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Effects of GnRH and the dual regulatory actions of GnIH in the pituitary explants and brain slices of *Astyanax altiparanae* males

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ABSTRACT

The pituitary gonadotropins, Fsh (follicle-stimulating hormone) and Lh (luteinizing hormone), regulate testicular development and functions in all vertebrates. At the pituitary, different signaling systems regulate the synthesis and secretion of the gonadotropins, such as the hypothalamic neuropeptides GnRH (gonadotropin-releasing hormone) and GnIH (gonadotropin-inhibitory hormone). While GnRH exerts stimulatory roles, the actions of GnIH remain controversial for many teleost species. Therefore, the aim of this study was to evaluate the *in vitro* effects of chicken GnRH2 (cGnRH2) and zebrafish GnIH-3 (zGnIH-3) on the male gonadotropin and GnRH system expression using pituitary explants and brain slices from a neotropical species with economical and ecological relevance, *Astyanax altiparanae*. Our results showed that in males, cGnRH2 increased *fshb* and *lhb* mRNA levels in the pituitary explants. Interestingly, zGnIH-3 has no effect on basal gonadotropin expression, however zGnIH-3 decreased the cGnRH2-induced *fshb* and *lhb* transcripts in male pituitary explants. In the male brain slices, zGnIH-3 showed stimulatory effects, increasing *gnrh2* mRNA levels. Overall, our results suggested that GnIH seems to have dual regulatory actions on gonadotropin and GnRH2 expression of *A. altiparanae* males. This study provided basic information on endocrine regulation of *A. altiparanae* reproduction, and the obtained results will expand our knowledge, improving the reproductive management of this economically important freshwater species.

1. Introduction

The reproductive process in vertebrates is derived from the close interactive relationship between environment (photoperiod, temperature, salinity, nutritional status, stress, among others) and internal physiological clues. In fish, similar to mammals, the brain-pituitary-gonadal (BPG) axis synchronously exerts overall control over the gonadal development and function (Nagahama, 1994; Schulz et al., 2001). The gonadotropin-releasing hormone (GnRH) secreted by preoptic hypothalamic neurons stimulates the synthesis and release of two gonadotropins, follicle-stimulating hormone (Fsh) and luteinizing hormone (Lh) (Nagahama, 1994). GnRH, first isolated in pigs (Matsuo et al., 1971) and sheeps (Amoss et al., 1971), is a neuropeptide composed of ten amino acid residues with different isoforms. Originally, the different isoforms were named according to the species from which they were isolated. Nevertheless, due to the increasing number of forms described, a phylogenetic classification has been adopted to distinguish them based on their location and function. Thus, three forms have been established: GnRH1, GnRH2, and GnRH3, where the latter one is exclusive to almost all teleosts (White and Fernald, 1998).

For many years, it was believed that GnRH was the only neuropeptide involved on gonadotropin synthesis and release. In 2000, however, Tsutsui and collaborators discovered a novel hypothalamic peptide which inhibited the gonadotropin release in Japanese quail. This peptide belongs to the LPXRFamide (X = L or Q) family and it was named as gonadotropin-inhibitory hormone (GnIH) (Tsutsui et al., 2000). Further studies have identified GnIH orthologs in mammalian species and showed that GnIH could also inhibit gonadotropin release

