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Characterization of the gonadotropin-releasing hormone system in the Neotropical teleost, *Steindachneridion parahybae* during the annual reproductive cycle in captivity

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ABSTRACT

This study evaluated by immunohistochemical and Western blot methods, the distribution of two distinct gonadotropin-releasing hormones (GnRHs), corresponding to catfish GnRH (cfGnRH or GnRH1) and chicken-II GnRH (cGnRH-II or GnRH2), in *Steindachneridion parahybae* females in captivity, focusing these analyses on the reproductive cycle by semi-quantification of optical density (OD). Further, we found that the GnRH neuronal systems co-localized with their respective GnRH-associated peptides (GAPs). A group of neurons immunoreactive (ir) to GnRH1 were identified along the ventral region of the olfactory bulb (vOB) in the telencephalon (vTel) and in the main areas of the diencephalon (especially the medial basal hypothalamus, HBM), including fibers extending into the pituitary gland. In contrast, GnRH2 neurons were confined to the midbrain tegmentum, close to the ventricular surface, without projections to the pituitary gland. Moreover, a cfGAP (GnRH1)-specific band (9 kDa) was identified in the brain and pituitary gland, while a cGAP-II (GnRH2)-specific band (26 kDa) was observed only in the brain extract. During the reproductive cycle, GnRH1-ir presented greater OD values at the vitellogenic and regression stages than at the previtellogenic stage and after artificially induced to spawn. Larger GnRH2-ir neurons were observed during the reproductive cycle, but a higher OD was identified only in the regression stage compared with the other maturation stages. Finally, GnRH1 axons were found to be directed towards the pituitary, and this GnRH type, which is probably the hypophysiotropic form, can contribute to the reproductive dysfunction that occurs in *S. parahybae* females in captivity, whereas GnRH2 may act as a neuromodulator and/or neurotransmitter.

1. Introduction

Reproduction is primarily regulated by the hypothalamic-pituitary-gonadal (HPG) axis. It is well established in the literature that in all vertebrates, the synthesis and release of gonadotropins (GtHs), follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland are modulated by hypothalamic peptides, mainly by gonadotropin-releasing hormone (GnRH) (Zohar et al., 2010; Golan et al., 2014; Honji and Moreira, 2017). Therefore, GnRH is considered as the key player in the modulation of reproduction.

The first fish GnRH variant was described in salmon (Sherwood et al., 1983), and several subsequent studies identified different forms of GnRH in other teleost species (Lethimonier et al., 2004; Kah et al., 2007; Okubo and Nagahama, 2008). At least two different forms have been observed in Siluriformes (Ngamvongchon et al., 1992; Bogerd et al., 1994; Zandbergen et al., 1995; Goos et al., 1997; Dubois et al., 2001) and Salmoniformes (Sherwood et al., 1983). Three different forms of GnRH have also been identified in several other groups of

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teleost species, such as Clupeiformes, Perciformes and Tetraodontiformes (Sherwood and Adams, 2005; Golan et al., 2014; Shahjahan et al., 2014), suggesting that this pattern (three forms) could be prevalent in the teleost group. Additionally, all GnRH neuropeptide originates from prepro-GnRH precursors that share a common structure: the cDNAs contain coding sequences for a signal peptide, a GnRH peptide, a conserved processing tripeptide, and a GnRH-associated peptide (GAP) (González-Martínez et al., 2002a,b). Studies performed with antibodies against GAP showed that a GAP antibody provides much greater specificity than can be achieved using antibodies against the smaller GnRH neuropeptide, as GAP is longer than the GnRH sequence, and decreases the cross-reactivity and false-positive detection observed when using GnRH antibodies (González-Martínez et al., 2002b; Pandolfi et al., 2005; Guilgur et al., 2006). Moreover, using GAP as an antigen, the diversity between different GnRH forms and between fish species is greater than GnRH decapeptide (González-Martínez et al., 2002a,b).

Generally, the distribution of GnRH in the fish brain indicates the existence of two major GnRH systems: a) a system along the ventral forebrain (olfactory bulb, terminal nerve area, ventral telencephalon preoptic area and hypothalamus), expressing different GnRH forms (commonly named GnRH1 and GnRH3); and b) a system in the synencephalon region, which is highly conserved and systematically expresses the GnRH2 form (Fernald and White, 1999). In species that express two forms of GnRH such as catfish (Siluriform group), the ventral forebrain, telencephalic preoptic area and hypothalamus regions express the same form (GnRH1 and GnRH3 are the same form), which is responsible for the modulation/regulation of reproduction (Zohar et al., 2010), while the GnRH2 system plays a possible neuromodulatory function (Amano et al., 1991; Dubois et al., 2001). Studies on fish possessing three GnRH forms (such as Perciformes) have shown that 1) the hypophysiotropic and reproductively relevant form (GnRH1) is expressed by the neuronal population distributed in the ventral telencephalic preoptic area and hypothalamus; 2) there is another neuronal population corresponding to a teleost-specific form (GnRH3 or salmon GnRH - sGnRH), which is expressed by populations distributed in the olfactory bulb and terminal nerve area and regulates sexual behavior, including spawning migration (Sherwood and Adams, 2005) and aggressive behavior (Tubert et al., 2012); and 3) the highly conserved form (GnRH2) (González-Martínez et al., 2001, 2002a,b, 2004), a neuropeptide with a neuromodulatory function (White et al., 1995; Soga et al., 2005), has been described as a melatonin-releasing factor in the pineal gland of teleost fish (Servili et al., 2010). GnRH2 neurons are involved in the feeding-related behavioral functions (Karigo and Oka, 2013), and intracerebroventricular injections with GnRH2 decrease food intake and hypothalamic orexin mRNA expression, even as treatment with orexin stimulate feeding, inhibit spawning behavior, and decreasing brain GnRH2 gene expression, suggesting a coordinated control of feeding and reproduction by the orexin and GnRH system (Hoskins et al., 2008; Volkoff, 2016).

The endangered Neotropical catfish *Steindachneridion parahybae* (Siluriformes: Pimelodidae) is a gonochoristic medium-sized, reophilic fish species (potamodromous), endemic to the Paraíba do Sul River Basin, seriously endangered in this basin and considered extinct in São Paulo State (Honji et al., 2009, 2016, 2017). The reproduction of this species in captivity occurs between November-March, presenting a group-synchronous ovary, and is extended seasonal spawners that undergo multiple-batch ovulations within this period (November-March), *i.e.,* these animals experience multiple spawning events during the annual reproductive cycle (Honji, 2011; Honji et al., 2015, 2016, 2017). Additionally, erstwhile studies of *S. parahybae* female in captivity showed that the maintenance of *S. parahybae* broodstocks is not completely successful because these animals exhibit reproductive dysfunction, involving failure to undergo final oocyte maturation, ovulation and spawning (Caneppele et al., 2009; Honji, 2011; Honji et al., 2011, 2012a,b, 2013a,b, 2015), suggesting the existence of endocrine dysfunction in these animals when reared in captivity. This absence of final maturation and ovulation in *S. parahybae* indicates disruption of the synthesis and/or release of LH (Honji et al., 2015), which is modulated by the GnRH system. Thus, an analysis of this GnRH system would be useful, in addition to the studies on steroid synthesis pathways and oocyte development that have already been performed (Honji et al., 2012b). In this context, data on GnRH systems (present study), gonadotropins and growth hormone family members (Honji et al. 2015), steroid synthesis pathways and oocyte development (preliminary study, Honji et al., 2012b) must be interpreted together to solve the puzzle of the reproductive dysfunction of captive *S. parahybae* females.

The aims of this study were: 1) for the first time in *S. parahybae* adult females, identify the different GnRH forms present in this threatened fish species, as well as the precise location and projections of different GnRH neurons and fibers in the brain and pituitary gland using immunohistochemical (IHC) and Western blot (WB) methods; 2) thus, we evaluate whether these different GnRH forms in *S. parahybae* brain change seasonally during the annual reproductive cycle and after artificial spawning using morphometric analyses and semi-quantification of immunoreactive cells, highlighting the possible relationships with reproductive disorders at the different levels of the HPG axis.

2. Materials and methods

2.1. Fish stocks, rearing conditions, and fish collection

The present study was performed at the *Unidade de Hidrobiologia e Aquicultura da Companhia Energética de São Paulo* (CESP) located in Paraibuna city, São Paulo State, Brazil. In December 2003, the first artificial breeding of wild broodstocks was conducted in captivity, according to the protocol described by Caneppele et al. (2009), and the fingerlings produced (first-generation offspring, F1) were stocked in the fish farm ponds for future broodstock captive rearing. These *S. parahybae* fingerlings (F1) produced were stocked from December 2003 to December 2007 in ponds (200 m^2), fed with commercial extruded feed for carnivorous fish (40% crude protein, TC40), and in early December 2007, after assessing the sexual maturation (according to Caneppele et al. (2009)), one hundred females of *S. parahybae* broodstock (F1) were randomly divided into two ponds (200 m^2) to reproductive cycle characterization. Annual water temperature at CESP fish farm was 21.10 ± 0.14 °C and dissolved oxygen was 7.58 ± 0.36 mg/L.

