Gonadotropins and Growth Hormone Family Characterization in an Endangered Siluriform Species, *Steindachneridion parahybae* (Pimelodidae): Relationship With Annual Reproductive Cycle and Induced Spawning in Captivity

RENATO MASSAAKI HONJI,[1,2] DANILO CANEPPELE,[3] MATIAS PANDOLFI,[2] FABIANA LAURA LO NOSTRO,[2] AND RENATA GUIMARÃES MOREIRA[1]*

[1]Departamento De Fisiologia, Instituto De Biociências, Universidade De São Paulo, Rua Do Matão, Travessa 14, 321, São Paulo 05508-090, SP, Brasil

[2]Departamento De Biodiversidad Y Biología Experimental, Facultad De Ciencias Exactas Y Naturales, Universidad De Buenos Aires & IBBEA, CONICET-UBA. Ciudad Universitaria (C1428EHA), Buenos Aires, Argentina

[3]Companhia Energética De São Paulo (CESP), Unidade De Hidrobiologia E Aquicultura, Rodovia Dos Tamoios, Km 38, 12260-000, Brasil

ABSTRACT

The aim of this study was to identify and characterize pituitary cells of *Steindachneridion parahybae* females in captivity, highlighting the possible relationship with reproductive disorders at this level, since this species shows oocyte final maturation, ovulation and spawning dysfunction in captivity. The localization and distribution of growth hormone (GH), prolactin (PRL), somatolactin (SL), β-luteinizing hormone (β-LH), and β-follicle stimulating hormone (β-FSH) immunoreactive (-ir) cells in the adenohypophysis was studied by immunohistochemical and Western blot methods. In addition, cellular morphometric analyses and semi-quantification of ir-cells optical density (OD) during the annual reproductive cycle and after artificial induced spawning (AIS) were performed. Results showed that the distribution and general localization of pituitary cell types were similar to that of other teleost species. However, the morphometrical study of adenohypophysial cells showed differences along the reproductive cycle and following AIS. In general, females at the vitellogenic stage presented greater OD values for GH, PRL and SL than at other maturation stages (previtellogenic and regression stages), probably

Abbreviations used: ADH = adenohypophysis; AIS = artificial induced spawning; BPG = brain-pituitary-gonad axis; GH = growth hormone; GnRH = gonadotropin-releasing hormone; GSI = gonadosomatic index; GtH = gonadotropin; HBM = medial basal hypothalamus; IHC = immunohistochemical; IL = inferior lobe; (ir-) = immunoreactive; NH = neurohypophysis; NRLI = nucleus lateral of the lateral recess; OD = optical density; PAS = periodic-acid-Schiff; PBS = phosphate buffered saline; PI = pars intermedia; Pi = Pituitary gland; PPD = proximal pars distalis; PRL = prolactin; PS = pituitary stalk; RPD = rostral pars distalis; RT = room temperature; SL = somatolactin; WB = Western blot; β-FSH = β-follicle stimulating hormone; β-LH = β-luteinizing hormone

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*Correspondence to: Renata Guimarães Moreira, Departamento De Fisiologia, Instituto De Biociências, Universidade De São Paulo. Rua Do Matão, Travessa 14, 321, São Paulo 05508-090, SP, Brasil. Fax: +55 11 30917568. E-mail: renatagm@ib.usp.br

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indicating an increased cellular activity during this stage. Conversely, β-LH OD did not vary during the annual reproductive cycle. After AIS, β-LH, SL and GH ir-cells showed an increase in OD values suggesting a possible involvement on oocyte final maturation, ovulation and spawning or a feedback control on the brain-pituitary-gonads axis. Reproductive dysfunction in *S. parahybae* females in captivity may be due to alteration of the synthesis pathways of β-LH. In addition, GH family of hormones could modulate associated mechanisms that influence the reproductive status in this species. Anat Rec, 298:1644–1658, 2015.

Key words: luteinizing hormone; follicle stimulating hormone; somatolactin hormone; prolactin hormone; growth hormone; reproductive dysfunction

INTRODUCTION

As shown by Lubzens et al. (2010), one of the major aims of commercial aquaculture is the production of a large number of viable eggs, as well as a high production and survival of fingerlings. However, to obtain viable gametes in captivity, the control of reproductive function is essential for sustainability of commercial aquaculture (Mylonas et al., 2010). The key component regulating fish reproduction is the endocrine system, primarily through the brain-pituitary-gonad axis (BPG). Furthermore, a new vision for aquaculture has gradually emerged from numerous studies, by applying it to the conservation of endangered fish species.

Fish reproduction in captivity can be modulated by manipulating environmental parameters, such as water temperature, salinity, photoperiod, social factors, among others (Zohar and Mylonas, 2001; Fiszbein et al., 2010; Mylonas et al., 2010; Ramallo et al., 2014). Although environmental requirements for many commercial fish today are well understood, the biology of several teleost species is not as well known. For that reason, sometimes it can be impractical or even impossible to simulate the required environmental conditions for natural reproduction in captivity. In addition, several important commercial fish are almost exclusively collected from the natural environment and transferred to captivity for broodstock maintenance and rearing. However, after their transference to fish farms, many species exhibit some form of reproductive dysfunction that force the use of exogenous hormones for successful reproduction (Zohar and Mylonas, 2001; Mylonas et al., 2010).

Generally speaking, reproductive dysfunctions in females involve failures in reaching final oocyte maturation, ovulation and/or spawning; and in males they comprise low quality or reduced milt production. Several researches have focused on the BPG axis to understand these disorders. Reproduction in farmed fish can be enhanced by the development of strategies which can modify the synthesis and release of pituitary hormones (Zohar and Mylonas, 2001; Mylonas et al., 2010; Zohar et al., 2010). However, manipulation of these hormones requires accurate identification of their producing cells. Despite the lack of specific antibodies for accurate identification of pituitary cell types in Neotropical, non-salmonid species, immunohistochemical (IHC) studies with heterologous antisera can be applicable to localize these pituitary cells, since their specificity and absence of cross-reactivity can be demonstrated by preadsorption tests and control assays (Shimizu et al., 2003a, 2003b; Pandolfi et al., 2005). Some IHC studies on pituitary cells have shown differences in morphology, size and optical density (OD) of immunostains associated with distinct physiological processes (Fiszbein et al., 2010; Ramallo et al., 2012; Honji et al., 2013a).

The Neotropical catfish *Steindachneridion parahybae* is an important migratory (potamodromous) ichthyophagous freshwater fish, endemic to the Paraíba do Sul River Basin, seriously endangered in this Basin and regionally extinct in São Paulo State (Honji et al., 2009a). Since 2003 a conservation action has been taking place involving a fish restocking program run by Companhia Energética de São Paulo (CESP), which has supported researches on *S. parahybae* biology, mainly on broodstock management, egg and sperm quality and successful larval rearing, to ensure successful controlled breeding programs.

In the last few years, we have advanced the understanding of fundamental aspects of the reproductive and developmental biology of *S. parahybae*. For instance, the protocol for induced spawning of wild (Caneppele et al., 2009) and captive (Honji et al., 2013b) *S. parahybae* broodstock (F1) was established, and the embryonic development and larval stages (Honji et al., 2012a) and sperm quality of *S. parahybae* (Sanches et al., 2013) have been described. These findings demonstrated that reproduction of this species in captivity is viable; however, it is necessary to improve broodstock management to enhance fertilization rates and obtain increased fingerling production for conservation programs. Unfortunately, the maintenance of *S. parahybae* broodstock in captivity is not completely successful as females fail to ovulate or spawn naturally (Caneppele et al., 2009; Honji et al., 2013b). As the endocrine reproductive regulation of *S. parahybae* is poorly understood, these facts highlight the importance of increasing the knowledge about the endocrine processes that control reproductive biology in this species under captivity breeding, since any new insights into the neural and hormonal processes that control the BPG axis can be applied in fish farming aimed at conservation.

TABLE 1. Biometrical parameters in ovarian maturation stage and after artificial induced to spawning (AIS) and GSI

Maturation stage	N	Standard length (cm)	Total weight (g)	GSI (%)
Previtellogenic	28	35.93 ± 0.64	698.52 ± 29.50	0.40 ± 0.14[a]
Vitellogenic	22	34.50 ± 0.55	596.66 ± 25.35	1.59 ± 0.24[b]
Regression	18	33.61 ± 0.46	603.85 ± 29.97	0.57 ± 0.09[a]
AIS	6	33.07 ± 0.09	541.67 ± 8.08	1.61 ± 0.14[b]

Values followed by different letters (a,b) are significantly different during the reproductive maturation stage ($P < 0.05$).

In this study we performed, for the first time in *S. parahybae* adult females, a characterization of gonadotropins (GtHs), β-follicle stimulating hormone (β-FSH) and β-luteinizing hormone (β-LH), and the growth hormone (GH)/prolactin (PRL)/somatolactin (SL) family of hormones using IHC analysis and Western blot (WB). Morphometric analyses and semi-quantification of immunoreactive (ir-) cells, along with comparison of these ir-cells during the annual reproductive cycle and after artificial induced spawning (AIS) in captivity were also performed.

MATERIAL AND METHODS

Experimental Design and Collection of Animals

The study was performed at the Hydrobiology and Aquaculture Unit of CESP (23°41′39″S and 45°60′42″W), located in Paraibuna city (Sao Paulo State, Brazil). In December 2007, one hundred adult *S. parahybae* females (Table 1), born and raised in the CESP (first generation, F1, Caneppele et al., 2009), were randomly divided into two ponds (200 m^2). During the annual experimental design the broodstock were fed with commercial extruded feed for carnivorous fish (40% crude protein, Purina®) at a feeding rate of 2% of the biomass/day, offered to the animals in two daily portions. The average water temperature and dissolved oxygen concentration (monitored with an oximeter, Horiba-ModU10) during the experimental period were 21.10 ± 0.14 (°C) and 7.58 ± 0.36 (mg/L) respectively.

Four animals were randomly sampled monthly, except during the winter time (Southern hemisphere), and transported to the CESP laboratory. Fish were anesthetized with 0.1% benzocaine (ethyl-p-aminobenzoate), total and standard length (cm) and total body weight (g) were registered for each animal, and then fish were killed by decapitation at the level of the operculum. Thereafter, ovaries were quickly removed and weighed. To confirm the maturation stage of ovarian development, gonads were fixed in Bouin's solution for 24 hr, and dehydrated through a series of increasing ethanol concentrations. Ovaries were then cleared in dimethylbenzene solution and embedded in Paraplast® according to routine histological procedures. Ovary sections (5 μm thick) were obtained, mounted on poly-L-lysine solution-coated slides, and stained with periodic-acid-Schiff (PAS)/haematoxylin/metanil yellow (Quintero-Hunter et al., 1991) or hematoxylin/eosin (Behmer et al., 1976). Sections were mounted with Erv-Mount®, and then examined and documented using a computerized image analyzer (Leica light microscope DM1000, Leica photographic camera DFC295, and image capture Leica Application Suite Professional software, LAS V3.6). Both the gonadosomatic index (GSI), expressed as the percentage of body weight due to the ovaries [GSI = (gonad weight/total weight) × 100] (Vazzoler, 1996), and the histological results were used to determine the maturity scale. The ovarian classification was as follows: previtellogenic, vitellogenic, and regression (Fig. 1), these stages having been previously identified by Honji (2011). The procedures used to sacrifice animals in this study are in agreement with the Animal Ethics Committee of the Institute of Biosciences, University of São Paulo (Protocol 072/2008).