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in these species (Kriegsfeld et al., 2006; Ubuka et al., 2012). In most of the vertebrates, the cDNA sequence that encodes the GnIH precursor polypeptide encompasses three peptides that have LPXRFamide or LPXRFamide-like sequences at the C termini (see review at Muñoz-Cueto et al., 2017). In teleost fish, it has been suggested three or two LPXRFamide or LPXRFamide-like peptides (Muñoz-Cueto et al., 2017). However, the mature GnIH in fish is known only for goldfish, and this mature peptide aligns to the human LPXRFamide-related peptide 3 (RFRP-3) (Sawada et al., 2002). With regards to GnIH physiological roles in fish, there are conflicting observations with respect to its actions on gonadotropin release and reproduction. For example, GnIH exerts stimulatory effects on gonadotropin release in sockeye salmon pituitary cells (Amano et al., 2006), tilapia (Biran et al., 2014) and goldfish (Moussavi et al., 2012, 2013). On the other hand, GnIH inhibited Fsh and Lh release in species, such as goldfish (Moussavi et al., 2013; Zhang et al., 2010), neotropical cichlid (Di Yorio et al., 2016), cinnamon clownfish (Choi et al., 2016) and sea bass (Paullada-Salmerón et al., 2016). According to Munõz-Cueto and collaborators (2017), these conflicting observations can be partially attributed to the endogenous GnIH which were not used in most of the studies, in addition to the nature of GnIH effects, which seems to be dependent on the species, sex, physiological status, concentration, route and time elapsed after administration. Gonadotropins (Fsh and Lh) are glycoproteins with 30–50 KDa chains composed of two different subunits: the alpha (α) subunit, which is common to both gonadotropins, and the beta (β) subunit, which is hormone-specific, and determines its biological activity (Schulz et al., 2001). Each subunit (Gpha, Fshβ and Lhβ) is encoded by a distinct gene (Levavi-Sivan et al., 2010). The role of gonadotropins and their endocrine regulation are still unknown for most of the Neotropical species, in particular for males. Therefore, studies to investigate the role of gonadotropins and their regulation are fundamental to improve our understanding on the hormonal mechanisms involved in the reproductive process of these species.

Astyanax altiparanae is a South American teleost species which is native to the Upper Paraná basin in Brazil. It belongs to the class Actinopterygii, order Characiformes, family Characidae (Garutti and Britski, 2000), and it is known locally as the lambari-do-rabo-amarelo (Nakatani et al., 2001). *A. altiparanae* is a fish whose traits include easy reproduction, good survival rate for larvae and juveniles, and rapid growth (Porto-Foresti et al., 2005). For these reasons, the species is of substantial commercial importance (Porto-Foresti et al., 2005). It also serves as a common study model in many topics of research (Adolfi et al., 2015; Camargo et al., 2017; Cassel et al., 2017; Chehade et al., 2015; Costa et al., 2014; Gomes et al., 2013; Jesus et al., 2017; Pereira-Santos et al., 2016; Siqueira-Silva et al., 2015; Yasui et al., 2015). In this context, the aim of this study is to evaluate the effects of chicken GnRH2 (cGnRH2) and zebrafish GnIH-3 (zGnIH-3) on *A. altiparanae fshb*, *lhb*, *gnrh2* and *gnrh3* expression using pituitary explants and brain slices from males. The cGnRH2 peptide was used in this study because it is the most conserved and ancient form of GnRH. Moreover, cGnRH2 seems to be more potent in inducing gonadotropin release in many species of teleosts (Chang et al., 1990; Goos et al., 1997; Habibi et al., 1992; Zohar et al., 1995), including *A. altiparanae* (Chehade et al., unpublished results). zGnIH-3 (or LPXRFa-3) was chosen because LPXRFa-3 is the mature peptide by mass spectrometry in goldfish brain (Sawada et al., 2002), and *in vivo* experiments demonstrated that zGnIH-3 was able to reduce the serum Lh levels in goldfish (Zhang et al., 2010).

2. Material and methods

2.1. Animals

The specimens used in this study were obtained from commercial stocks and kept at the animal facility of the Reproductive and Molecular Biology Group, in the Department of Morphology, Institute of Biosciences of São Paulo State University (UNESP), Botucatu, São Paulo. The animals were acclimated in round 250 L tanks for 40 days before the experiments. For the species identification, genomic DNA extracted from fins was used for COI gene sequencing and DNA amplification protocols. The sequences obtained were analyzed in the Geneious software and compared against the NCBI database. This step was performed in the Fish Genetics and Biology Group, in the Department of Morphology, Institute of Biosciences of UNESP Botucatu. Animal housing and experimentations were consistent with Brazilian national regulations and were approved by São Paulo State University (IBB-UNESP) animal use and care committee (1030-CEUA-IBB/UNESP).

2.2. Histology

The reproductive phase of each animal was assessed by histological analysis of their gonads in all experiments. The animals were anesthetized with 0.1% benzocaine solution and their gonads were collected and fixed overnight in Karnovsky (4% paraformaldehyde and 2% glutaraldehyde in a Sorensen phosphate buffer, 0.1 M at pH 7.2). The testes were embedded in historesin (Leica), sectioned with 3 μm thickness using a Leica Surgipath DB80 LS microtome blade, stained with hematoxylin and eosin and analysed using an image capture system attached to a Leica DMA 4000B photomicroscope.