Four animals were randomly sampled monthly from January 2008 to March 2009, except during the winter time (Southern hemisphere). During this period fish were also fed with the same diet already described. To confirm the maturation stages of ovarian development, histological and gonadosomatic index (GSI) were used to determine the maturity scale. These maturation stages were previously identified by Honji (2011) and Honji et al. (2015). The experimental groups evaluated in the present study through morphometric analysis and semi-quantification of immunoreactive (ir) neurons during the annual reproductive cycle were as follows: fish in previtellogenic (n = 28; May-October; GSI = 0.40 ± 0.14%), vitellogenic (n = 22; November-February; GSI = 1.59 ± 0.24%), and regression (n = 18; March-April; GSI = 0.57 ± 0.09%) stages. As *S. parahybae* broodstock females do not breed naturally 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 artificial spawning using carp pituitary extract was performed in December (Caneppele et al., 2009; Honji et al., 2012a,b, 2013b, 2015). In this experimental group, referred as the "artificially induced spawning" (AIS) group (n = 6; December; 1.61 ± 0.14%), the females were manually stripped for gamete collection and sampled immediately.

Animals sampled in the CESP tanks were 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, and then fish were killed by decapitation at the level of the operculum. Brain and pituitary gland were quickly removed, and then were fixed in Bouin's solution for 24 h (for histological and IHC analyzes), and dehydrated through a series of increasing ethanol concentrations. Samples were cleared in dimethylbenzene solution and embedded in Paraplast® according to routine histological procedures. Serial sections (12 μm thick) were obtained, mounted on Poly-L-Lysine solution-coated slides, and followed for histological or IHC analyzes according to the procedures described below. All procedures used in the sampling of the animals were in agreement with the Animal Ethics Committee of the Institute of Biosciences, University of São Paulo (Protocol 072 /2008).

2.2. Histological analysis of the brain and pituitary

Histological slides of the brain and pituitary were prepared with haematoxylin/eosin, acid haematoxylin, periodic acid-Schiff (PAS) or Masson's trichrome staining and were examined and documented using a computerized image analyzer (Leica light microscope DM1000, Leica photographic camera DFC295 and image capture Leica Application Suite Professional, LAS V3.6). For precise localization of the various brain and nucleus areas, we relied on the detailed atlases of other species, including *Clarias gariepinus* (Zandbergen et al., 1995; Dubois et al., 2001), *Danio rerio* (Wullimann et al., 1996), *Oryzias latipes* (Ishikawa et al., 1999), and *Dicentrarchus labrax* (Cerdá-Reverter et al., 2001a, 2001b, 2008), as well as an atlas that is currently being constructed for *S. parahybae* (Honji et al., in preparation).

2.3. Single immunohistochemical localization and Western blot characterization of GnRH in the brain and pituitary gland

For IHC analysis, tissue sections were immunostained using the Catalyzed Signal Amplification System (CSA Amplification System kit-Code: k1500, Dako) and visualized using 3.3′-diaminobenzidine (DAB) in a chromogen solution and DAB substrate buffer (DAKO kit), following the manufacturer's instructions. The sections were incubated overnight at 4 °C with specific primary antisera (the antisera and dilutions used are detailed in Table 1) (antibody). To confirm the specificity of the IHC reactions, control sections were carried out by: 1) preadsorbed of the primary antisera to GnRH or GAP with 1 μg of corresponding antigen per ml of primary antisera at the working dilution for 24 h before use (Table 1); 2) the primary antibody was replaced with PBS buffer (phosphate buffered saline, pH7.4); 3) by substitution of normal biotinylated secondary antibody in the same dilution as the primary antisera (Table 1). No positive structures or cells were detected in these sections (Supplementary Figure). 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*, Brazil.

To confirm the specificity of heterologous antisera against GAPs (the antisera and dilutions used are detailed in Table 1) in the *S. parahybae* brain and pituitary gland and to estimate the molecular weights of these peptides in this species, some brain (n = 5) and pituitary gland (n = 5) samples were quickly removed, and then were frozen and stored at −80 °C until the analysis. We performed electrophoresis of brain and pituitary homogenates in 15% sodium dodecylsulfate-polyacrylamide gels (SDS – PAGE). After electrophoresis, the proteins and molecular markers (ColorBrustTM Electrophoresis Markers) were transferred to nitrocellulose membranes. These membranes were then incubated with different primary antisera (the antisera and dilutions used are detailed in Table 1), and the reaction was visualized using 3.3′-diaminobenzidine in a chromogen solution and DAB substrate buffer (DAKO kit). Finally, the membranes were dried and digitized (Fig. 1), and the molecular weights of ir-bands were estimated using Image Gauge software (Fuji, Photo Film, version 3.12). As described for the IHC reactions,

Table 1
Primary (including characteristics of antisera) and secondary antisera dilutions used in the immunohistochemical and Western blot methods to gonadotropin-releasing hormone (GnRH). cfGnRH: catfish GnRH; cfGAP: catfish GAP; sGnRH: salmon GnRH; sGAP: salmon GAP; cGnRH-II: chicken-II GnRH; cGAP-II: chicken-II GAP; NP: not performed.

Antiserum raised against	Immunohistochemistry			Western blot			Antibody data			
	Dilution (antisera)	Secondary antibody and dilution	(+) positive (−) negative	Dilution (antisera)	Secondary antibody and dilution	(+) positive (−) negative	Source	Lot or accession (GenBank®) number	References
Clarias gariepinus cfGnRH (1)	1:1000	anti-rabbit 1:600	+	NP	NP	NP	Dr. H. Goos	n°41-2	Schulz et al. (1993)
Clarias gariepinus cfGAP (1)	1:500	anti-rabbit 1:600	+	1:4000	anti-rabbit 1:600	+	Dr. H. Goos	X78049.1*	Bogerd et al. (1994)
Oncorhynchus mykiss sGnRH (3)	1:1000	anti-rabbit 1:600	–	NP	NP	NP	Dr. I. Parhar	Lot. 2	Soga et al. (2005)
Dicentrarchus labrax sGAP (3)	1:500	anti-guinea pig 1:600	–	1:4000	anti-guinea pig 1:600	–	Dr. J.A. Muñoz-Cueto	AAF62898*	González-Martínez et al. (2002a)
Oncorhynchus mykiss cGnRH-II (2)	1:1000	anti-rabbit 1:600	+	NP	NP	NP	Dr. I. Parhar	ISP2	Soga et al. (2005)
Dicentrarchus labrax cGAP-II (2)	1:500	anti-guinea pig 1:600	+	1:4000	anti-guinea pig 1:600	+	Dr. J.A. Muñoz-Cueto	AAF62900*	González-Martínez et al. (2002a)
LRH13 (monoclonal)	1:1500	anti-mouse (kit DAKO)	+	NP	NP	NP	Dr. I. Parhar	LRH13	Park and Wakabayashi (1986)

All the antisera used in this study were kindly provided by Drs: H. Goos (Utrecht University, Utrecht, The Netherlands), K. Wakabayashi (Gunma University, Maebashi, Japan), I. Parhar (Nippon Medical School, Tokyo, Japan), J. Muñoz-Cueto (Universidad de Cádiz, Puerto Real, Spain). To run the preadsorbed test, cfGnRH and cfGAP antigens were kindly provided by Dr. G. Somoza (IIB-INTECH – CONICET-UNSAM); sGnRH and sGAP antigens by Dr. I. Parhar; cGnRH-II and cGAP-II provided by Dr. J. A. Muñoz-Cueto; LRH13 was preadsorbed with mGnRH (mammalian) (Sigma-Aldrich).

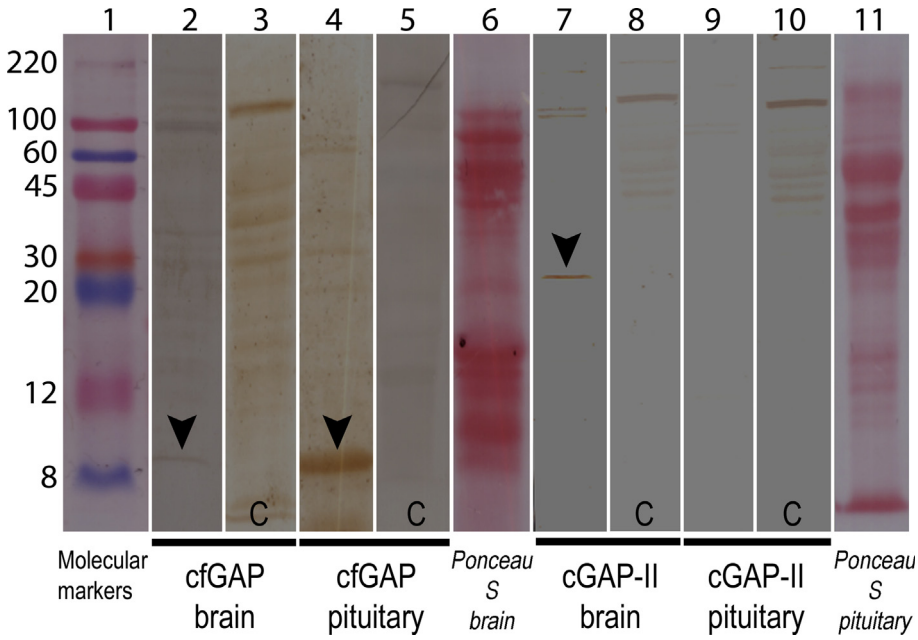

Fig. 1. Western blot analysis of brain and pituitary homogenates of *Steindachneridion parahybae* incubated with primary antisera: catfish gonadotropin-releasing hormone-associated peptide (cfGAP) brain (line 2) and pituitary (line 4); chicken-II gonadotropin-releasing hormone-associated peptide (cGAP-II) brain (line 7) and pituitary (line 9). Control (C) lines (3, 5, 8, 10) incubated with preadsorbed antibodies. Molecular markers (line 1) and *Ponceau S* (line 6 for brain and line 11 for pituitary).