Since *S. parahybae* broodstock females do not breed in captivity, six females were selected on the basis of the typical morphological characteristics of sexual maturity, according to the principles previously established for *S. parahybae*, and the protocol for AIS with carp pituitary extract (Fish Braz) was performed (100% survival and positive response for induced spawning) (Caneppele et al., 2009; Honji et al., 2012a, 2013b). This experimental group was referred to as "artificial induced spawning" (AIS), in which females were manually stripped for gamete collection and then were sampled immediately.

Histological Analysis

For this analysis, the pituitary gland was dissected and fixed for 24 hr in Bouin's solution and dehydrated through a series of increasing ethanol concentrations. As described for ovarian histology, samples were cleared in dimethylbenzene solution, and embedded in Paraplast®, according to routine histological procedures. Serial sections (12-μm thick) were obtained and stained with haematoxylin/eosin, acid haematoxylin, periodic acid-Schiff (PAS) or Masson's trichrome. Sections were analyzed using a computerized image analysis (described above).

Single Immunohistochemistry

For IHC analyses, pituitary sections were immunostained by means of the Catalyzed Signal Amplification System (CSA Amplification System Kit-Code: K1500, Dako), following manufacturer's instructions. Briefly, samples were deparaffinized in xylene, rehydrated through graded ethanol concentrations, washed in PBS buffer (phosphate buffered saline, pH 7.4), treated with 0.3% hydrogen peroxide (H_2O_2) in PBS for 15 min at room temperature (RT) to block endogenous peroxidase activity, washed in PBS again and treated with 5% non-fat dry milk in PBS buffer for 30 min at RT to inactivate non-specific sites (blocking solution). Next, sections were incubated overnight at 4°C with the specific primary antiserum (detail of antisera and dilutions used in Table 2). After incubation with the primary antibody, sections were washed in PBS buffer for 5 min (RT), and

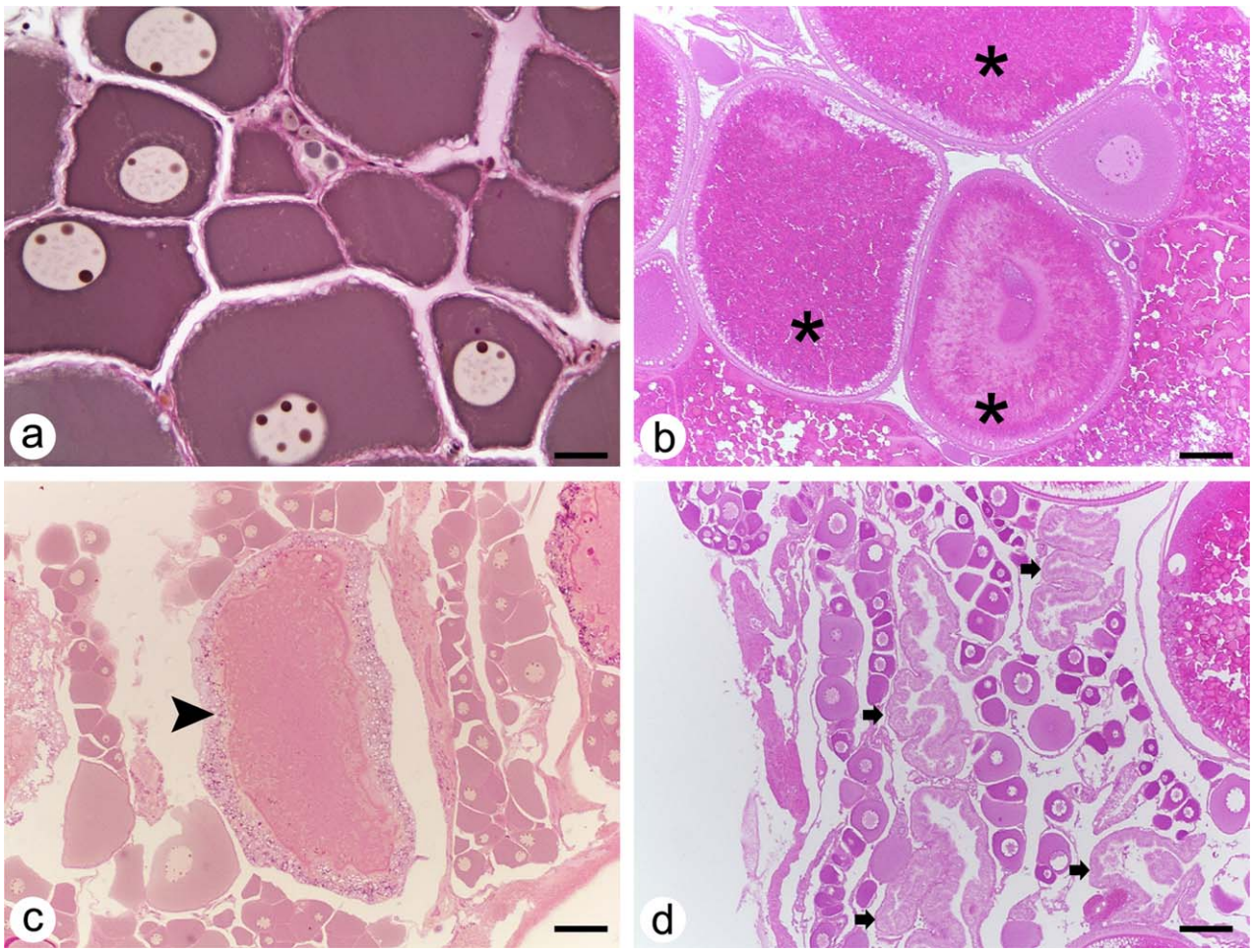

Fig. 1. Micrographs of the ovarian maturation stage of *Steindachneridion parahybae*. (**a**) previtellogenic stage, characterized mainly by the presence of perinucleolar oocytes; (**b**) vitellogenic stage, characterized mainly by the presence of vitellogenic oocytes (asterisk); (**c**) regression stage, characterized mainly by the presence of atretic oocytes (arrowhead); (**d**) after artificial induced to spawning (AIS) group, characterized mainly by the presence of postovulatory follicles (arrow). Periodic-acid-Schiff (PAS)/hematoxylin/metanil yellow (a,c) and hematoxylin/eosin staining (b,d). Bars: 100μm (a); 300μm (b,c,d).

TABLE 2. Primary and secondary antisera dilutions used in the immunohistochemical and Western blot methods

	Immunohistochemistry		Western blot	
Antiserum raised against	Dilution	Secondary antibody (rabbit)	Dilution	Secondary antibody (rabbit)
Oncorhynchus keta (β-FSH – code 2684)	1:4,000	1:600	NP	1:300
Oncorhynchus keta (β-FSH – code 8510)	1:1,000		NP	
Fundulus heteroclitus (β-FSH)	1:1,000		1:1,000	
Fundulus heteroclitus (β-LH)	1:1,000		1:3,000	
Oncorhynchus keta (GH)	1:2,000		1:3,000	
Oncorhynchus keta (PRL)	1:2,000		1:2,000	
Sparus aurata (SL)	1:2,000		1:2,000	

All the antisera used in this study were kindly provided by Drs: A. Shimizu (National Research Institute of Fisheries Sciences, Fisheries Research Agency, Kanazawa, Yokohama, Japan), H. Kawauchi (School of Fisheries Sciences, Kitasato University, Iwate, Japan), and A. Astola (Facultad de Ciencias, Universidad de Cádiz, Spain).
NP–not performed.

then incubated for 45 min (RT) with a biotinylated secondary antibody (dilutions used are detailed in Table 2). To amplify the signal, sections were washed in PBS buffer (5 min at RT), and afterwards sequentially incubated in streptavidin, tyramide and peroxidase-conjugated streptavidin solutions (CSA Amplification

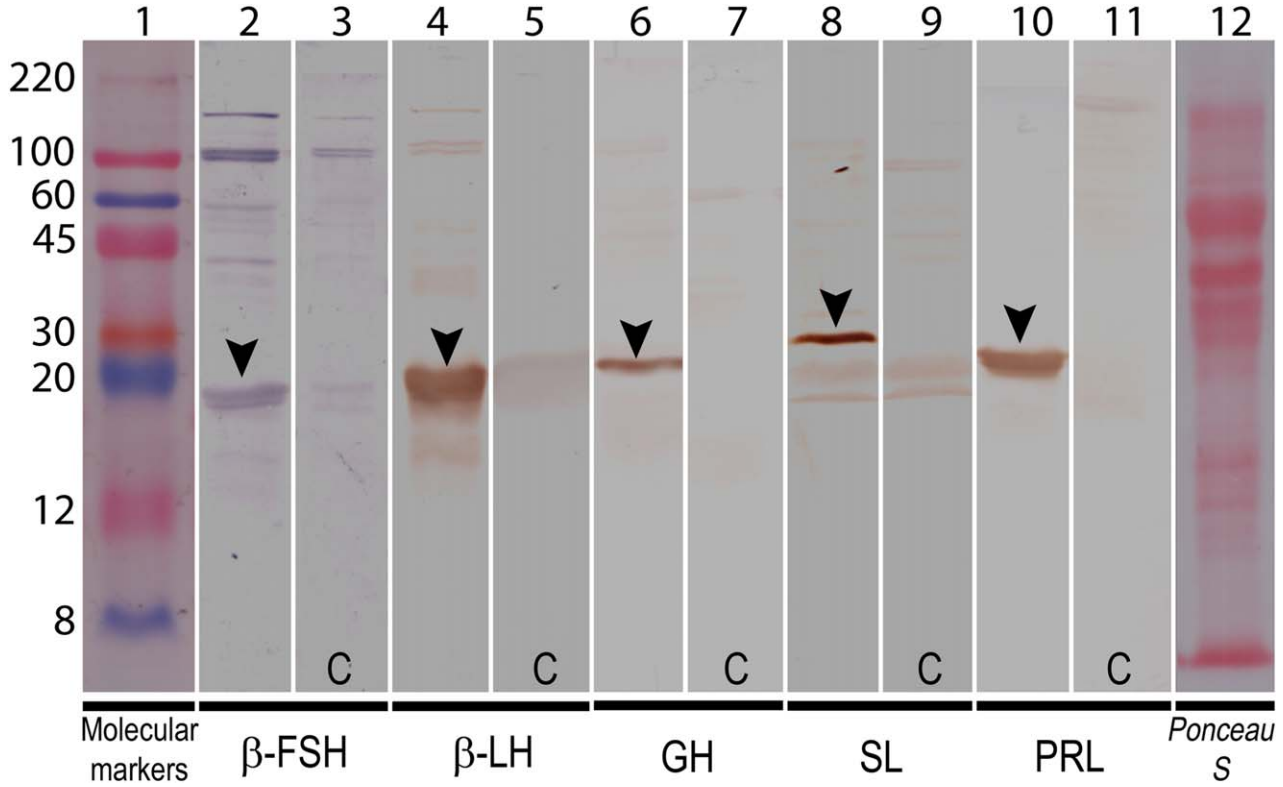

Fig. 2. Western blot analysis of pituitary homogenates of *Steindachneridion parahybae* incubated with primary antisera: β-Follicle-stimulating hormone, β-FSH (line 2), β-Luteinizing hormone, β-LH (line 4), Growth hormone, GH (line 6), Somatolactin hormone, SL (line 8), and Prolactin hormone, PRL (line 10). Control lines (3, 5, 7, 9, 11) incubated with preadsorbed antibodies. Molecular markers (line 1) and Ponceau S (line 12).