2.3. Pituitary explant standardization

The *in vitro* assessment of gonadotropin expression was performed using a pituitary explant system adapted from Cánepa et al. (2008). The animals (males: n = 24; females: n = 24) were anesthetized in 0.1% benzocaine solution, and their pituitary glands were dissected out and washed rapidly in a saline solution. Each pituitary gland was cultivated in a 96-well plate containing 200 μL per well of Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 0,01 M HEPES (Merck, Darmstadt, Germany), 0.5% w/v bovine serum albumin fraction V (Roche, Mannheim, Germany), 0.25 μg/mL amphotericine B (Fungizone; Invitrogen), 200 U/ml penicillin (Invitrogen), and 200 ug/ml streptomycin (Invitrogen); pH was adjusted to 7.4. To evaluate whether the culture conditions could affect the basal gonadotropin expression and pituitary morphology, the pituitary glands were kept in the culture up to 72 h at 26 °C; medium was replaced every 24 h. The pituitaries were collected at time zero (immediately before the pituitary glands were placed in the culture medium), and after 24, 48, and 72 h of cultivation. After each period, the pituitary glands (n = 3 per period) were snap-frozen in liquid nitrogen and kept at −80 °C until total RNA extraction. Some pituitaries were also collected for histological analysis.

2.4. Medium-term effects of cGnRH2 and zGnIH-3 on gonadotropin and GnRH expression

To study the medium-term *in vitro* effects of cGnRH2 and zGnIH-3 on gonadotropin and GnRH expression, five males in the same reproductive phase were used per treatment. The males were anesthetized in 0.1% benzocaine solution, and their brains and pituitary glands were dissected out and washed rapidly in a saline solution. For the brains, we isolated the forebrain, identified the hypothalamus at the bottom of the forebrain, and sliced coronally, ensuring that each slice included the hypothalamus and the optic lobes (tectum). Each pituitary gland and each brain slice were cultivated in Leibovitz's L-15 supplemented either in the absence or presence of hormonal treatment.

The chicken GnRH (cGnRH2) was obtained from Bachem (H-4288) and zebrafish GnIH-3 (zGnIH-3 SGTGPSATLPQRFa or zf LPXRFa-3) was synthesized at the peptide services center of the University of Calgary (Calgary, AB, Canada). The treatments for the pituitaries were as follows: (1) negative control with only the L-15 medium; (2) cGnRH2 (10, 100, 1000 nM); and (3) zGnIH-3 (10, 100, 1000 nM); (4) co-treatment

with cGnRH2 (100 nM) and zGnIH-3 (100 nM). In the co-treatment, the chosen concentration for cGnRH2 was based on its stimulatory effects on gonadotropin expression (see Results), while the one for zGnIH-3 was based on Moussavi et al. (2012). Brain slices were cultivated as follows: (1) negative control with only the L-15 medium; (2) cGnRH2 (100 nM); (3) zGnIH-3 (100 nM); and (4) co-treatment with cGnRH2 (100 nM) and zGnIH-3 (100 nM). The chosen concentrations for cGnRH2 and zGnIH-3 were based on its stimulatory and inhibitory effects on gonadotropin expression, respectively.

Each pituitary gland and brain slice were cultivated for 12 h at 26 °C. After this period, tissue were collected for gene expression analysis, as described below. The period of incubation for the pituitary explants (12 h) was based in our previous experiments (see 2.3 Pituitary Explant Standardization), which showed no changes on gonadotropin expression up to 48 h of incubation when compared to the time zero. Moreover, previous studies with goldfish primary pituitary cell culture used 12 h of incubation and showed stimulatory/inhibitory actions of GnIH peptide (Moussavi et al., 2012). Therefore, we decided to use a medium-term exposure (12 h) to evaluate the acute effects (< 24 h) of cGnRH2 and zGnIH-3 in pituitaries and brain slices from *A. altiparanae* males. For the brain slices, we did not perform any standardization experiment, however previous studies in fathead minnow (*Pimephales promelas*) have standardized these culture conditions, showing high tissue viability until 48 h of culture (Johnston et al., 2016).