both positive and negative controls were also performed in the WB analysis, and no ir band were detected. Additionally, all WB analyses were performed with brain and pituitary extract samples, along with molecular marker (including the *Ponceau S*) in each batch of WB reactions to further control for differences in marking (WB method and control, with different antibodies were described previously by our research group: Pandolfi et al. (2005); Cánepa et al. (2006); Honji et al. (2015)).

Furthermore, as mentioned before, IHC and WB methods analyzes employed in this study were identical to those described in Honji et al. (2015), with the exceptions reported here.

2.4. Morphometric and statistical analysis

For the morphometrical analysis of GAP ir-neurons in *S. parahybae* during the annual reproductive cycle and after AIS in captivity, the following parameters were considered: optical density (OD) of immunostaining (a.u.) and cellular and nuclear areas. For analysis of the OD of immunostaining, images captured for each sample using the LAS system (1260 pixels by 960 pixels) were analyzed with Image Gauge software. Thus, representative area of this image with ir-neurons was selected and the OD of immunostaining was quantified. Eight ir-neurons with detectable nuclei and cellular areas (μm^2) were analyzed and measured in each sampled animal using the software Image-Pro Plus software (Media Cybernetics). For these analyses, the same images employed for OD measurements were used. To reduce the variability in the results regarding the intensity of immunostaining between tissues processed separately, representative females in all maturation stages and from the AIS group were included in each batch of IHC reactions, to further control of differences in staining. Similar analyses have been performed in other teleost species (Pandolfi et al., 2009; Honji et al., 2013b), including a study examining *S. parahybae* pituitary cells (Honji et al., 2015).

All values were expressed as the mean ± standard error of the mean (M ± SEM). Statistical analysis of data related to the 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 the Dunn's or Tukey's test, for non-parametric or parametric analysis, respectively (Zar, 2010). Statistical differences were considered to be significant when $P < 0.05$. All statistical analyses were performed using the statistical software SigmaStat for Windows (version 3.10).

3. Results

3.1. Histological, immunohistochemical and Western blot analysis of the brain and pituitary gland

Macroscopically and microscopically (data not shown) the major brain regions and nuclei identified in *S. parahybae* were: the most rostral part of the brain is represented by the olfactory bulbs (OBs) and is connected with the telencephalon (Tel) through the medial olfactory tract (TOM). Tel can be divided into two main regions: dorsal and ventral. The preoptic area (POA) of *S. parahybae* extends from the ventral Tel region to the beginning of the diencephalon and surrounds the anterior diencephalic ventricle portion, also known as the third ventricle. Moreover, the main nuclei of the POA were identified: nucleus preopticus periventricularis (NPP) and nucleus anterioris periventricularis (NAP). In the next region, the hypothalamus emerges ventral to the caudal pole of the POA. The hypothalamus is positioned ventral to the thalamus and caudal to the optic region in *S. parahybae* and forms part of the diencephalon. Furthermore, the regions of the diencephalon were identified in the present study: medial basal hypothalamus (HBM), lateral part of the nucleus of the lateral recess (NRLI) and subcommissural organ (SCO). The pituitary gland of *S. parahybae* appeared to be attached to the HBM, being connected by a thin pituitary stalk (Ps). More caudally and dorsally to the HBM, the midbrain tegmentum (MB) was identified, adjacent to the third ventricle.

Seven GnRH antisera were used in this study, but only five recognized the cell bodies and fibers of GnRH-ir neurons in different neuronal areas of the *S. parahybae* brain (Table 1; Figs. 2 and 3). The GnRH1 antibody recognized specific neuron-ir and did not show cross-reactivity with the antigens of any other GnRH forms (Fig. 2a–d). The GnRH2 antibody also recognized specific neuron-ir (Fig. 2e–h) but showed cross-reaction with GnRH1 (Fig. 3a, b). The monoclonal antibody LRH_{13} recognized only neuron-ir in the ventral region of the brain (Fig. 3c, d), the region corresponding to GnRH1. Anti-cfGAP (Fig. 2c, d) and cGAP-II (Fig. 2g, h) similarly co-localized with anti-GnRH1 (Fig. 2a, b) and anti-GnRH2 (Fig. 2e, f) (and did not show a cross-reaction), respectively, and we preferred to use the GAP antisera rather than GnRH antisera. Additionally, sGnRH (Supplementary Figure) and sGAP