System Kit, Dako) for 45 min each at RT. Samples were finally washed in PBS buffer, and peroxidase activity was visualized with 3.3'-diaminobenzidine in a chromogen solution and DAB substrate buffer (imidazole-HCl buffer, pH 7.5, containing hydrogen peroxide and an anti-microbial agent). Subsequently, sections were lightly counterstained with hematoxylin for 2–3 min, mounted with Erv-Mount®, and analyzed using a computerized image analysis (described above).

For β-FSH and β-LH, to recover antigen immunoreactivity, samples were treated with citrate buffer for epitope unmasking. After blocking of endogenous peroxidase activity, samples were heated for 10 min at 90°C with citrate buffer, cooled at RT, washed in distiller water and then followed the IHC method described above. In addition, to confirm the specificity of IHC reactions, control sections were incubated with the primary antibody preadsorbed with an excess of its respective antigen, as was previously performed in other species: *Cichlasoma dimerus* (Pandolfi et al., 2001a, 2001b, 2006, 2009a, 2009b), *Salminus hilarii* (Honji et al., 2013a), *Fundulus heteroclitus*, *Pagrus major*, *Micropterus dolomieu* (Shimizu et al., 2003a) and other fish species (Shimizu et al., 2003b). To avoid false positive reactions the primary antibody was replaced with PBS or normal biotinylated secondary antibody (instead of primary antibody). No positive structures or cells were detected in these sections.

Histological slides were deposited in the collection of the Laboratório de Metabolismo e Reprodução de Organismos Aquáticos (LAMEROA), Instituto de Biociências, Universidade de São Paulo, Brasil.

Western Blot Analysis

To confirm the specificity of heterologous antisera against PRL, GH, SL, β-FSH and β-LH in *S. parahybae* pituitary gland, and also estimate the molecular weights of these hormones in this species, we performed electrophoresis on 15% sodium dodecylsulfate-polyacrylamide (SDS–PAGE) of pituitary homogenates. After electrophoresis, proteins and molecular markers (ColorBrust™ Electrophoresis Markers) were transferred into nitrocellulose membranes, and incubated with the different primary antisera (Table 2); immunodetection was visualized using an alkaline phosphatase developing kit (BCIP/NBT, Vector Blue, DAKO, to Alkaline Phosphatase kit) or with 3.3'-diaminobenzidine in a chromogen solution and DAB substrate buffer (DAKO kit), depending on the primary antiserum. Finally, membranes were dried and digitalized (Fig. 2), and molecular weights of immunoreactive bands were estimated using Image Gauge software (Fuji, Photo Film, V. 3.12). In addition, as described for IHC reactions, both positive and negative controls were also performed for WB analysis.

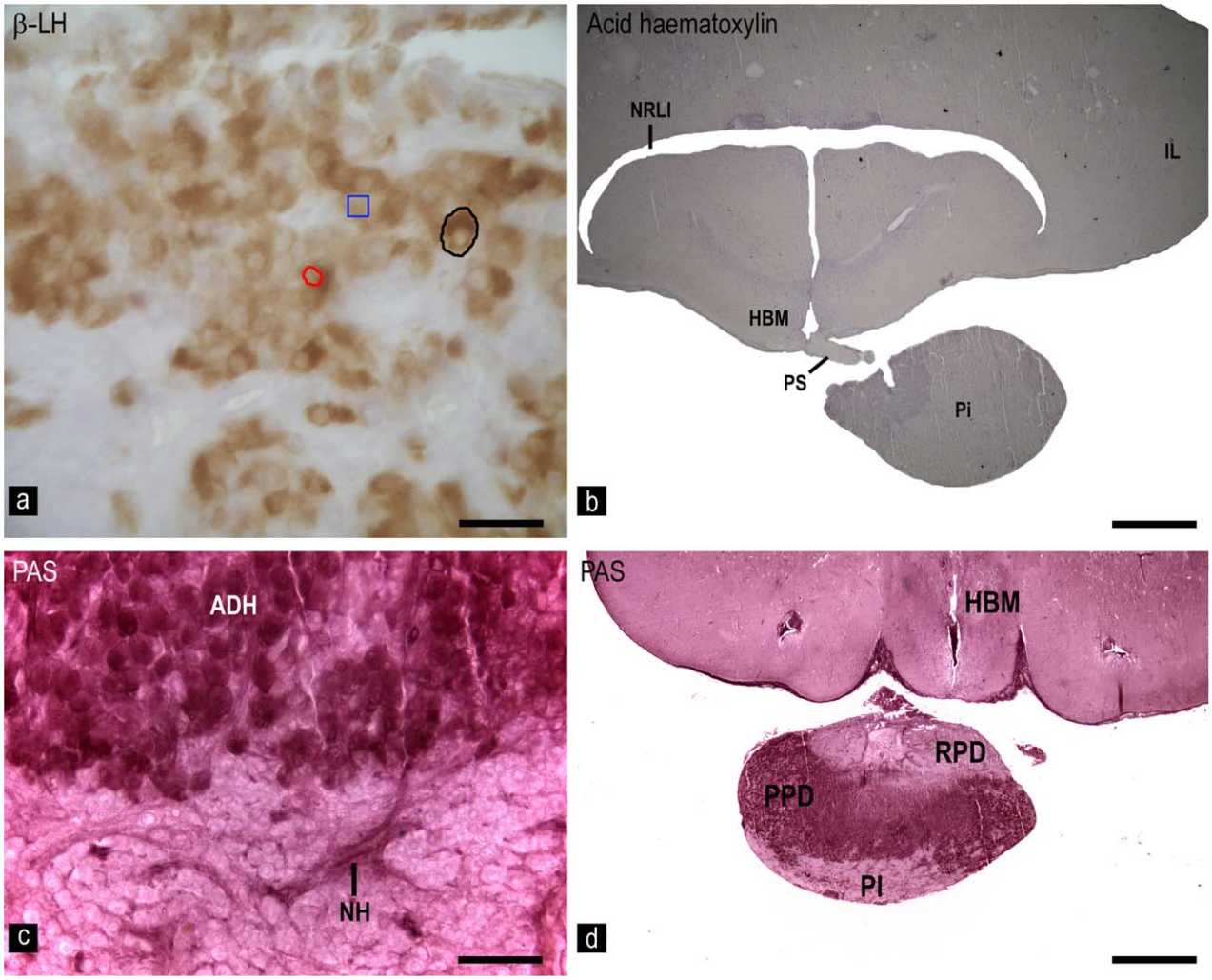

Fig. 3. Transversal sections through the pituitary gland and brain of *Steindachneridion parahybae*. (**a**) Photomicrograph of the stained (immunoblot) with β-Luteinizing hormone (β-LH) cells. Example of measured OD (blue square) and cellular area (black circle) and nuclear area (red circle) were indicated; b,c,d) Transversal sections of pituitary and brain stained using acid hematoxylin (b) and periodic acid-Schiff (PAS) (c,d). (**b**) Medial basal hypothalamus (HBM) region showing the direct connection from the brain to the pituitary gland (Pi) through pituitary stalk (PS). Others brain region: nucleus lateral of the lateral recess (NRLI) and inferior lobe (IL); (**c**) Two components of pituitary gland named adenohypophysis (ADH) and neurohypophysis (NH); (**d**) ADH subdivided into RPD, PPD, and PI. Bars: 10μm (a,c); 400μm (b,d).

Morphometric Analysis

For analysis of the OD of immunostain, images captured for each sample using the LAS system (1260 pixels by 960 pixels) were analyzed by means of the Image Gauge software. Ten images per animal were analyzed; fifteen randomly selected cells in each image were measured (Fig. 3a). To reduce variability in the results regarding immunostain intensity between tissues processed separately, representatives of females in all maturation stages and of AIS were included in each batch of IHC reactions to further control for staining differences. For analysis of pituitary cellular and nuclear area (Fig. 3a), the same images used for OD measurements were used to estimate these parameters. As cell bodies are irregular in shape and most are split during histological section, we only analyzed those cells where the nucleus was clearly visible. To assess cellular and nuclear area (μm^2) we used the Image Pro Plus software (Media Cybernetics).

Statistical Analyses

Statistical analysis of data related to morphometric analysis, taking into account the maturation stage (previtellogenic, vitellogenic, regression stages) and "AIS" group was performed using one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls (SNK) test for parametric analysis or Dunn's or Tukey's test for non-parametric analysis. Statistical differences were considered to be significant when $P < 0.05$. Data were expressed as mean ± standard error of the mean ($M \pm$ SEM). All statistical analyses were performed using the statistical software SigmaStat for Windows (version 3.10).