2.5. RNA extraction and RT-qPCR

The total RNA of each pituitary gland was extracted using the PureLink® RNA Mini Kit (Code 12183018A), while total RNA of each brain slice was extracted using Trizol. The concentration (ng/μl) was evaluated using a NanoDrop One spectrophotometer (ThermoFisher). The samples obtained were treated with DNase in order to eliminate genomic DNA using Invitrogen DNase I Amplification Grade following standard procedures. After verifying RNA integrity, cDNA was synthesized using random hexamers and Superscript II Reverse Transcriptase Kit (ThermoFisher) according to the manufacturer's instructions.

To evaluate gonadotropin and GnRH expression, real-time quantitative polymerase chain reaction (RT-qPCR) was performed. Forward and reverse primers (Supplementary Table 1) were designed according to specific sequences of *A. altiparanae fshb*, *lhb, elfa1* (Jesus et al., 2017) and *gnrh* (Chehade et al., unpublished results). Specificity and validation for *fshb*, *lhb* and *elfa1* primers are provided by Jesus et al. (2017) (Supplementary Table 1). Data concerning the validation of *gnrh2* and *gnrh3* primers are presented in the Supplementary Table 1. The 10 μL reactions were performed using SYBR Green (Applied Biosystem), 1.125 nM for each of the primers (forward and reverse) and 100 ng of total cDNA. The elongation factor 1 alpha (*ef1a*) was used as endogenous reference gene. The Cts were determined using a StepOnePlus Real-Time PCR System (Applied Biosystems). The reactions underwent to 10 min at 95 °C (Holding stage), 15 s at 95 °C followed by 1 min at 60 °C (40 denaturation cycles – Cycling stage), followed by a melting curve from 60 °C to 95 °C. The relative mRNA levels of the target genes were determined using the $2^{-(\Delta\Delta CT)}$ method, in which the expression of the studied genes were normalized using *ef1a* as the reference gene and subsequently calibrated to the expression values of the negative control group.

2.6. Statistical analyses

The results were expressed as mean ± standard error (SE). The differences between two groups were identified using Student's unpaired *t*-test ($p < 0.05$). Comparisons between the means (more than two groups) were performed using one-way ANOVA, followed by Student-Newman-Keuls method ($p < 0.05$). The GraphPad Prism software, version 4.0 was used for all of the statistical analyses.

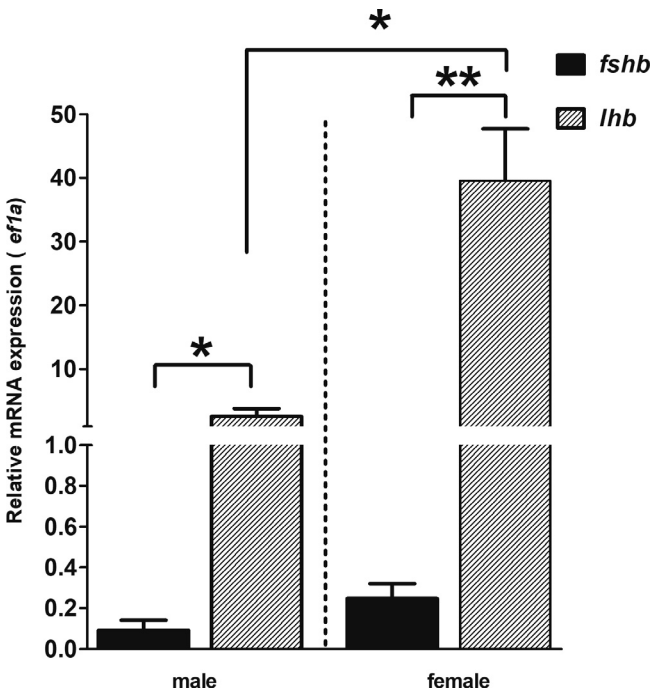

Fig. 1. Relative expression of *A. altiparanae fshb* and *lhb* in pituitaries of males and females. The males were in the developing phase of the reproductive cycle, while females were in the spawning capable phase. The mRNA levels of *fshb* and *lhb* were normalized with *ef1a* (*elongation factor 1α*) and expressed as relative values. The asterisks denote significant differences among the groups, where $^{*}p < 0.05$ and $^{**}p < 0.01$. Values are expressed as mean ± standard error.