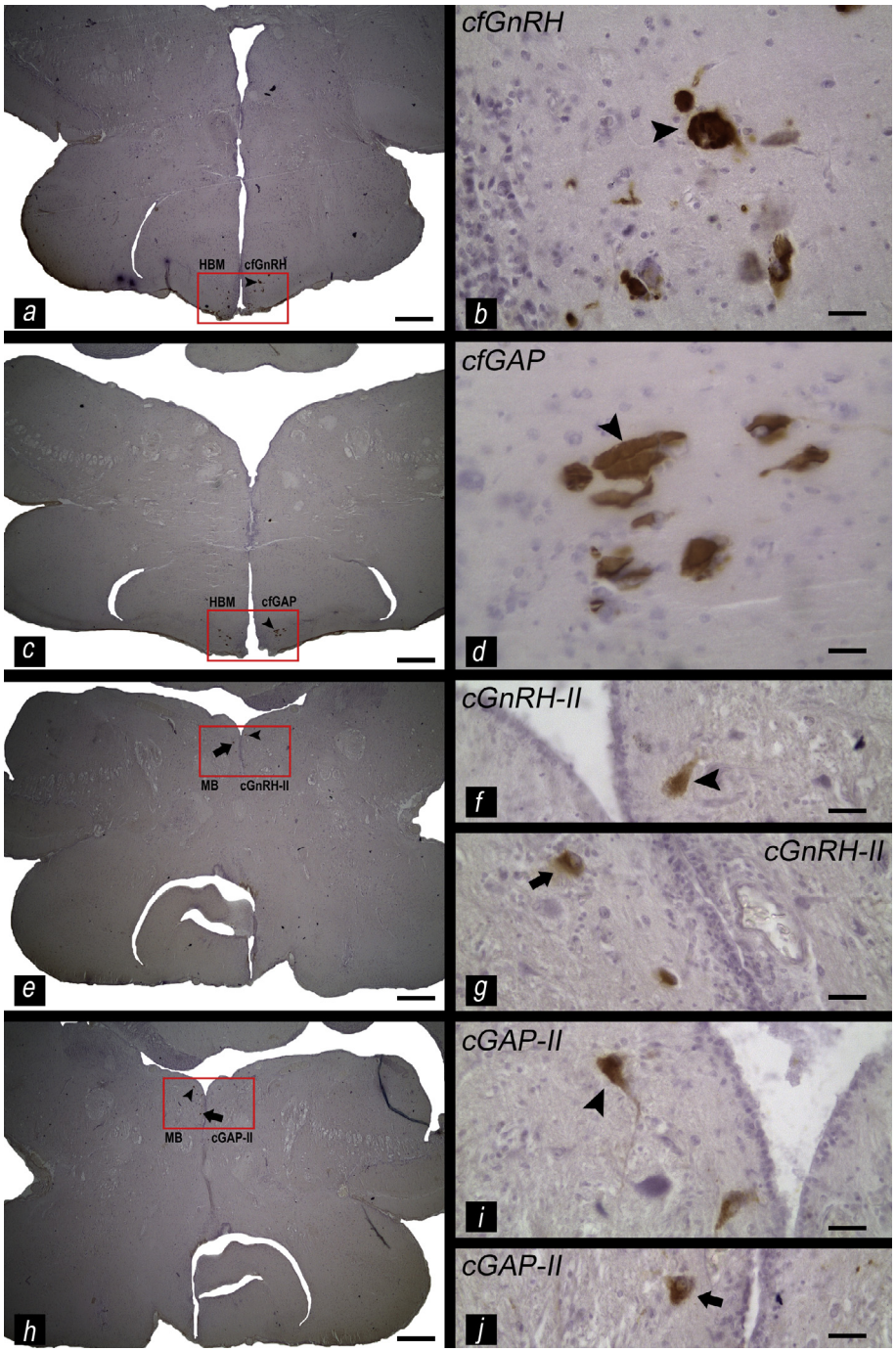

Fig. 2. Transversal sections through the brain of *Steindachneridion parahybae*. a, b) catfish gonadotropin-releasing hormone (cfGnRH) immunoreactive (ir) neurons in the medial basal hypothalamus (HBM) (arrowhead in red square in [a]) and details of cfGnRH-ir (arrowhead in [b]); c, d) catfish gonadotropin-releasing hormone-associated peptide (cfGAP) neurons-ir in the HBM (arrowhead in red square in [c]) and details of cfGAP-ir (arrowhead in [d]); e, f, g) chicken-II gonadotropin-releasing hormone (cGnRH-II) neurons-ir in the midbrain tegmentum (MB) (arrowhead and arrow in red square in [e]) and details of cGnRH-II-ir (arrowhead in [f] and arrow in [g]); h, i, j) chicken-II gonadotropin-releasing hormone-associated peptide (cGAP-II) neurons-ir in the MB (arrowhead and arrow in red square in [h]) and details of cGAP-II-ir (arrowhead in [i] and arrow in [j]). Since the neurons-ir were sparse to cGnRH-II and cGAP-II (respectively [e] and [h]), we only indicated more than one neuron-ir in different images ([f] and [g] to cGnRH-II; [i] and [j] to cGAP-II). Scale bar: 400 μm (a, c, e, g); 30 μm (b, d, f, g, i, j).

(Supplementary Figure) did not recognize any specific GnRH antigen in the *S. parahybae* brain.

In general, GnRH1-ir cell bodies and fibers were identified along the ventral region of the OB, Tel, and POA (NPP and NAP) and in the MBH, including fibers extending into the pituitary gland, while GnRH2-ir neurons were confined to the MB, close to the ventricular surface, without projections to the pituitary gland. The ventral part of the OB, Tel and POA (NPP and NAP) contained small and scattered GnRH1-ir cell bodies (Fig. 4a–d) throughout the reproductive cycle, in contrast to the moderate number of larger GnRH1-ir cell bodies observed in the HBM (Fig. 4e). The GnRH1-ir cell bodies in the Tel and POA regions were smaller in size (Fig. 4d) (cellular area: 16.54 ± 0.32 μm^2; nuclear area: 9.16 ± 0.53 μm^2) than the GnRH1-ir cell bodies in the HMB region (Fig. 4e) (cellular area: 25.11 ± 0.58 μm^2; nuclear area: 12.13 ± 0.45 μm^2) (Table 2) ($P < 0.01$). In the MB (Fig. 4f), a moderate number of larger GnRH2-ir cell bodies (cellular area: 24.02 ± 0.42 μm^2; nuclear area: 9.72 ± 0.28 μm^2) (Table 2) were identified.

GnRH1-ir fibers were observed mainly in the ventral region of Tel (Fig. 5a–d). Strong GnRH1-ir fibers innervation was detected in the TOM (Fig. 5a), which connects the OB with Tel, as well as the optic nerve (NO) (Fig. 5b), optic chiasma (OC), POA, NPP (Fig. 5c) and NAP (Fig. 5d). At the diencephalic level, GnRH1-ir fibers were more evident in the HBM (Fig. 5e), and conspicuous innervation was also observed in the optic tectum (OT) (Fig. 5f). GnRH1-ir fibers were numerous in the HBM and were strongly related to pituitary gland innervation through Ps (Fig. 6a–c). The pituitary gland received a relatively moderate number of GnRH1-ir fibers (Fig. 6c, d), mainly at the proximal pars

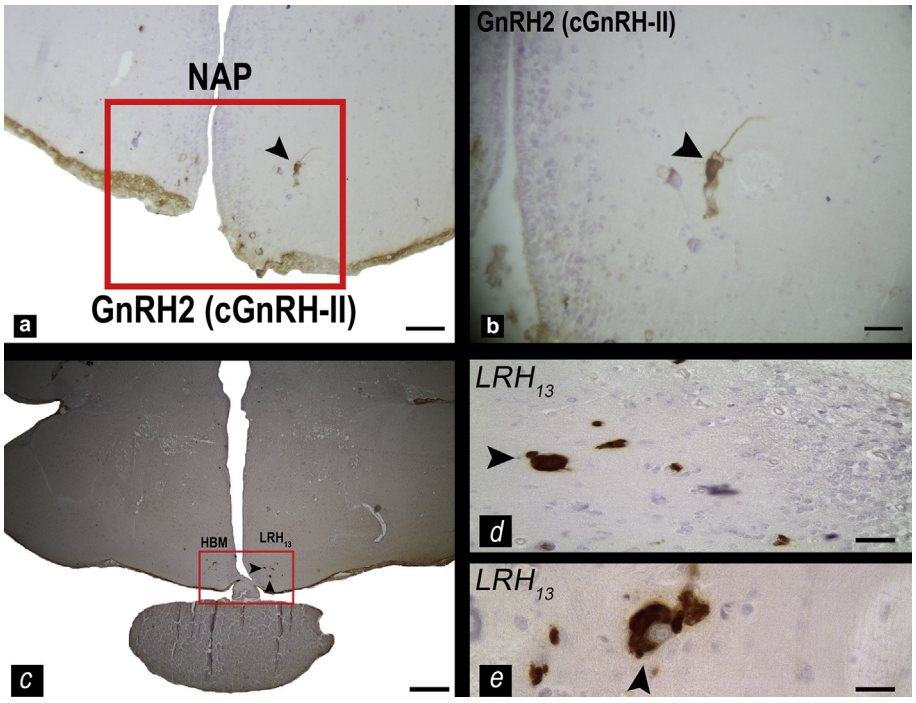

Fig. 3. Transversal sections through the brain of *Steindachneridion parahybae*. a, b) Cross-reaction of chicken-II gonadotropin-releasing hormone (cGnRH-II) antisera immunoreactive (ir) neurons with catfish gonadotropin-releasing hormone (cfGnRH) in the nucleus anterioris periventricularis (NAP) (arrowhead in red square in [a]) and details of cGnRH-II antisera neurons-ir in the same place of cfGnRH neurons (arrowhead in [b]); c, d, e) gonadotropin-releasing hormone (GnRH) neurons-ir using monoclonal antibody LRH_{13} in the medial basal hypothalamus (HBM) (arrowhead in red square in [c]) and details of GnRH-ir (arrowhead in [d] and [e]). Neurons-ir with LRH_{13} corresponding to cfGnRH. Scale bar: 400 μm (a, c); 30 μm (b, d, e).