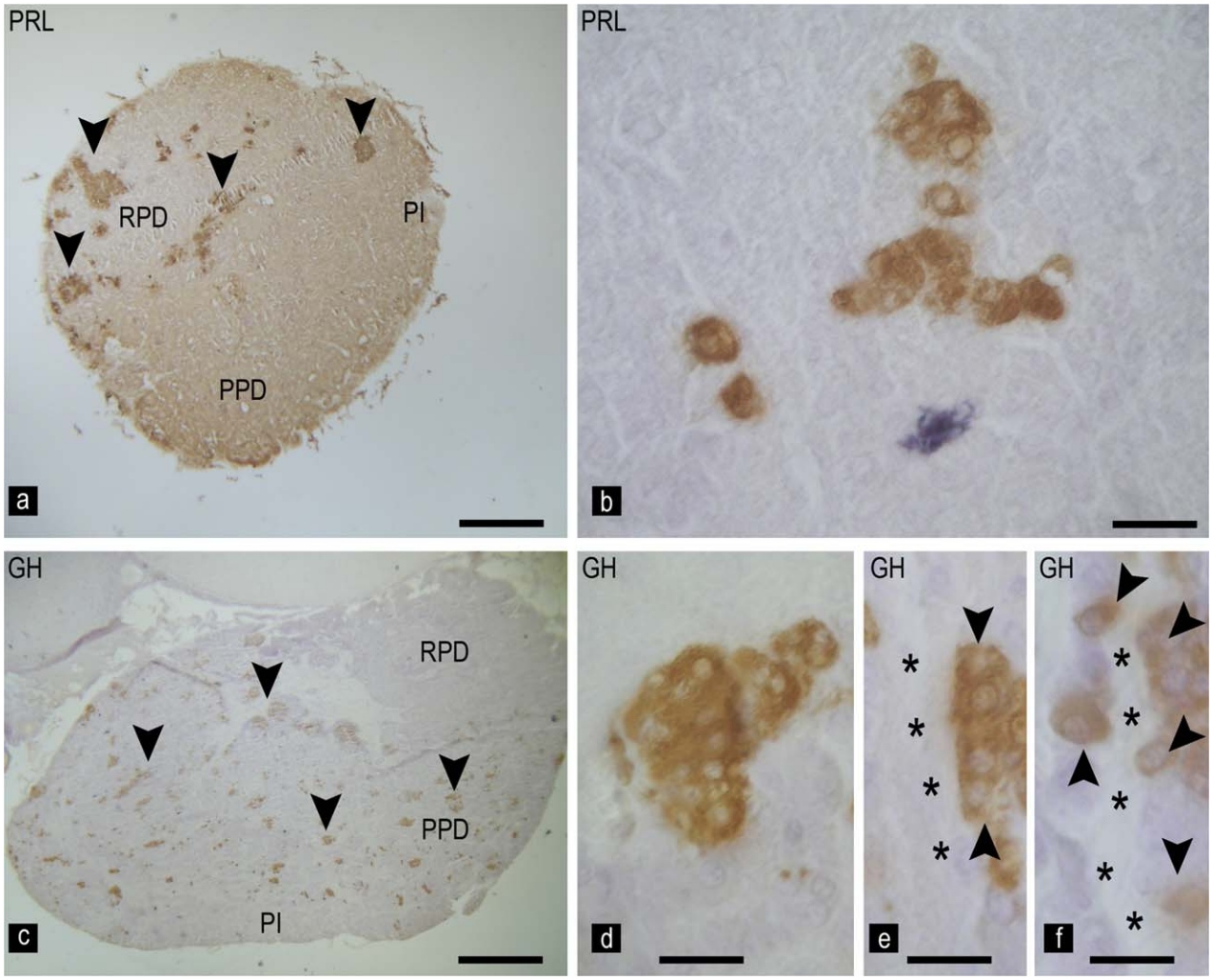

Fig. 4. Transversal or sagittal sections through the pituitary gland and/or brain of *Steindachneridion parahybae*. (**a**,**b**) Prolactin hormone immunoreactive (PRL-ir) cells (a, arrowhead) in the RPD and details (b) of PRL-ir using anti-chum salmon antisera; (**c–f**) Growth hormone immunoreactive (GH-ir) cells (c, arrowhead) along in the PPD and details (d) of GH-ir using anti-chum salmon antisera. GH-ir cells (e and f, arrowhead) were arranged near the NH branches (e and f, *). Bars: 400μm (a,c); 10μm (b,d,e,f).

RESULTS

After assessing oocyte development, the maturation stages were identified based on ovarian histology. The following ovarian maturation stages were assigned, considering overall oocyte developmental phase: previtellogenic stage (presence of perinucleolar oocytes) (Fig. 1a); vitellogenic stage (presence of vitellogenic oocytes) (Fig. 1b); and regression stage (presence of atretic oocytes) (Fig. 1c). In the "AIS" group several postovulatory follicles (Fig. 1d) were observed. Furthermore, GSI values for the vitellogenic stage were higher than in previtellogenic and regression stages ($P < 0.01$) (Table 1), and similar to the "AIS" group.

Histological, Immunohistochemical, and Western Blot Analyses of the Pituitary Gland

The pituitary gland in *S. parahybae* is located in the ventral region of the mediobasal hypothalamus, connected by a thin pituitary stalk (Fig. 3b). This gland consisted of two components, neurohypophysis (NH) and adenohypophysis (ADH) (Fig. 3c), the latter subdivided into rostral pars distalis (RPD), proximal pars distalis (PPD), and pars intermedia (PI) (Fig. 3d).

The RPD contained weakly acidophilic cells, which were not easily identified by histological methods (Masson's trichrome). These cells were PAS-negative and arranged in cords. They positively immunostained with anti-chum salmon PRL antiserum (Fig. 4a,b). No cross-reactivity with other antisera was observed. Thin branches of NH could be observed in this region.

The NH also extended several ramified branches into the PPD. Only two different cellular types were identified within the purpose of this study in the PPD, with presence of GH ir- (Fig. 4c–f) and β-LH ir-cells (Fig. 5a,b). Multiple GH ir-cells were identified in the PPD with anti-chum salmon GH antiserum, restricted to the central region; no other cells in other regions of the ADH showed immunoreactivity for GH. These cells were

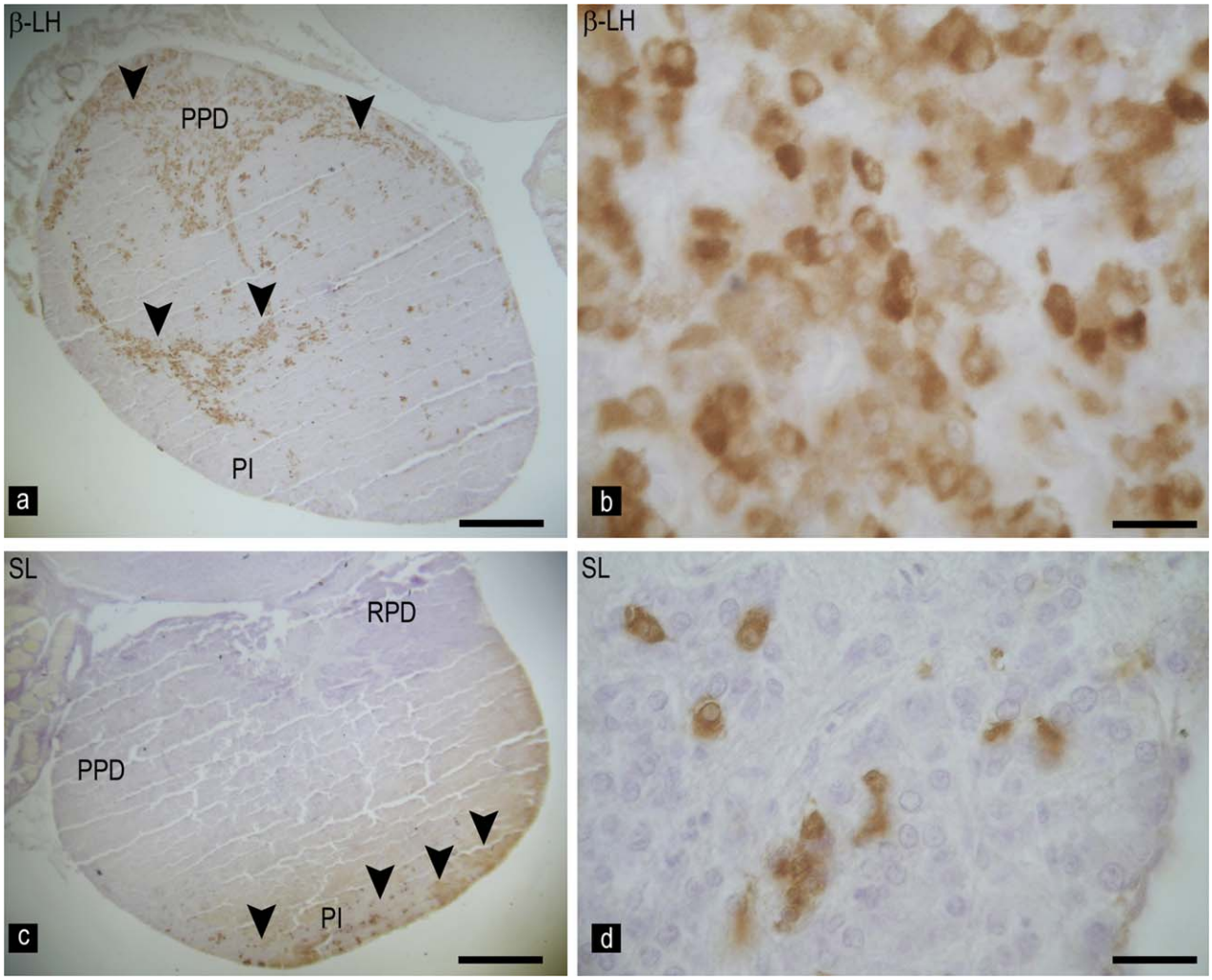

Fig. 5. Transversal or sagittal sections through the pituitary gland and/or brain of *Steindachneridion parahybae*. (**a**,**b**) β-Luteinizing hormone immunoreactive (β-LH-ir) cells (a, arrowhead) along in the PPD and details (b) of β-LH-ir using anti-mummichog antisera; (**c**,**d**) Somatolactin hormone immunoreactive (SL-ir) cells (c, arrowhead) in the PI and details (d) of SL-ir using anti-chum salmon antisera (d). Bars: 400μm (a,c); 10μm (b,d).

PAS-negative, acidophilic, oval in shape, with a large excentric and irregular nucleus, and were arranged near the NH branches (Fig. 4e,f) and blood vessels. Gonadotrophs were the most abundant cell type in the PPD region and easily recognizable by histological methods, as they were PAS-positive, basophilic and contained vacuoles. Only LH producing cells were immunoreactive with anti-mummichog β-LH antiserum (Fig. 5a,b). In contrast, FSH producing cells were not immunodetected with anti-mummichog β-FSH antiserum either anti-salmon β-FSH antiserum by IHC; however, an ir-band of the expected molecular weight was detected by WB with anti-mummichog β-FSH antiserum.

The PI was characterized by the presence of numerous deep NH branches and several slightly acidophilic and PAS-positive cells. These cells are responsible for the synthesis and release of SL hormone, showing positive immunostaining with anti-seabream SL antiserum (Fig. 5c,d). No cross-reactivity with any other cell type in the ADH was observed. Somatolactin ir-cells were not detected outside the PI.

Figure 2 summarizes the results obtained by WB to confirm the specificity of the immunostain using heterologous antisera against PRL, GH, SL, β-FSH and β-LH in *S. parahybae* pituitary gland. The estimated molecular weights for these peptides were: PRL: 22 kDa, GH: 21 kDa, SL: 26 kDa, β-FSH: 18 kDa, and β-LH: 19 kDa.

