthesis and release. In migratory fish, the major cause of reproductive impairments has been generally attributed to the absence of Lh release by gonadotropic cells (Mylonas et al., 2010). However, our data corroborates recent findings in other South American migratory species (Honji et al., 2015; Moreira et al., 2015), that showed that reproductive dysfunctions can occur due to other disturbances upstream of gonadotropin synthesis as well as their downstream signaling in the gonads.

5. Conclusion

The cloning of the full-length *gpha*, *fshb*, and *lhb* cDNA sequences in *A. altiparanae* allowed us to develop new molecular tools for Characiformes. Thus, we observed that gonadotropin expression was restricted to the pituitary, in two distinct gonadotropic cells. Several endocrine/reproductive parameters were assessed suggesting that the reproductive dysfunction in *A. altiparanae* is probably due to a lack of increase of Lh synthesis during spawning season. However, experimental studies *in vivo* must be performed to clarify this issue. In addition, it is possible that a differential regulation among *fshb* transcripts and E_2 levels in this model might be related to asynchronous ovarian development and multiple spawning. Altogether, such data support the need for further basic and applied studies with potential biotechnological applications in *A. altiparanae* fish farming.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2016.12.004.

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