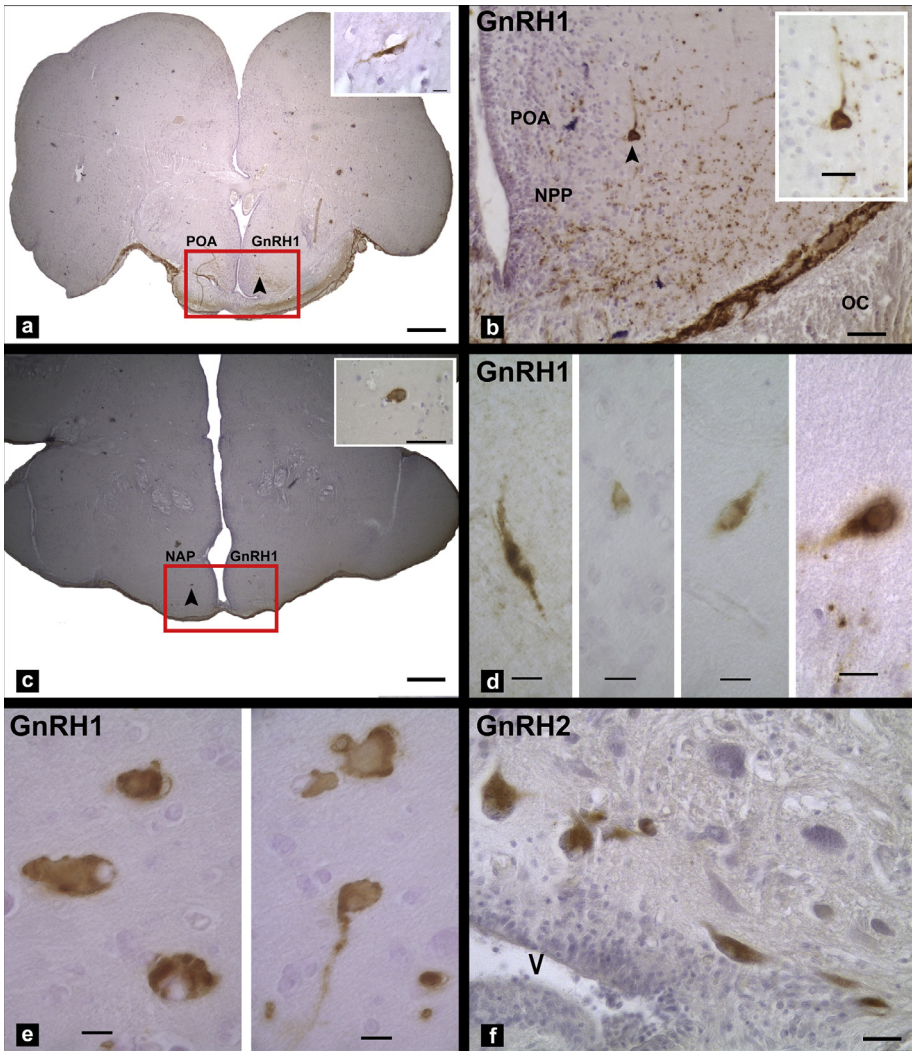

Fig. 4. Transversal sections through the brain of *Steindachneridion parahybae*. a-e) Brain section shows the presence of positive immunoreactive (ir) neurons in the preoptic area (POA) (arrowhead in red square in [a] and neuron-ir in superior detail) and in two main nuclei of POA, nucleus preopticus periventricularis (NPP) (arrowhead in [b] and neuron-ir in superior detail) and nucleus anterioris periventricularis (NAP) (arrowhead in red square in [c] and neuron-ir in superior detail) using catfish gonadotropin-releasing hormone-associated peptide (cfGAP/GnRH1). Anterior region of the brain (NPP and NAP nuclei) contained small and scattered GnRH1-ir cells bodies (detail in [d]) unlike the moderate number and larger GnRH1-ir cells bodies (detail in [e]) in the medial basal hypothalamus; f) midbrain tegmentum shows moderate number and larger with chicken-II gonadotropin-releasing hormone-associated peptide (cGAP-II/GnRH2) next to ventricle (V). Scale bar: 400 μm (a, c); 100 μm (b); 30 μm (e, f); 10 μm (d; and all insets in a, b, c).

Table 2
Average of cellular and nuclear area and number of gonadotropin-releasing hormone (GnRH) immunoreactive neurons in various brain zones (GnRH1 = catfish GnRH; GnRH2 = chicken-II GnRH). The values expressed the average area (μm^2) ± standard error or the mean (M ± SEM). Tel: telencephalon; HBM: medial basal hypothalamus; MB: midbrain; NE: no expression.

Brain zones	GnRH1 (n = 8 neurons/animal)		GnRH2 (n = 8 neurons/animal)	
	Cellular area n = 8	Nuclear área n = 8	Cellular area n = 8	Nuclear area n = 8
Anterior (Tel)	16.54 ± 0.32*	9.16 ± 0.53*	NE	NE
Posterior (HB-M)	25.11 ± 0.58**	12.13 ± 0.45**	NE	NE
Posterior (MB)	NE	NE	24.02 ± 0.42	9.72 ± 0.28

Values followed by different symbols (*, **) are significantly different between brain zones ($P < 0.05$).

distalis (PPD) (Fig. 6d). GnRH2-ir fibers were abundant in the TO and MB, adjacent to the third ventricle (Fig. 6e, f, respectively). No GnRH2-ir fibers were detected in the pituitary gland of *S. parahybae*.

Fig. 1 summarizes the results obtained via WB to confirm the specificity of immunostaining using heterologous antisera against cfGAP, sGAP and cGAP-II in the *S. parahybae* brain and pituitary gland. The estimated molecular weights of these peptides and their locations were as follows: cfGAP (9 kDa) was found in the brain and pituitary; a cGAP-II (26 kDa)-specific band was identified only in the brain; and no specific band for sGAP was recognized (data not shown). This specificity was reinforced by the absence of immunostaining and cross-reactivity observed under both the WB and IHC methods when the primary antibodies were incubated with an excess of GAP antigens (for cfGAP and cGAP-II). The brain distribution patterns of different GnRH bodies, based on the expression of the various GAPs, are summarized in Fig. 7.

3.2. Semiquantitative analysis of the optical density of staining and cellular and nuclear areas

The highest concentration of GnRH1-ir neurons was found in the HBM region, which is larger than the ventral part of the OB, Tel and POA. Only these neurons were considered in the semiquantitative analysis (OD, cellular and nuclear areas) during the annual reproductive cycle and will be discussed below.

Fig. 8 and Table 3 summarize the results obtained via semiquantitative analysis. The GnRH1 OD values were lower in the previtellogenic stage (124.86 ± 2.06 a.u.) than the vitellogenic stage (163.32 ± 3.13 a.u.) ($P < 0.01$) and remained high in regression stages (156.12 ± 4.32 a.u.) ($P < 0.01$). In the AIS group, the GnRH1 OD value (118.80 ± 2.91 a.u.) was lower (27.26%) compared with the vitellogenic and regression stages ($P < 0.01$) (Fig. 8a). The GnRH2 OD values showed little variation throughout the reproductive cycle

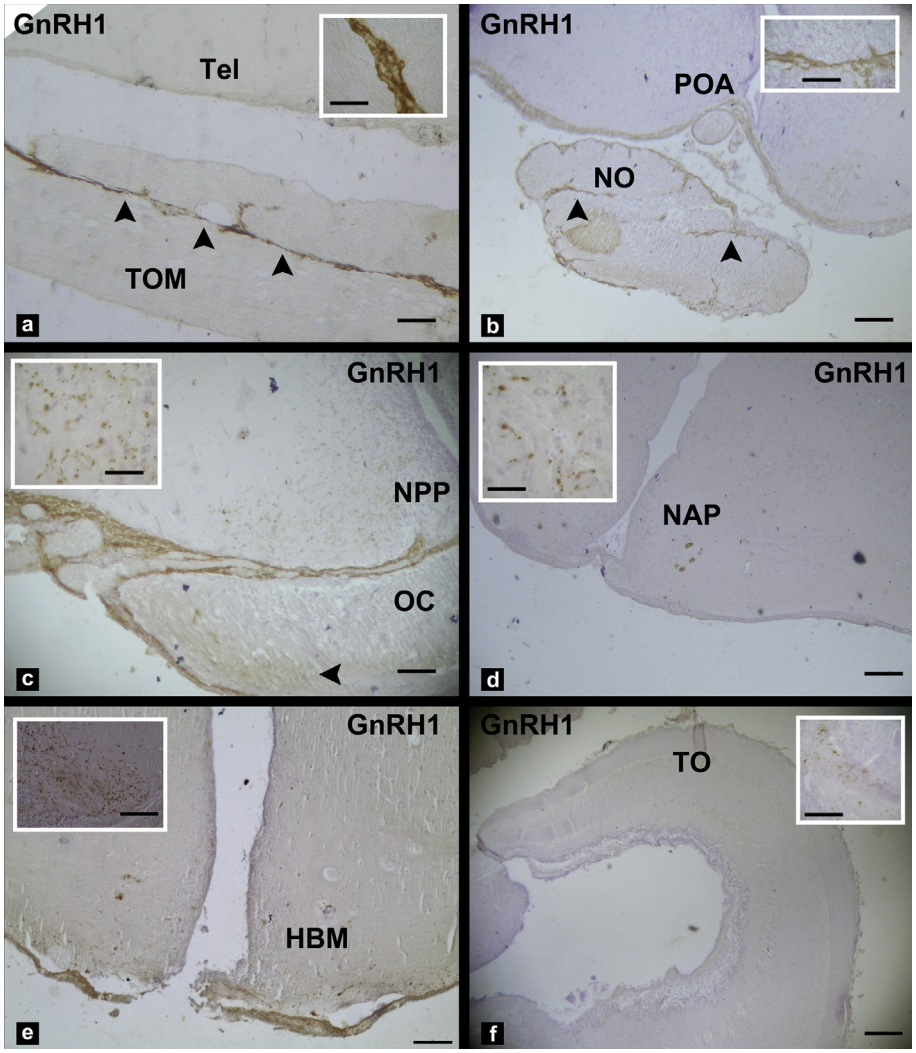

Fig. 5. Transversal and sagittal sections through the brain of *Steindachneridion parahybae*. a–f) Distribution of fibers immunoreactive (ir) with catfish gonadotropin-releasing hormone-associated peptide (cfGAP/GnRH1) antisera. a) details of strong fibers-ir along the medial olfactory tract (TOM) below the telencephalon (Tel) (aspect of fibers-ir in inset); b–d) main nuclei of preoptic area (POA): [b] optic nerve (NO) (arrowhead and superior detail of fibers-ir in inset); [c] nucleus preopticus periventricularis (NPP) and optic chiasma (OC) (arrowhead and superior detail of fibers-ir in inset); [d] nucleus anterioris periventricularis (NAP) (superior detail of fibers-ir in inset); e) strong and several fibers-ir lengthways in the medial basal hypothalamus (HBM) (superior detail of fibers-ir in inset); f) some fibers-ir showed present in the optic tectum (OT) (superior detail of fibers-ir in inset). Scale bar: 400 μm (a–f); 30 μm ([a–f] in insets).