Cellular and Nuclear Areas and Semiquantitative Analysis of Immunostain Optical Density

Some significant differences were identified regarding the cellular and nuclear areas and the OD of the immunostain (IHC) during the annual reproductive cycle and after AIS of *S. parahybae* in captivity. OD values for β-LH were similar throughout the reproductive cycle: previtellogenic, 113.19 ± 6.50 a.u.; vitellogenic, 120.53 ± 1.10 a.u.; and

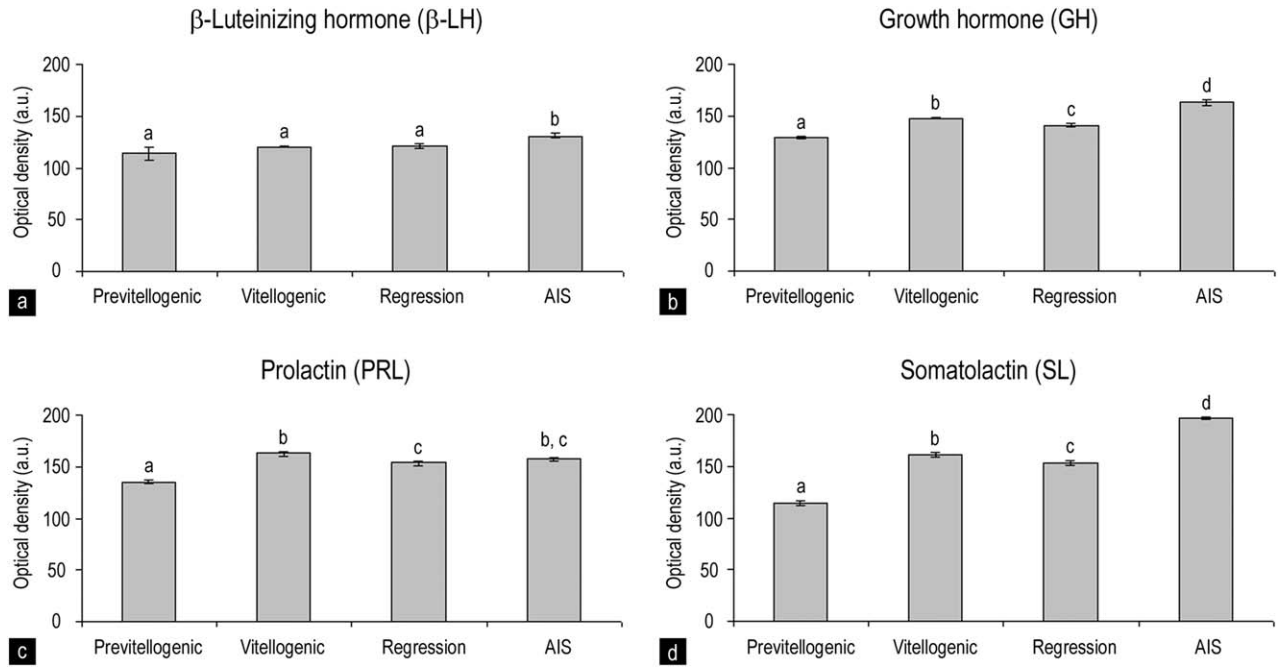

Fig. 6. Semiquantitative analysis (optical density, a.u.) of immunohistochemistry of pituitary gland of *Steindachneridion parahybae* during annual reproductive cycle and after the artificial induction spawning (AIS): (**a**) β-Luteinizing hormone (β-LH); (**b**) Growth hormone (GH); (**c**) Prolactin hormone (PRL); (**d**) Somatolactin hormone (SL). Values followed by different letters (a,b) are significantly different during the reproductive cycle ($P < 0.05$).

regression, 120.68 ± 1.34 a.u. (Fig. 6a). However, the "AIS" group (130.97 ± 1.66 a.u.) showed higher OD for β-LH when compared to all other stages ($P < 0.05$) (Fig. 6a). Cellular and nuclear areas of β-LH did not differ during the annual reproductive cycle or for the "AIS" group (respectively, previtellogenic: 11.85 ± 0.17 μm^2 and 6.75 ± 0.09 μm^2; vitellogenic: 12.26 ± 0.21 μm^2 and 7.03 ± 0.10 μm^2; regression: $12\ 54 \pm 0.21$ μm^2 and 6.99 ± 0.12 μm^2; "AIS" group: 12.73 ± 0.30 μm^2 and 6.71 ± 0.13 μm^2) (Fig. 7a).

For GH, OD values were significantly higher for the vitellogenic stage (147.48 ± 0.82 a.u.) than for the previtellogenic (128.94 ± 1.43 a.u.; $P < 0.01$) and regression stages (141.07 ± 1.48 a.u.; $P < 0.01$) (Fig. 6b). In the "AIS" group, the OD values (162.21 ± 2.40 a.u.) were higher than all other stages ($P < 0.01$) (Fig. 6b). Cellular area of GH ir-cells showed significant differences during the annual reproductive cycle, with higher values at the regression stage (12.51 ± 0.35 μm^2) than at other reproductive stages: previtellogenic (10.94 ± 0.32 μm^2) and vitellogenic (11.19 ± 0.26 μm^2; $P < 0.05$), and similar to those for the "AIS" group ($11.27 \pm 0\ .33$ μm^2) (Fig. 7b). In addition, the nuclear area showed no significant differences during the reproductive cycle.

In the case of PRL, the OD values during the reproductive cycle showed the same pattern as described for GH, lower at previtellogenic (134.70 ± 1.61 a.u.; $P < 0.01$) and regression stages (152.99 ± 1.51 a.u.; $P < 0.01$) than at the vitellogenic stage (162.17 ± 1.95 a.u.) (Fig. 6c). In the "AIS" group, OD values (157.05 ± 1.77 a.u.) were only significantly higher than those of the previtellogenic stage ($P < 0.01$) (Fig. 6c). The cellular area of PRL cells, was significantly higher in the vitellogenic stage (15.13 ± 0.21 μm^2) than in the previtellogenic (12.98 ± 0.23 μm^2) and regression stages (12.89 ± 0.53 μm^2), as well as in the "AIS" group (13.30 ± 0.47 μm^2; $P < 0.01$) (Fig. 7c). The nuclear area showed no significant differences during the reproductive cycle.

Semi-quantitative analysis for SL ir-cells again showed higher OD values for the vitellogenic stage (160.67 ± 1.56 a.u.) when compared to the previtellogenic (114.15 ± 1.46 a.u.; $P < 0.01$) and regression stages (152.85 ± 1.46 a.u.; $P < 0.01$). In the "AIS" group OD values were higher than all maturation stages (196.35 ± 0.92 a.u.; $P < 0.01$) (Fig. 6d). Somatolactin cellular diameter also showed differences between maturation stages and the "AIS" group. The vitellogenic (12.16 ± 0.30 μm^2) and regression stages (12.56 ± 0.37 μm^2) showed a higher SL ir-cell area than the previtellogenic stage (10.66 ± 0.24 μm^2) and the "AIS" group (10.91 ± 0.20 μm^2) ($P < 0.01$) (Fig. 7d). In addition, the nuclear diameter of these SL cells showed no significant differences during the reproductive cycle.

As FSH producing cells were not immunodetected with anti-mummichog β-FSH antiserum by IHC, semiquantitative analysis for FSH was performed during the reproductive cycle by the same way applied by IHC, however with immunodetected bands obtained by WB. FSH ir-bands showed higher OD values in the previtellogenic stage (15.53 ± 2.92 a.u.) when compared to the vitellogenic (8.26 ± 0.19 a.u.) and regression stages (8.67 ± 0.81 a.u.; $P < 0.01$) (Fig. 8).

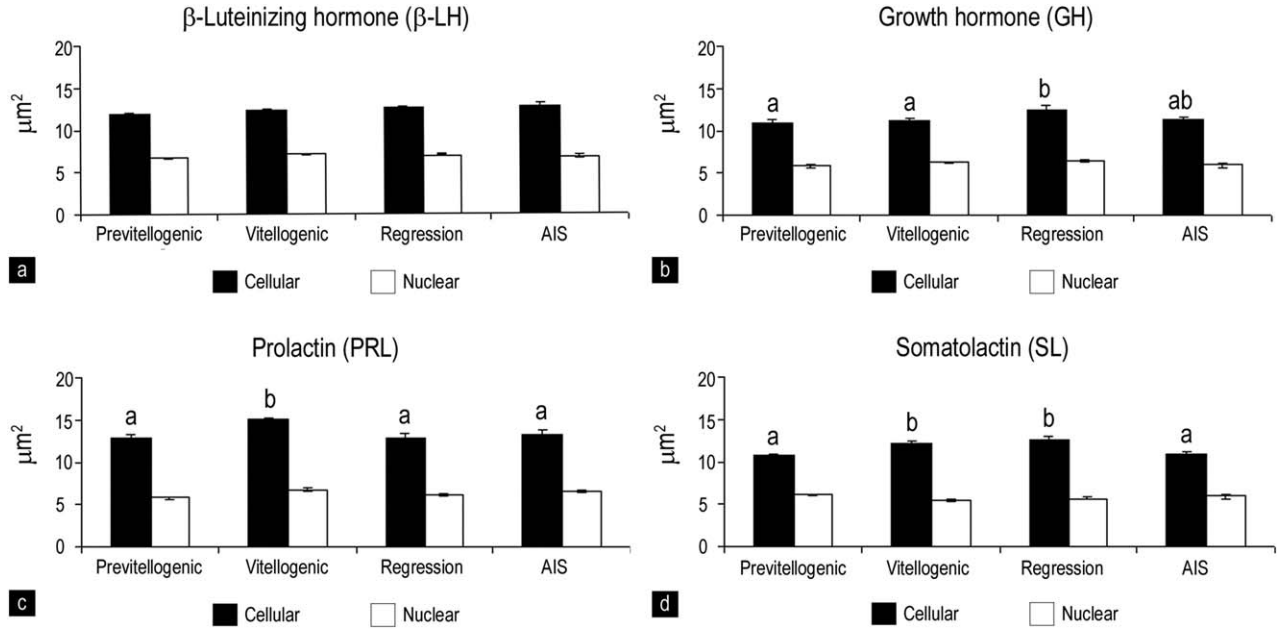

Fig. 7. Morphometric analysis of the pituitary cell types of *Steindachneridion parahybae* during annual reproductive cycle and after the artificial induction spawning (AIS): (**a**) β-Luteinizing hormone (β-LH); (**b**) Growth hormone (GH); (**c**) Prolactin hormone (PRL); (**d**) Somatolactin hormone (SL). Values followed by different letters (a,b) are significantly different during the reproductive cycle ($P < 0.05$). Black bars indicate cellular area and white bars indicate nuclear area.

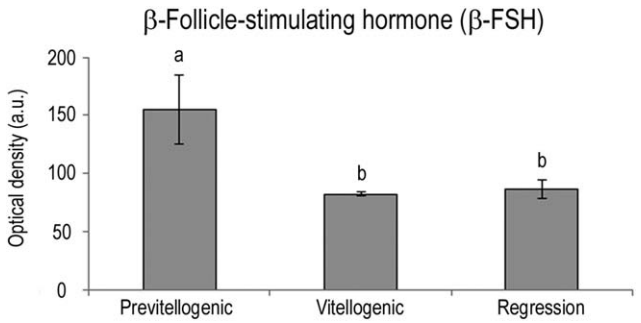

Fig. 8. Semiquantitative analysis (optical density, a.u.) of immunoblots (Western blot) of pituitary gland of *Steindachneridion parahybae* during annual reproductive cycle for β-Follicle-stimulating hormone (β-FSH). Values followed by different letters (a,b) are significantly different during the reproductive cycle ($P < 0.05$).

DISCUSSION

In this study, histological analysis of *S. parahybae* gonads during the reproductive cycle suggests that, even in captivity, these broodstocks undergo full gametogenesis (primary growth) and ovarian development until the vitellogenic oocyte phase. Therefore, broodstock maturation occurred from previtellogenic to vitellogenic stages. However, despite the complete ovarian maturation observed, *S. parahybae* females did not exhibit oocyte final maturation, ovulation or spontaneous spawning in captivity, probably due to dysfunction of the endocrine system (Honji, 2011; Honji et al., 2012b, 2013b).

As expected, GSI values increased as the stage of gonad maturation progressed, with the largest GSI values being recorded when the broodstock had fully developed ovaries (vitellogenic stage) and lowest GSI values observed at the previtellogenic stage, indicating a normal ovarian cycle, even in captivity. Furthermore, the regression stage observed in *S. parahybae* is commonly found in captive fish, mainly in species that fail to spawn naturally. This GSI pattern of variation is observed in several teleost fish species (Vazzoler, 1996; Rocha and Rocha, 2006; Honji et al., 2009b). After AIS, GSI values remained high, even in those *S. parahybae* females that had been induced and had spawned, suggesting that these animals experience multiple spawning events during the annual reproductive cycle.

Morphometric analysis (nuclear and cellular diameter) and OD of immunostain are widely used in medical practice, and increasingly improved accuracy of these analyses has been developed by use of automated image analysis methods (Smith et al., 2005; Theodosiou et al., 2007; Filippi-Chiela et al., 2012). A change in nuclear and cellular morphology occurs in many physiological conditions (Filippi-Chiela et al., 2012) and the OD is a useful tool to assess antigen abundance in different patterns of antigen localization (Smith et al., 2005; Theodosiou et al., 2007). Even though we do not know with certainty whether these substances are being released or accumulated in the cytoplasm, it is generally accepted that, at least in the case of GtHs, hormones are rapidly released after synthesis (Maruska et al., 2011). Since there are no tests available for measuring pituitary hormones in the plasma of non-salmonid species, the methods used are a valid substitute to evaluate pituitary hormones during the annual reproductive cycle in *S. parahybae*. Similar analyses were performed in other teleost species: *C. dimerus* (Fiszbein et al., 2010; Piazza et al., 2011; Tubert et al., 2012), *S. hilarii* (Honji et al.,

2013a). Moreover, despite nuclear diameter being a useful tool to analyze neuron activity (Fiszbein et al., 2010; Piazza et al., 2011), as no differences in nuclear diameter were observed in this study, the morphometric analysis was based mainly on the OD and the cellular diameter as indicative of higher or lower cellular activity during the reproductive cycle of *S. parahybae*.

Several cytochemical investigations focused on the identification and localization of ADH cells types in teleost species, including representative species of the following orders: Cyprinodontiformes (Batten, 1986), Osteoglossiformes (Borella et al., 2009), Perciformes (García-Ayala et al., 1997; Villaplana et al., 2000; Pandolfi et al., 2001a, 2001b); Clupeiformes (Laiz-Carrión et al., 2003), Salmoniformes (Naito et al., 1993), Siluriformes (Volckaert et al., 1999), Cypriniformes (Chapman et al., 2005), Osmeriformes (Saga et al., 1999), Pleuronectiformes (Einarsdóttir et al., 2006), and Atheriniformes (Vissio et al., 1997). Among the reophilic Neotropical species, only *S. hilarii* (Honji et al., 2013a), *S. brasiliensis* (Jesus et al., 2014) and *Piaractus mesopotamicus* (Borella et al., 1997), all Characiformes, were previously analyzed, adding for the first time to the list the tropical catfish *S. parahybae* in this study. Although we found similar distribution and localization patterns for ADH cell types in *S. parahybae* with other teleost species (Agulleiro et al., 2006; Kawauchi and Sower, 2006; Takei and Loretz, 2006; Levavi-Sivan et al., 2010), this is the first detailed and accurate identification of ADH cells in a threatened South American fish species, which exhibits endocrine disorders in captivity, reinforcing the importance of this study.

As showed by Zohar and Mylonas (2001) and Mylonas et al. (2010) three reproductive dysfunctions in teleost fish are frequently observed in many species when breeding in captivity. Briefly, (1) female vitellogenesis and male spermatogenesis fail completely when broodstocks are maintained in captivity; (2) cultured females fail to spawn at the end of the reproductive cycle (oocytes undergo normal vitellogenesis, final maturation, and ovulation; however, ovulated eggs are not released to the water); (3) vitellogenesis appears to progress normally in cultured females, but oocytes fail to reach final maturation, resulting in neither ovulation nor spawning. These reproduction disorders have been identified to be due to dysfunctions in GtHs synthesis and/or release pathways from the pituitary gland (Zohar et al., 2010). *Steindachneridion parahybae* females exhibit the third problem listed here when reared in captivity.

Regarding gonadotropins (β-FSH and β-LH), both GtHs were characterized in *S. parahybae* pituitary with heterologous antibodies (*F. heteroclitus*). Only β-LH was identified by both IHC and WB methods. A β-FSH ir-band of the expected molecular weight was detected by WB analysis, but no ir-cells could be detected. In addition, anti-chum salmon antisera were used for studying GtHs in several fish and have been described to give different ir-cells among diverse teleost species studied so far (Agulleiro et al., 2006; Kasper et al., 2006), however, in this study this antibody showed no positive ir-cells. The difference between β-FSH and β-LH immunodetection in pituitary sections could be due to the different molecular structure, phylogeny and evolution of GtHs observed in many teleost fish (Levavi-Sivan et al., 2010). Furthermore, epitopes for β-FSH could have been partially masked or degraded in histological sections by fixation, dehydration, and embedding steps (typical for IHC procedures) (Shimizu et al., 2003a). If that was the case, a further treatment by heating the samples with epitope unmasking solution to denature the protein could have been required for positive detection with the antibody. Although heterologous antisera were used to localize GtHs producing cells, their specificity was established in our species by the absence of cross-reactivity, by the WB analysis and also by the negative control. Moreover, the estimated molecular weights of *S. parahybae* GtHs are similar to that of other teleost fish (Levavi-Sivan et al., 2010).

Vitellogenesis is likely to be controlled by FSH, as observed in salmonid and non-salmonid species (Swanson et al., 1991; Levavi-Sivan et al., 2010). In *S. parahybae*, during the reproductive cycle, the data herein found suggest a higher amount of this hormone during the previtellogenic than vitellogenic and regression stages, displaying a close relationship with the seasonality of the ovarian development, i.e., vitellogenesis (Honji et al., 2012b). In addition, unlike salmonid species, due to absence of an accurate method to measure plasma levels of FSH in Neotropical siluriform, semiquantitative analysis was performed in this study to detect differences in the pattern of FSH in *S. parahybae*.

The absence of final maturation and ovulation indicates that spawning appears to be strictly modulated by the synthesis and release of β-LH, as observed in other species (Zohar and Mylonas, 2001). In *S. parahybae*, OD values did not differ between previtellogenic and vitellogenic stages, indicating in the latter a lower cellular activity than required to stimulate final oocyte maturation, ovulation and spawning. This OD values were higher in the "AIS" group that reached final oocyte maturation and spawning. As described to FSH, there is no protocol (i.e., specific antibodies) to measure plasma levels of LH in *S. parahybae*, therefore, the development of assays to measure these GtHs in siluriform species plasma can be useful. Furthermore, other preliminary studies in our laboratory are focused on the steroid synthesis pathways and oocyte development (Honji et al., 2012b) throughout the reproductive cycle and after AIS in these females, together with the study of neurohormonal modulation at the brain by gonadotropin-releasing hormone (GnRH) (Honji et al., 2011). In this regard, data about GnRH systems, steroid synthesis pathways and oocyte development must be interpreted together to assemble the puzzle that is reproductive dysfunction in *S. parahybae* females in captivity.

Although several studies have been conducted on the growth hormone family (GH, PRL and SL), most of these investigations have focused only on identification, distribution and characterization of these hormones in the pituitary gland of teleost fish (Agulleiro et al., 2006; Kawauchi and Sower, 2006; Takei and Loretz, 2006, among other studies). Moreover, few studies have analyzed the possible seasonal changes of GH family hormones, especially in non-salmonid species. These studies include seasonal changes of GH (Pérez-Sánchez et al., 1994; Riley et al., 2003), PRL hormone (Figueroa et al., 1994, 1997) or all three members of the GH family (Vargas-Chacoff et al., 2009; Fiszbein et al., 2010); only one study was conducted with a reophilic South American species, *S. hilarii* (Honji et al., 2013a), in which

hormones were compared between wild and captive broodstock females during the annual reproductive cycle. Distribution of GH, PRL and SL in the pituitary gland of *S. parahybae* females was similar to those previously described for other teleosts, and so were the estimated molecular weights for GH family hormones (Berghman et al., 1996; Stefano et al., 1999; de Celis et al., 2004; Pandolfi et al., 2009a, 2001a).

Somatolactin function in teleost fish is not yet fully established, since mode of action depends on the species considered (Kaneko, 1996). Some studies suggested that SL is involved in sexual maturity and smoltification in salmonid species (Rand-Weaver et al., 1992; Bhandari et al., 2003; Onuma et al., 2010); and in adaptation to background and photoperiod changes (Zhu and Thomas, 1997; Ayson and Takemura, 2006); metabolism and acid-base balance (Kakizawa et al., 1993, 1996; Zhu and Thomas, 1995), stress response (Rand-Weaver et al., 1993), and reproductive physiology in non-salmonid species (Mousa and Mousa, 1999, 2000; Honji et al., 2013a). Furthermore, environmental variations, such as photoperiod and temperature changes, can influence the SL profile (Vargas-Chacoff et al., 2009; Fiszbein et al., 2010). In this study, SL cells were PAS-positive and distributed in clusters surrounding the NH. Using the PAS stain method in other teleost fish species has revealed two different SL cell types in the PI region, based on whether they expressed non-glycosylated (PAS-negative) or glycosylated SL (PAS-positive) variants (Rand-Weaver et al., 1991a, 1991b; Kaneko, 1996; Villaplana et al., 1997; Borella et al., 2009; Honji et al., 2013a, and other studies). Even though all SL cells immunostained with anti-seabream antisera in the PI region were PAS-positive and only one ir-band was identified by WB, the presence in *S. parahybae* of both SL variants cannot be ruled out.

Mousa and Mousa (1999, 2000) observed morphometrical variations in SL cells of *Oreochromis niloticus* and *Mugil cephalus* during the reproductive period, with higher synthetic and secretory activity during sexual maturation and spawning, suggesting that SL is involved in some reproductive processes. Furthermore, the larger nuclear area and higher OD observed in vitellogenic *S. hilarii* females (Honji et al., 2013a) also suggests higher cellular activity during the reproductive period in this species. In this sense, during the annual reproductive cycle of *S. parahybae*, SL-producing cells showed a more intense OD at the vitellogenic stage than at the previtellogenic and regression stages, suggesting a possible physiological role of this hormone during the reproductive period in this species as well. After artificial induction, the highest OD was observed, also indicating that SL is related to the reproductive cycle, and to spawning in captivity following induction. Therefore, in agreement with previous studies, our results also suggest that SL may be linked to reproductive physiology in *S. parahybae*.