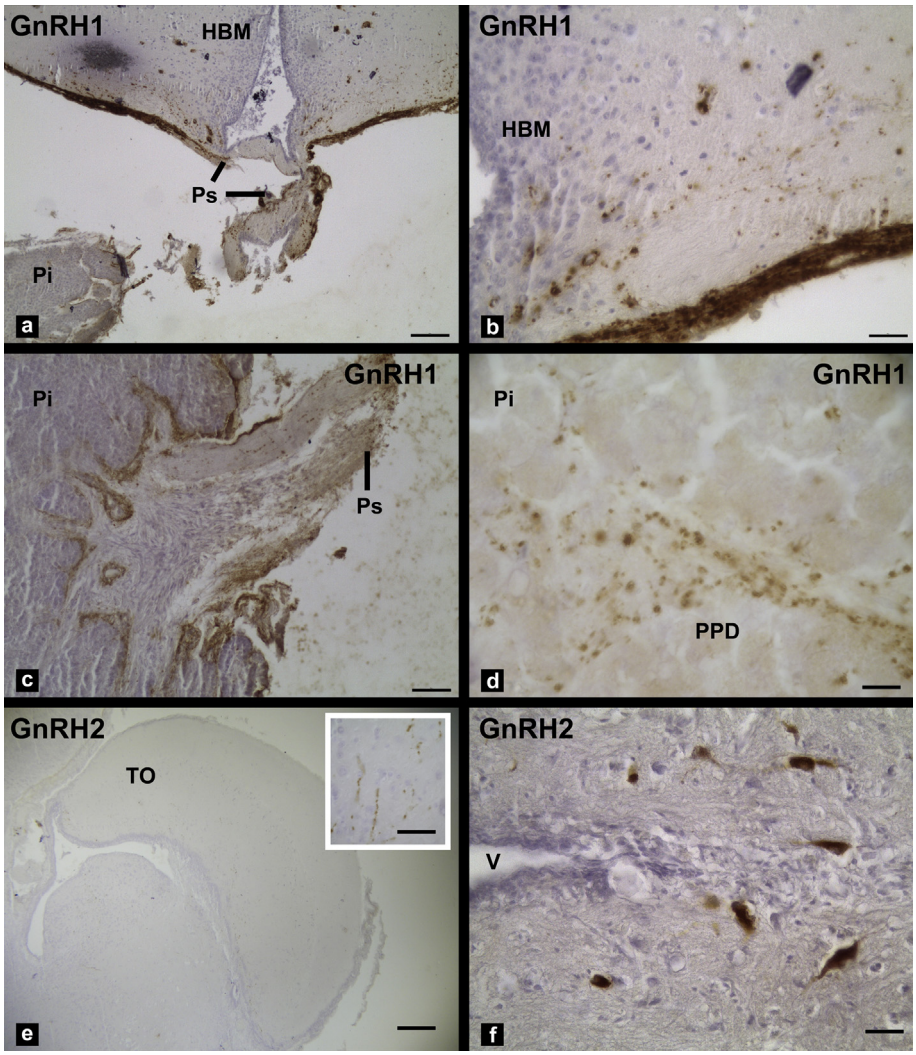

Fig. 6. Transversal sections through the brain and pituitary of *Steindachneridion parahybae*. a–d) distribution of fibers immunoreactive (ir) with catfish gonadotropin-releasing hormone-associated peptide (cfGAP/GnRH1) antisera; a) details of strong and several fibers-ir lengthways to GnRH1 in the medial basal hypothalamus (HBM and detail in [b]), pituitary stalk (Ps and detail in [c]), pituitary gland (pituitary and detail in [c]), and in the proximal pars distalis (PPD and detail in [d]) of pituitary gland subdivision; e–f) distribution of fibers-ir with chicken-II gonadotropin-releasing hormone-associated peptide (cGAP-II/GnRH2); e) details of several fibers-ir to GnRH2 in the optic tectum (OT and superior detail in [e]) and [f] midbrain region next to ventricle (V). Scale bar: 400 μm (a, e); 100 μm (b, c); 30 μm (d, f, and inset in [e]).

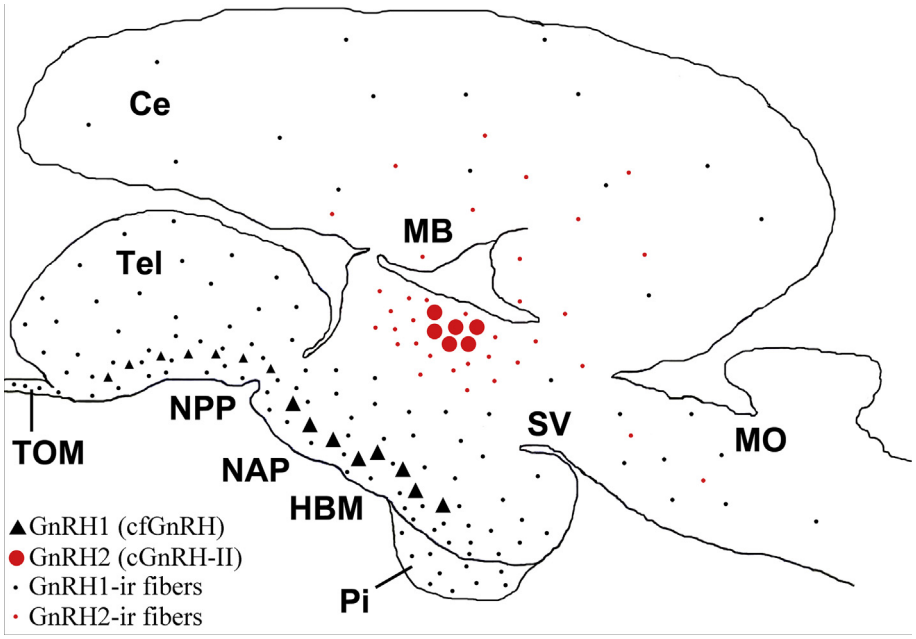

Fig. 7. Sagittal drawing of *Steindachneridion parahybae* brain summarizing: distribution of small catfish gonadotropin-releasing hormone (cfGnRH/GnRH1) neurons (small triangles) immunoreactive (ir) in the anterior region than larger cfGnRH/GnRH1 neurouns-ir (larger triangles) in the posterior region and chicken-II gonadotropin releasing hormone (cGnRH-II/GnRH2) (larger red circles) in the midbrain region. Distribution of major immunoreactive fibers for GnRH1 (black circles) and GnRH2 (small red circles) in different brain regions of *S. parahybae*. Ce, cerebellum; HBM, medial basal hypothalamus; MB, midbrain; MO, medulla oblongata; NAP, nucleus anterioris periventricularis; NPP, nucleus preopticus periventricularis; Pi, pituitary gland; SV, saccus vasculosus; Tel, telencephalum; TOM, medial olfactory tract. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