In *S. parahybae* females, our data also suggest that GH is involved in the reproductive process. Cells expressing GH showed higher OD values at the vitellogenic stage than at the previtellogenic and regression stages, which may be reflecting a higher cellular activity. In addition, in the "AIS" group even higher GH OD values were observed. This pattern of variation in cellular activity during the reproductive cycle was also observed in another South American fish, *S. hilarii* (Honji et al., 2013a), i.e, higher cellular activity during the reproductive period. Vargas-Chacoff et al. (2009), studying gene expression of GH in a *S. aurata* broodstock during the reproductive cycle, found a high profile of expression during the summer (reproductive period) rather than the winter season (non reproductive period). The change in pattern observed by the authors was related with increased feeding rates during summer and/or to reproduction. In addition, in *Odontesthes bonariensis*, GnRH binding sites were identified in cells that expressed GH (Stefano et al., 1999), suggesting a relationship between GnRH and GH, in addition to the well established relationship between GtHs and GnRH. In *S. parahybae* we also identified this relationship between GnRH neuron (Honji et al., in preparation) and GH ir-cells and GtHs cells. Based on these evidences, our results were consistent with a variation of the GH profile during the reproductive cycle of *S. parahybae*.

In teleosts, several studies showed that PRL is mostly related to osmoregulation; particularly it has a role in ion uptake and ion and water permeability pathways in freshwater and euryhaline fish (Manzon, 2002; Sakamoto and McCormick, 2006). Environmental variations in photoperiod and temperature are the main factors that influence PRL gene expression profiles in fish (Manzon, 2002; Sakamoto and McCormick, 2006; Vargas-Chacoff et al., 2009; Onuma et al., 2010). In *Cyprinius carpio* and *C. dimerus* photoperiod was considered an important neuromodulator in the hormonal pathways that activate PRL synthesis and release in the pituitary gland, mainly through modulation of PRL secretion by melatonin (Falcón et al., 2007; Fiszbein et al., 2010). In *S. aurata*, higher PRL gene expression was observed during spring and autumn, the breeding season for this species, and when considerable differences in temperature (up to 8°C) are recorded, when compared to winter, a non breeding season (Vargas-Chacoff et al., 2009). Although *S. parahybae* females were exposed to a natural photoperiod and temperature in captivity, some considerations can be observed: PRL ir-cells showed lower OD and cellular area during winter/autumn (non-breeding season), when temperatures were low (19°C) and days short, when compared to the higher OD and cellular area of animals during summer/spring (breeding season), when temperatures were higher (28°C) and days longer. However, no differences were observed after AIS, suggesting PRL involvement on reproductive status (modulation of synthesis and/or release by environmental changes) but not directly on oocyte final maturation.

In summary, in this study we characterized by IHC and WB methods the different cells types in the ADH of *S. parahybae* females throughout the annual reproductive cycle in captivity. These results could contribute to a better reproductive performance in captivity, since their endangered status deserves special attention and urgent action, especially by increasing the knowledge of their reproductive physiology, which is the basic premise for the program of fish restocking in the Paraíba do Sul River Basin.

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LITERATURE CITED

Agulleiro B, Garcia-Hernández MP, García-Ayala A. 2006. Teleost adenohypophysis: morphofunctional and developmental aspects. In: Reinecke M, Zaccone G, Kappor BG, editors. Fish endocrinology. Science Publishers. p 287–323.

Ayson FG, Takemura A. 2006. Daily expression patterns for mRNAs of GH, PRL, SL, IGF-I and IGF-II in juvenile rabbitfish, *Siganus guttatus*, during 24-h light and dark cycles. Gen Comp Endocrinol 149:261–268.

Batten TF. 1986. Immunocytochemical demonstration of pituitary cell types in the teleost *Poecilia latipinna*, by light and electron microscopy. Gen Comp Endocrinol 63:139–154.

Behmer OA, Tolosa EMC, Neto AGF. 1976. Manual de técnicas para histologia normal e patológica. EDART, São Paulo: Livraria Editora Ltda.

Berghman LR, Lescroart O, Roelants I, Ollevier F, Kühn ER, Verhaert PD, De Loof A, van Leuven F, Vandesande F. 1996. One-step immunoaffinity purification and partial characterization of hypophyseal growth hormone from the African catfish, *Clarias gariepinus* (Burchell). Comp Biochem Physiol 113:773–780.

Bhandari RK, Taniyama S, Kitahashi T, Ando H, Yamauchi K, Zohar Y, Ueda H, Urano A. 2003. Seasonal changes of responses to gonadotropin-releasing hormone analog in expression of growth hormone/prolactin/somatolactin genes in the pituitary of masu salmon. Gen Comp Endocrinol 130:55–63.

Borella MI, Gazola R, Valsella MV, Fava-de-Moraes F. 1997. Histochemical and immunohistochemical study of the pituitary gland of the South-American teleost fish pacu *Piaractus mesopotamicus* (Cypriniformes, Characidae). Braz J Morph Sci 14:219–225.

Borella MI, Venturieri R, Mancera JM. 2009. Immunocytochemical identification of adenohypophyseal cells in the pirarucu (*Arapaima gigas*), an amazonian basal teleost. Fish Physiol Biochem 35:3–16.

Caneppele D, Honji RM, Hilsdorf AWS, Moreira RG. 2009. Induced spawning of the endangered neotropical species *Steindachneridion parahybae* (Siluriformes: Pimelodidae). Neotropic Ichthyol 7: 759–762.

Chapman SC, Sawitzke AL, Campbell DS, Schoenwolf GC. 2005. A three-dimensional atlas of pituitary gland development in the zebrafish. J Comp Neurol 487:428–440.

de Celis SV, Gómez-Requeni P, Pérez-Sánchez J. 2004. Production and characterization of recombinantly derived peptides and antibodies for accurate determinations of somatolactin, growth hormone and insulin-like growth factor-I in european sea bass (*Dicentrarchus labrax*). Gen Comp Endocrinol 139:266–277.

Einarsdóttir IE, Silva N, Power DM, Smáradóttir H, Björnsson BT. 2006. Thyroid and pituitary gland development from hatching through metamorphosis of a teleost flatfish, the atlantic halibut. Anat Embryol 211:47–60.

Falcón J, Besseau L, Sauzet S, Boeuf G. 2007. Melatonin effects on the hypothalamo-pituitary axis in fish. Trends Endocrinol Metab 18:81–88.

Figueroa J, Molina A, Alvarez M, Villanueva J, Reyes A, Léon G, Krauskopf M. 1994. Prolactin gene expression and changes of prolactin pituitary levels during the seasonal acclimatization of the carp. Comp Biochem Physiol 108:551–560.

Figueroa J, Reyes A, Ríos M, Vera MI, Krauskopf M. 1997. Effect of temperature and photoperiod on prolactin transcription in *Cyprinus carpio*. Zool Sci 14:353–357.

Filippi-Chiela EC, Oliveira MM, Jurkovski B, Callegari-Jacques SM, da Silva VD, Lenz G. 2012. Nuclear morphometric analysis (NMA): screening of senescence, apoptosis and nuclear irregularities. PLoS One 7:e42522-

Fiszbein A, Cánepa M, Vázquez GR, Maggese C, Pandolfi M. 2010. Photoperiodic modulation of reproductive physiology and behaviour in the cichlid fish *Cichlasoma dimerus*. Physiol Behav 99: 425–432.

García-Ayala A, García-Hernández MP, Quesada JA, Agulleiro B. 1997. Immunocytochemical and ultrastructural characterization of prolactin, growth hormone, and somatolactin cells from the mediterranean yellowtail (*Seriola dumerilii*, Risso 1810). Anat Rec 247:395–404.

Honji RM, Caneppele D, Hilsdorf AWS, Moreira RG. 2009a. Threatened fishes of the world: *Steindachneridion parahybae* (steindachner, 1877) (Siluriformes: Pimelodidae). Environ Biol Fish 85:207–208.

Honji RM, Caneppele D, Moreira RG. 2013b. Caracterização macroscópica das gônadas durante a reprodução induzida em cativeiro do surubim-do-paraíba. Pesq Agrop Bras 48:1110–1114.

Honji RM, Caneppele D, Pandolfi M, Lo Nostro FL, Moreira RG. 2011. The brain-pituitary axis structure in captivity reared females of *Steindachneridion parahybae* (Siluriformes). In: Proceeding of the 9th International Symposium on Reproductive Physiology of Fish. Vol. 4: p 30–31.

Honji RM, Caneppele D, Pandolfi M, Moreira RG. 2012b. Studies on the brain-pituitary-ovaries axis of *Steindachneridion parahybae* (Siluriformes: Pimelodidae) females when reproductive migration is blocked. In: 7th International Symposium on Fish Endocrinology. Vol. 1: p 161.

Honji RM, Narcizo AM, Borella MI, Romagosa E, Moreira RG. 2009b. Patterns of oocyte development in natural habitat and captive *Salminus hilarii* Valenciennes, 1850 (Teleostei: Characidae). Fish Physiol Biochem 35:109–123.

Honji RM, Nóbrega RH, Pandolfi M, Shimizu A, Borella MI, Moreira RG. 2013a. Immunohistochemical study of pituitary cells in wild and captive *Salminus hilarii* (Characiformes: Characidae) females during the annual reproductive cycle. SpringerPlus 2: 460–474.

Honji RM, Tolussi CE, Mello PH, Caneppele D, Moreira RG. 2012a. Embryonic development and larval stages of *Steindachneridion parahybae* (Siluriformes: Pimelodidae) -implications for the conservation and rearing of this endangered neotropical species. Neotrop Ichthyol 10:313–327.

Honji RM. 2011. Controle do eixo hipotálamo-hipófise-gônadas do surubim do Paraíba *Steindachneridion parahybae* (Siluriformes: Pimelodidae) em relação ao ciclo reprodutivo e à reprodução induzida em cativeiro. Unpublished Ph.D Dissertation, Universidade de São Paulo. São Paulo.

Jesus LWO, Chehade C, Costa FG, Borella MI. 2014. Pituitary gland morphogenesis and ontogeny of adenohypophyseal cells of *Salminus brasiliensis* (Teleostei, Characiformes). Fish Physiol Biochem 40:897–909.

Kakizawa S, Kaneko T, Hasegawa S, Hirano T. 1993. Activation of somatolactin cells in the pituitary of the rainbow trout *Oncorhynchus mykiss* by low environmental calcium. Gen Comp Endocrinol 91:298–306.

Kakizawa S, Kaneko T, Hirano T. 1996. Elevation of plasma somatolactin concentrations during acidosis in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol 199:1043–1051.

Kaneko T. 1996. Cell biology of somatolactin. Int Rev Cytol 169:1–24.

Kasper RS, Shved N, Takahashi A, Reinecke M, Eppler E. 2006. A systematic immunohistochemistry survey of the distribution patterns of GH, prolactin, somatolactin, β-TSH, β-FSH, β-LH, ACTH, and α-MSH in the adenohypophysis of *Oreochromis niloticus*, the nile tilapia. Cell Tissue Res 325:303–313.

Kawauchi H, Sower SA. 2006. The dawn and evolution of hormones in the adenohypophysis. Gen Comp Endocrinol 148:3–14.

Laiz-Carrión R, del Mar Segura-Noguera M, del Pilar Martín del Río M, Mancera JM. 2003. Ontogeny of adenohypophyseal cells in the pituitary of the American shad (*Alosa sapidissima*). Gen Comp Endocrinol 132:454–464.

Levavi-Sivan B, Bogerd J, Mañanós EL, Gómez A, Lareyre JJ. 2010. Perspectives on fish gonadotropins and their receptors. Gen Comp Endocrinol 165:412–437.

Lubzens E, Young G, Bobe J, Cerdà J. 2010. Oogenesis in teleosts: how eggs are formed. Gen Comp Endocrinol 165:367–389.

Manzon LA. 2002. The role of prolactin in fish osmoregulation: a review. Gen Comp Endocrinol 125:291–310.

Maruska KP, Levavi-Sivan B, Biran J, Fernald RD. 2011. Plasticity of the reproductive axis caused by social status change in an African cichlid fish: I. Pituitary gonadotropins. Endocrinology 152: 281–290.

Mousa MA, Mousa SA. 1999. Immunocytochemical study on the localization and distribution of the somatolactin cells in the

pituitary gland and the brain of *Oreochromis niloticus* (Teleostei, Cichlidae). Gen Comp Endocrinol 113:197–211.

Mousa MA, Mousa SA. 2000. Immunocytochemical studies of the gonadotropic cells in the pituitary gland of male mullet, *Mugil cephalus*, during the annual reproductive cycle in both natural habitat and captivity. J Appl Ichthyol 16:98–103.

Mylonas CC, Fostier A, Zanuy S. 2010. Broodstock management and hormonal manipulations of fish reproduction. Gen Comp Endocrinol 165:516–534.

Naito N, De Jesus EG, Nakai Y, Hirano T. 1993. Ontogeny of pituitary cell-types and the hypothalamo-hypophyseal relationship during early development of chum salmon, *Oncorhynchus keta*. Cell Tissue Res 272:429–437.

Onuma TA, Ban M, Makino K, Katsumata H, Hu W, Ando H, Fukuwaka MA, Azumaya T, Urano A. 2010. Changes in gene expression for GH/PRL/SL family hormones in the pituitaries of homing chum salmon during ocean migration through upstream migration. Gen Comp Endocrinol 166:537–548.

Pandolfi M, Cánepa M, Meijide FJ, Alonso F, Vázquez GR, Maggese MC, Vissio PG. 2009b. Studies on the reproductive and developmental biology of *Cichlasoma dimerus* (Perciformes, Cichlidae). Biocell 33:1–18.

Pandolfi M, Lo Nostro FL, Shimizu A, Pozzi AG, Meijide FJ, Vázquez GR, Maggese MC. 2006. Identification of immunoreactive FSH and LH cells in the cichlid fish *Cichlasoma dimerus* during the ontogeny and sexual differentiation. Anat Embryol 211:355–365.

Pandolfi M, Muñoz-Cueto JA, Lo Nostro FL, Downs JL, Paz DA, Maggesse MC, Urbanski HF. 2005. GnRH systems of *Cichlasoma dimerus* (Perciformes, Cichlidae) revisited: a localization study with antibodies and riboprobes to GnRH-associated peptides. Cell Tissue Res 321:219–232.

Pandolfi M, Paz DA, Maggese C, Meijide FJ, Vissio PG. 2001b. Immunocytochemical localization of different cell types in the adenohypophysis of the cichlid fish *Cichlasoma dimerus* (Heckel, 1840). Biocell 25:35–42.

Pandolfi M, Paz DA, Maggese C, Ravaglia M, Vissio PG. 2001a. Ontogeny of immunoreactive somatolactin, prolactin and growth hormone secretory cells in the developing pituitary gland of *Cichlasoma dimerus* (Teleostei, Perciformes). Anat Embryol 203: 461–468.

Pandolfi P, Pozzi A, Cánepa M, Vissio PG, Shimizu A, Maggese MC, Lobo G. 2009a. Presence of β-follicle-stimulating hormone and β-luteinizing hormone transcripts in the brain of *Cichlasoma dimerus* (Perciformes: Cichlidae). Neuroendocrinology 89:27–37.

Pérez-Sánchez J, Marti-Palanca H, Le Bail PY. 1994. Seasonal changes in circulating growth hormone (GH), hepatic GH-binding and plasma insulin-like growth factor-I-immunoreactivity in a marine fish, gilthead sea bream, *Sparus aurata*. Fish Physiol Biochem 13:199–208.

Piazza Y, Pandolfi M, Lo Nostro FL. 2011. Effect of the organochlorine pesticide endolsulfan on GnRH and gonadotrope cell populations in fish larvae. Arch Environ Contam Toxicol 61:300–310.

Quintero-Hunter I, Grier HJ, Muscato M. 1991. Enhancement of histological detail using metanil yellow as counterstain in periodic acid schiff's hematoxylin staining of glycol methacrylate tissue sections. Biotech Histochem 66:169–172.

Ramallo MR, Grober M, Cánepa MM, Morandini L, Pandolfi M. 2012. Arginine-vasotocin expression and participation in reproduction and social behavior in males of the cichlid fish *Cichlasoma dimerus*. Gen Comp Endocrinol 179:221–231.

Ramallo MR, Morandini L, Alonso F, Birba A, Tubert C, Fiszbein A, Pandolfi M. The endocrine regulation of cichlids social and reproductive behavior through the eyes of the chanchita, *Cichlasoma dimerus* (Percomorpha; Cichlidae). J Physiol Paris, 108:194–202.

Rand-Weaver M, Baker BJ, Kawauchi H. 1991b. Cellular localization of somatolactin in the pars intermedia of teleost fishes. Cell Tissue Res 263:207–215.

Rand-Weaver M, Noso T, Muramoto K, Kawauchi H. 1991a. Isolation and characterization of somatolactin, a new protein related to growth hormone and prolactin from atlantic cod (*Gadus morhua*) pituitary glands. Biochemistry 30:1509–1515.

Rand-Weaver M, Pottinger TG, Sumpter JP. 1993. Plasma somatolactin concentrations in salmonid fish are elevated by stress. J Endocrinol 138:509–515.

Rand-Weaver M, Swanson P, Kawauchi H, Dickhoff WW. 1992. Somatolactin, a novel pituitary protein: purification and plasma levels during reproductive maturation of coho salmon. J Endocrinol 133:393–403.

Riley LG, Hirano T, Grau EG. 2003. Effects of transfer from seawater to fresh water on the growth hormone/insulin-like growth factor-I axis and prolactin in tilapia, *Oreochromis mossambicus*. Comp Biochem Physiol 136:647–655.

Rocha MJ, Rocha E. 2006. Morphofunctional aspects of reproduction from synchronous to asynchronous fishes—an overview. In: Reinecke M, Zaccone G, Kappor BG, editors. Fish endocrinology. Science Publishers. p 570–624.

Saga T, Yamaki K, Doi Y, Yoshizuka M. 1999. Chronological study of the appearance of adenohypohysial cells in the aya (*Plecoglossus altivelis*). Anat Embryol 200:469–475.

Sakamoto T, McCormick SD. 2006. Prolactin and growth hormone in fish osmoregulation. Gen Comp Endocrinol 147:24–30.

Sanches EA, Marcos RM, Okawara RY, Caneppele D, Bombardelli RA, Romagosa E. 2013. Sperm motility parameters for *Steindachneridion parahybae* based on open-source software. J Appl Ichthyol 29:1114–1122.

Shimizu A, Sakai T, Nashida K, Honda H. 2003b. Universal antisera for immunocytochemical identification of two different gonadotrophs in acanthopterygian fishes. Fish Physiol Biochem 29:275–287.

Shimizu A, Tanaka H, Kagawa H. 2003a. Immunocytochemical applications of specific antisera raised against synthetic fragment peptides of mummichog GtH subunits: examining seasonal variations of gonadotrophs (FSH cells and LH cells) in the mummichog and applications to other acanthopterygian fishes. Gen Comp Endocrinol 132:35–45.

Smith PD, McLean KJ, Murphy MA, Wilson Y, Murphy M, Turnley AM, Cook MJ. 2005. A brightness-area-product-based protocol for the quantitative assessment of antigen abundance in fluorescent immunohistochemistry. Brain Res Protoc 15:21–29.

Stefano AV, Vissio PG, Paz DA, Somoza GM, Maggese MC, Barrantes GE. 1999. Colocalization of GnRH binding sites with gonadotropin-, somatotropin-, somatolactin-, and prolactin-expressing pituitary cells of the pejerrey, *Odontesthes bonariensis*, in vitro. Gen Comp Endocrinol 116:133–139.

Swanson P, Suzuki K, Kawauchi H, Dickhoff WW. 1991. Isolation and characterization of two coho salmon gonadotropins, GTH I and GTH II. Biol Reprod 44:29–38.

Takei Y, Loretz CA. 2006. Endocrinology. In: Evans DH, Claiborne JB, editors. The physiology of fish. 3rd ed. Taylor & Francis Group. p 271–318.

Theodosiou Z, Kasampalidis IN, Livanos G, Zervakis M, Pitas I, Lyroudia K. 2007. Automated analysis of FISH and immunohistochemistry images: a review. Cytometry 71:439–450.

Tubert C, Lo Nostro F, Villafañe V, Pandolfi M. 2012. Aggressive behavior and reproductive physiology in females of the social cichlid fish *Cichlasoma dimerus*. Physiol Behav 106:193–200.

Vargas-Chacoff L, Astola A, Arjona FJ, Martín del Río MP, García-Cózar F, Mancera JM, Martínez-Rodríguez G. 2009. Gene and protein expression for prolactin, growth hormone and somatolactin in *Sparus aurata*: seasonal variations. Comp Biochem Physiol 153:130–135.

Vazzoler AEAM. 1996. Biologia da reprodução de peixes teleósteos: teoria e prática. Maringá: EDUEM.

Villaplana M, García-Ayala A, Chaves Pozo E, Agulleiro B. 2000. Identification of mammosomatotropes, growth hormone cells and prolactin cells in the pituitary gland of the gilthead sea bream (*Sparus aurata* L., Teleostei) using light immunocytochemical methods: an ontogenetic study. Anat Embryol 202:421–429.

Villaplana M, García-Ayala A, García-Hernández MP, Agulleiro B. 1997. Ontogeny of immunoreactive somatolactin cells in the pituitary of gilthead sea bream (*Sparus aurata* L., Teleostei). Anat Embryol 196:227–234.

Vissio PG, Somoza GM, Maggese C, Paz DA, Strussmann CA. 1997. Structure and cell type distribution in the pituitary

gland of pejerrey *Odontesthes bonariensis*. Fish Sci 63: 64–68.

Volckaert FAM, Mugoyo JWM, Lescroart O, Grisez L, Ollevier F. 1999. Immunohistochemically detected ontogeny of prolactin and growth hormone cells in the African catfish *Clarias gariepinus*. Comp Biochem Physiol 122:423–431.

Zhu Y, Thomas P. 1995. Red drum somatolactin: development of a homologous radioimmumoassay and plasma levels after exposure to stressors of various backgrounds. Gen Comp Endocrinol 99:275–288.

Zhu Y, Thomas P. 1997. Studies on the physiology of somatolactin secretion in red drum and Atlantic croaker. Fish Physiol Biochem 17:271–278.

Zohar Y, Muñoz-Cueto JA, Elizur A, Kah O. 2010. Neuroendocrinology of reproduction in teleost fish. Gen Comp Endocrinol 165: 438–455.

Zohar Y, Mylonas CC. 2001. Endocrine manipulations of spawning in cultured fish: from hormones to genes. Aquaculture 179: 99–136.