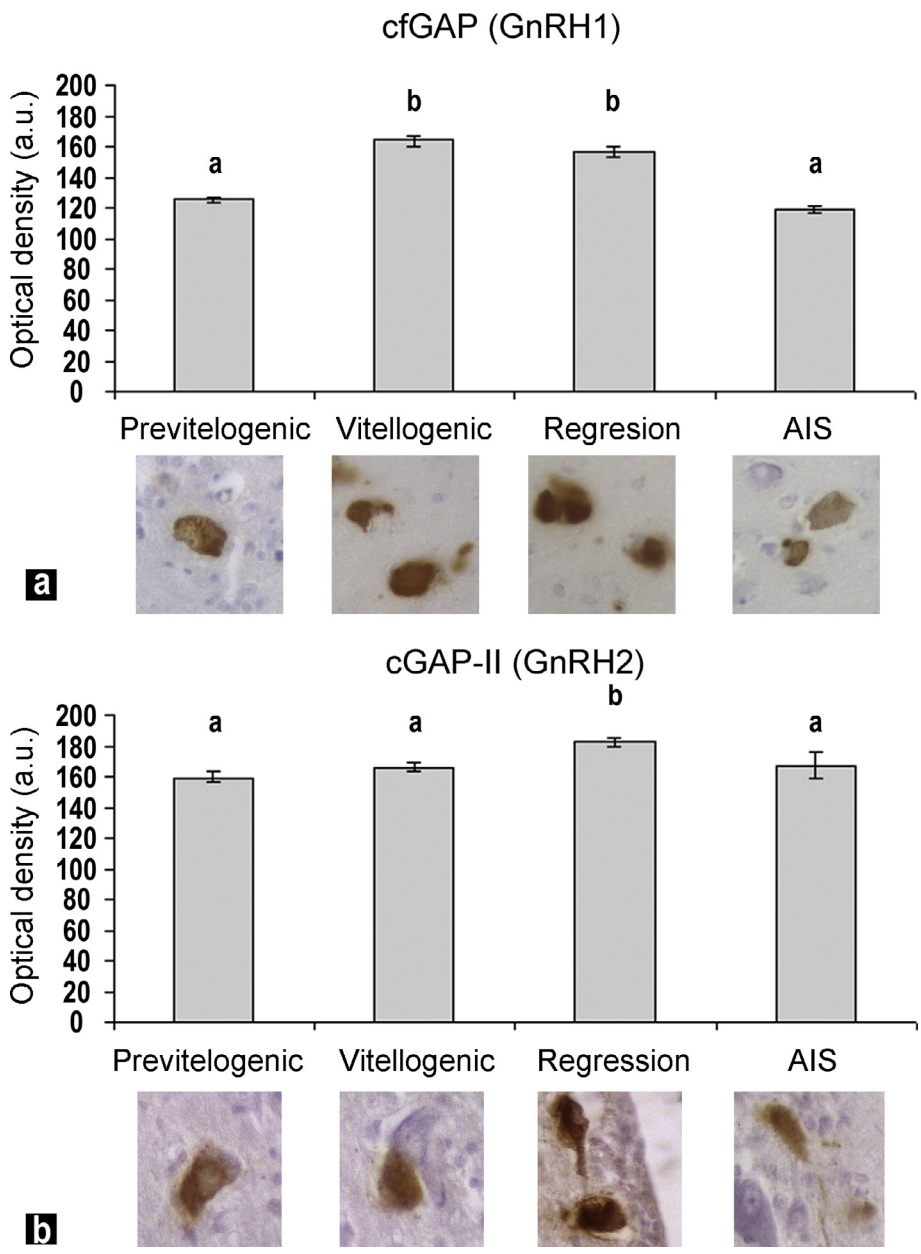

Fig. 8. Semiquantitative analysis (optical density, a.u.) of immunohistochemistry of the gonadotropin-releasing hormone of *Steindachneridion parahybae* brain during annual reproductive cycle and after the artificial induction spawning (AIS): catfish gonadotropin-releasing hormone-associated peptide (cfGAP/GnRH1) antisera (a) and chicken-II gonadotropin-releasing hormone-associated peptide (cGAP-II/GnRH2) (b). Values followed by different letters (a, b) are significantly different during the reproductive cycle ($P < 0.05$).

Table 3
Average of cellular and nuclear area of gonadotropin-releasing hormone (GnRH) immunoreactive neurons (GnRH1 = catfish GnRH; GnRH2 = chicken-II GnRH) during annual reproductive cycle and after artificial induced to spawning (AIS). The values expressed the average area (μm^2) ± standard error or the mean (M ± SEM).

Ovarian maturation stages	GnRH1 (n = 8 neurons/animal)		GnRH2 (n = 8 neurons/animal)	
	Cellular area	Nuclear area	Cellular area	Nuclear area
Previtellogenic	25.30 ± 0.99	11.62 ± 1.15	24.53 ± 0.99	9.84 ± 0.23
Vitellogenic	26.04 ± 1.53	14.43 ± 1.33	22.66 ± 0.84	9.60 ± 0.53
Regression	23.44 ± 1.20	11.41 ± 0.95	23.73 ± 0.66	10.63 ± 1.51
AIS	24.90 ± 5.65	11.98 ± 0.44	25.77 ± 0.88	9.24 ± 0.58

(Fig. 8b). The GnRH2 OD value was higher in the regression stage (181.62 ± 2.50 a.u.) ($P < 0.01$) compared with the other stages (previtellogenic: 158.89 ± 3.31 a.u.; vitellogenic: 165.76 ± 2.75 a.u.; and AIS group: 167.04 ± 4.82 a.u.) (Fig. 8b).

The cellular and nuclear areas corresponding to GnRH1 did not differ during the annual reproductive cycle of *S. parahybae*

(previtellogenic stage: 25.30 ± 0.99 μm and 11.62 ± 1.15 μm; vitellogenic stage: 26.04 ± 1.53 μm and 14.43 ± 1.33 μm; regression stage: 23.44 ± 1.20 μm and 11.41 ± 0.95 μm; AIS group: 24.90 ± 5.65 μm and 11.98 ± 0.58 μm, respectively) (Table 3). The same finding was obtained for GnRH2 (previtellogenic stage: 24.53 ± 0.99 μm and 9.84 ± 0.23 μm; vitellogenic stage: 22.66 ± 0.84 μm and 9.60 ± 0.53 μm; regression stage: 23.73 ± 0.66 μm and 10.63 ± 1.51 μm; AIS group: 25.77 ± 0.88 μm and 9.24 ± 0.58 μm, respectively) (Table 3). It is important to note that the same results were obtained when we used cfGnRH-ir or cfGAP-ir and cGnRH-II-ir or cGAP-II-ir in these morphological analyses.

4. Discussion

The microscopic description of the brain nuclei was not the purpose of the present study, but it was necessary for the correct localization of the GnRH in *S. parahybae*, and to an atlas is currently being constructed for *S. parahybae* (Honji et al., in preparation). Two different forms of GnRH neurons were identified in *S. parahybae*: (1) one group of small neurons ir to the GnRH1 antibody was identified along the ventral region of the vOB and vTel (similar to GnRH3 in species with three different form), and another group ir to large GnRH1 was found in the main areas of the diencephalon, especially in the POA region and HBM, differing only in terms of the cell body size of GnRH neurons, including fibers extending into the pituitary gland (responsible for the modulation/regulation of reproduction); and (2) large GnRH2-ir neurons were confined to the midbrain tegmentum, close to the ventricular surface, without projections to the pituitary gland (representing the conserved GnRH in vertebrates). These results corroborate previous IHC findings indicating that Siluriformes species only possess two different GnRH forms (Subhedar and Rama-Krishna, 1988; Bogerd et al., 1992, 1994; Ngamvongchon et al., 1992; Schulz et al., 1993; Zandbergen et al., 1995; Dubois et al., 2001), including two populations of GnRH1 neurons (an anterior population of smaller neurons and another larger population in the posterior region) and a GnRH2 system (Dubois et al. 2001). The sGnRH (GnRH3) antibody did not recognize any specific neurons in *S. parahybae*, and this form has also not been observed in any other catfish species (Sherwood and Adams, 2005; Kah et al., 2007; Okubo and Nagahama, 2008).

Moreover, the LRH_{13} antibody (monoclonal) used in the present study recognized only one type of GnRH in the ventral Tel region, POA and HBM, the same location identified for GnRH1 (cfGnRH), and did not recognize any neurons in the MB region (GnRH2). These results corroborate the data obtained by Park and Wakabayashi (1986), who reported that this antibody binds with to GnRH neurons in the forebrain region with higher affinity and to GnRH neurons in the MB region with lower affinity. In contrast, the LRH_{13} monoclonal antibody recognizes three GnRH forms in *C. dimerus* adults and larvae (Pandolfi et al., 2002) and *O. bonariensis* larvae (Miranda et al., 2003), including GnRH2 and GnRH3. Additionally, adjacent sections of the *S. parahybae* brain were immunostained with LRH_{13} + GnRH3, GnRH3 (sGnRH), LRH_{13} + GnRH1 and GnRH1 (cfGnRH) antibodies, and positive GnRH-ir neurons and fibers were recognized only by the LRH_{13} + GnRH1 and GnRH1 (cfGnRH) antibodies. These data further support the hypothesis that the *S. parahybae* brain contains only two different forms of GnRH.

As catfish anti-GAP and chicken-II anti-GAP were similarly localized to anti-GnRH1 and anti-GnRH2, respectively, in the *S. parahybae* brain, we preferred to use GAP antibodies as an alternative to GnRH antibodies, because GAPs are oligopeptides, are more divergent, and show lower homology among different forms compared with decapeptide GnRHs (González-Martínez et al., 2002a,b; Guilgur et al., 2007; Zohar et al., 2010). These molecular characteristics of different GAPs confer greater specificity compared with different GnRH types, suggesting that these GAPs are valuable tools for identifying the distinct GnRH form, since they avoid the cross-reaction observed when GnRH antibodies are used. Such cross-reactions are often observed in IHC reactions (Zandbergen et al., 1995; Dubois et al., 2001; González-Martínez et al., 2001, 2002a, 2002b; Pandolfi et al., 2005) and were also observed in the present study when we employed anti-GnRH antibodies in the *S. parahybae* brain but were not detected when anti-GAP antibodies were employed. Additionally, sGAP did not recognize any specific GnRH-ir neurons, which were also not detected using sGnRH (GnRH3).

Positive GnRH1-ir fibers were observed to project widely over different brain areas and to run through the pituitary gland. In contrast, GnRH2-ir fibers did not reach the pituitary gland. The GnRH1 fiber pathways extending to the pituitary gland represent the main source of pituitary innervation (via neurohypophysis) in *S. parahybae*, and a significant number of GnRH1-ir fibers have also been observed in the PPD, adjacent to GtH cells. This result corroborates physiological evidence suggesting a main role for GnRH1 (cfGnRH) in stimulation of the secretion of GtHs in siluriform species. Nevertheless, the specific physiological functions and sites of GnRH2 projections of still unclear. Recently, Servili et al. (2010) demonstrated the presence of GnRH2 receptors in pineal gland cells of *D. labrax*, providing the first evidence in a vertebrate species supporting the role of GnRH2 in modulating the functions of this gland, probably in melatonin secretion. Moreover, in several teleost species, it has been suggested that extra-hypothalamic areas of GnRH2 act as neuromodulators, especially in relation to reproductive performance (Amano et al., 2002; Miranda et al., 2003).

Beyond the main role of GnRH1 in the stimulation of GtH secretion, previously reported data concerning the characterization of GtHs and growth hormone family members in *S. parahybae* (Honji et al., 2015) suggest that in addition to reaching GtH cells in the PPD region, GnRH1-ir fibers also project to growth hormone (GH) cells. This possibility has been investigated in some studies, in which the results indicate that GnRH modulates GH, either stimulating (Lin et al., 1993; Melamed et al., 1996) or inhibiting GH (Bosma et al., 1997; Holloway and Leatherland, 1998). Thus, further mechanistic studies (e.g., evaluation of gene expression and GnRH receptors in GH-expressing cells) are necessary to establish whether GnRH1 (cfGnRH) is involved in GH synthesis in *S. parahybae*, reinforcing this concept of GnRH as a neuropeptide that control different pituitary hormones.

The specificity of heterologous the cfGAP (*C. gariepinus*) and cGAP-II (*D. labrax*) antibodies in the *S. parahybae* brain and pituitary gland was clear, as the absence of immunoblotting in WB lanes with an excess of antigen reinforced the assumption of the specificity of this reaction. A weak cfGAP band was detected in the *S. parahybae* brain compared with the pituitary gland. This weak band in the brain was probably due to the presence of many proteins, which resulted in many nonspecific bands, unlike the pituitary gland, which contains only a small amount of protein (compared with the brain), and the result of WB was “cleaner”. Furthermore, cfGAP has not been previously studied in catfish species via WB; therefore, no available data exist in the specific literature for comparison with the molecular weight observed in this study. For cGAP-II, a clear specific band was detected only in the *S. parahybae* brain, suggesting that GnRH2 did not reach the pituitary gland, as found in other siluriform species (Zandbergen et al., 1995; Dubois et al., 2001). In addition, molecular weights similar to that observed in *S. parahybae* have been identified in other teleosts, such as, *C. dimerus* (34 kDa) (Pandolfi et al., 2002), *D. labrax* (34 kDa) (Gonzalez-Martinez et al., 2002a). Therefore, SDS-PAGE added IHC results, it is clear that cfGnRH (GnRH1) is the physiologically relevant isoform related to GtH modulation in siluriform species, unlike GnRH2 that does not reach the pituitary gland, acting as a neuromodulator in *S. parahybae*.

In *S. parahybae*, the posterior GnRH1 (cfGnRH) and restricted GnRH2 neurons were larger compared with the anterior GnRH1 neurons. This variation in GnRH cell diameter has also been observed in other teleost species, such as *C. gariepinus*: anterior cfGnRH (mean diameter of 7 μm), posterior cfGnRH (16 μm in length and 8 μm in height), and GnRH2 (mean diameter between 25 and 40 μm) (Dubois et al., 2001); *D. labrax*, 10–25 μm (anterior GnRH3), 15–23 μm

(GnRH2), and 5–20 μm (posterior GnRH1) (González-Martínez et al., 2002a); and *C. dimerus*, 5–20 μm (anterior GnRH3), 4–17 μm (posterior GnRH1), and 21 μm (GnRH2) (Pandolfi et al., 2005). According to Grober et al. (1994), the sexual maturation stage and/or transitions between animal life cycle stages (youth/adult) could explain the variations in the diameter of neurons. On the other hand, since we only use adult animals (and from the same breeding batch), it is not possible to discuss this variation in GnRH cell diameter, and further experimental studies are required to answer this question.

The examination of changes in cellular morphology, together with relative optical density, is a useful tool for assessing antigen abundance among different patterns of antigen localization (Smith et al., 2005; Theodosiou et al., 2007); such changes could occur under different physiological conditions. These methods are valid for evaluating pituitary hormones during the annual reproductive cycle of *S. parahybae* (Honji et al., 2015) and were applied here to evaluate the changes in GnRH neurons. GnRH1 showed higher OD values in the vitellogenic stage compared with the previtellogenic stage, which coincided with increased gonadosomatic index of *S. parahybae* (Honji et al., 2015), and the observation that the axons of GnRH1 neurons project to the pituitary gland suggests that GnRH1 modulates the synthesis and release of GtHs (FSH and LH). Vitellogenesis is likely controlled by FSH, and GnRH1 is likely synthesized and released in sufficient amounts to modulate FSH, thus promoting vitellogenesis (Honji et al., 2015); these phenomena display a close relationship with the seasonality of ovarian development of *S. parahybae* (Honji et al., 2012b). Similar results were found in *Paralichthys olivaceus*, in which GnRH increases during early maturation, confirming that the HPG axis is required for this physiological process (Pham et al., 2006). Additionally, vitellogenesis appears to progress normally in cultured *S. parahybae* females, but oocytes fail to undergo final maturation, resulting in neither ovulation nor spawning (Caneppele et al., 2009; Honji et al., 2012a,b, 2013a,b, 2015). These reproductive disorders have been shown to be due to dysfunctions in the GtH (mainly LH) synthesis and/or release pathways from the pituitary gland. Nevertheless, the fact that the secretion of GtHs is regulated by GnRH shows the relevance of studying the AIS group.

The higher OD values of GnRH1 observed during vitellogenic and regression stages, in contrast to the AIS group, suggest that the impairment of final oocyte maturation and, consequently, ovulation and spawning in this species is due to a failure of GnRH1 release, together with the lack of other physiological modulators, such as kisspeptin (van Arle et al., 2008; Zohar et al., 2010) or feedback sex steroids (Colledge, 2008; Zohar et al., 2010), among other physiological neurohormones and neuromodulators that influence GnRH neurons (Zohar and Mylonas, 2001; Mylonas et al., 2010; Zohar et al., 2010). Additionally, the present study showed that the higher OD values of GnRH1 detected during the vitellogenic stage and, likely, the accumulation of this GnRH during the regression stage may be affected LH release from the pituitary gland (Honji et al., 2015), causing a failure of 17α,20β-dihydroxy-4-pregnen-3-one (MIS) synthesis by the ovaries, preventing the final oocyte maturation, ovulation, and spawning when these animals are in captivity (Honji et al., in preparation). High GnRH1 values are also found in *Paralichthys olivaceus* at the end of the reproductive period, when the animals are in regression (Pham et al., 2006). Similar results have been obtained in *Pagrus major* (Senthilkumaran et al., 1999), *Scophthalmus maximus* (Anderson et al., 2001), and *Sebastes rastrelliger* (Collins et al., 2001).

Regarding GnRH2, higher OD values were observed only in the regression stage, suggesting a physiological role during this ovarian maturation stage. In *C. auratus*, it has been demonstrated that GnRH2 can increase DNA fragmentation (apoptosis) in mature testes (Andreau-Vieyra and Habibi, 2001; Andreau-Vieyra et al., 2005); further suggesting that this GnRH form plays an autocrine and/or paracrine role during testicular development. The GnRH2 form likely has a similar function during ovarian development in *S. parahybae*, since the regression stage is characterized by many atretic oocytes, indicating absorption and/or apoptosis (Honji et al., in preparation). Several previous studies have suggested the presence of GnRH in the gonads of fish (von Schalburg et al., 1999; Gray et al., 2002; Sherwood and Adams, 2005), as shown in *S. parahybae* females via IHC methods (data not published). Although studies with GnRH gene expression have been extensively done, the OD of immunostain and morphometric analysis (nuclear and cellular diameter) used in the present study, are also widely used, and increasingly improved accuracy of these analyses has been developed by the use of automated image analysis methods (Smith et al., 2005; Theodosiou et al., 2007; Filippi-Chiela et al., 2012; Tubert et al., 2012; Ramallo et al. 2012). GnRH2 has also been implicated in the regulation of feeding behavior, acting as an anorexigenic factor in *C. auratus* (Hoskins et al., 2008) and *D. rerio* (Nishiguchi et al., 2012). In *C. auratus*, this action is due to the orexin inhibition of the GnRH system pathways (Hoskins et al., 2008). Already to *D. rerio,* GnRH2 administration act on food intake, suggesting that this GnRH suppresses food consumption (Nishiguchi et al., 2012).

In summary, the results obtained in this study clearly demonstrated that the *S. parahybae* brain system presents two different GnRH forms (GnRH1 or cfGnRH and GnRH2 or cGnRH-II) and that GnRH1 neurons and fibers are intimately related to Pi regulation, while GnRH2 neurons and fibers (which do not innervate the pituitary gland) are probably related to neuromodulation and/or reproductive behavior in this species. Moreover, the colocalizations of cfGAP with cfGnRH and cGAP-II with GnRH2 emphasize that these GAPs are valuable tools for studies on the GnRH system of any species, even in phylogenetically distant taxa. Additionally, the changes in OD values during the reproductive cycle and after artificial induction of spawning suggest that when *S. parahybae* females are transferred to fish farms, they exhibit endocrine reproductive dysfunction, especially in terms of GnRH1 modulation of pituitary, and consequently dysfunctions in LH synthesis or release (Honji et al., 2015), culminating in failure of final oocyte maturation, ovulation and spawning. Furthermore, these results can be useful tools for fulfilling the proposed scheme for understanding the physiological basis of the reproductive disorders exhibited by many migratory fish (rheophilic species) when they are transferred to a fish farm.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2018.05.007.

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