3. Results

3.1. Pituitary explant standardization

First we evaluated whether the culture conditions could affect the basal *fshb* and *lhb* expression over the period of incubation. We compared the *fshb* and *lhb* mRNA levels between males and females immediately before the pituitary glands were placed in the culture medium (Fig. 1). In this analysis, although males and females were at different stages of the reproductive cycle (see below), *lhb* was always more expressed than *fshb*, and *lhb* mRNA levels were ~50 times higher in females than males (Fig. 1).

The temporal analysis of *fshb* and *lhb* under basal culture conditions revealed that mRNA levels for both gonadotropins remained constant over the period with tendency toward decrease after 72 hculture (Fig. 2A, B). A temporal analysis of *fshb* expression in females was also performed, and no significant difference in its expression over time was found (Fig. 2C). On the other hand, there was a significant decrease of mRNA levels of *lhb* in all culture periods when compared to time zero (Fig. 2D). Histological analysis of pituitary gland under culture conditions confirmed that, even after 72 h in the culture medium, the tissue maintained its standard structural characteristics; the adenohypophysis and neurohypophysis were preserved (Fig. 3A).

For the pituitary explant standardization experiments, all animals were at the same stage of the reproductive cycle (terminology adopted according to Brown-Peterson et al., 2011). The males were at the developing phase of the reproductive cycle, which is characterized by the presence of a continuous germinal epithelium (GE) and cysts at several stages of spermatogenesis (Fig. 3B). The females were at spawning capable phase (Brown-Peterson et al., 2011), exhibiting ovaries with few oocytes at primary growth stage and many vitellogenic oocytes, including full-grown oocytes (Supplementary Fig. 1).

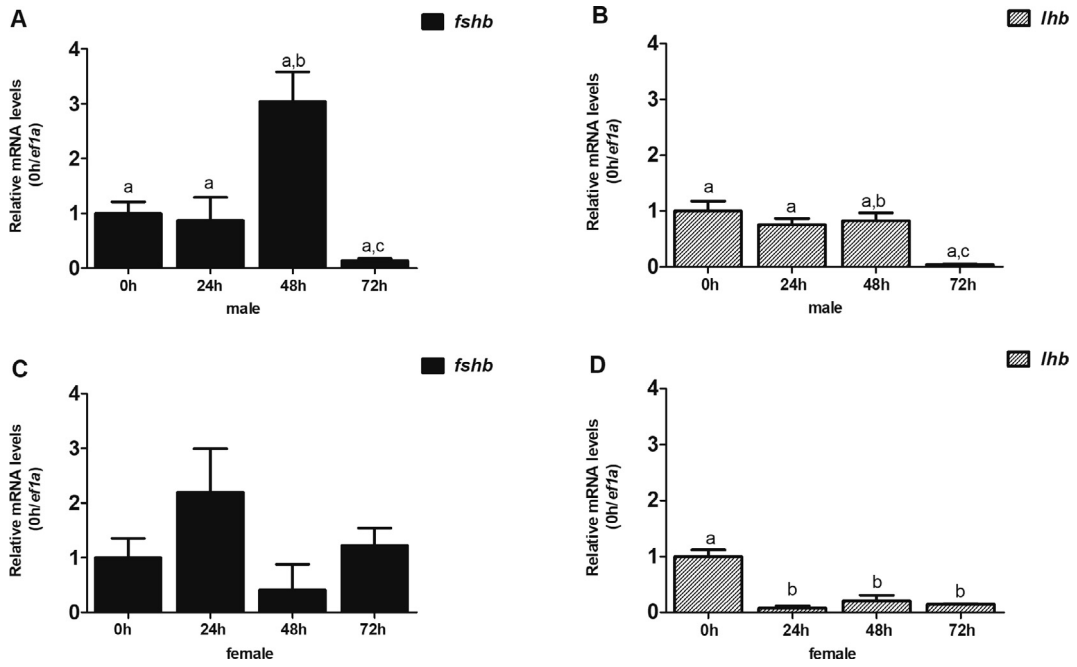

Fig. 2. Temporal expression analysis of *A. altiparanae fshb* and *lhb* under basal culture conditions. *fshb* and *lhb* mRNA levels were normalized with the mRNA levels of *ef1a* (*elongation factor 1α*) and expressed as relative values of the time zero. Bars indicate mean ± standard error (SE); different letters denote significant differences among the groups ($p < 0.05$). Males and females were at the same reproductive phase (males – developing phase; females – spawning capable phase).

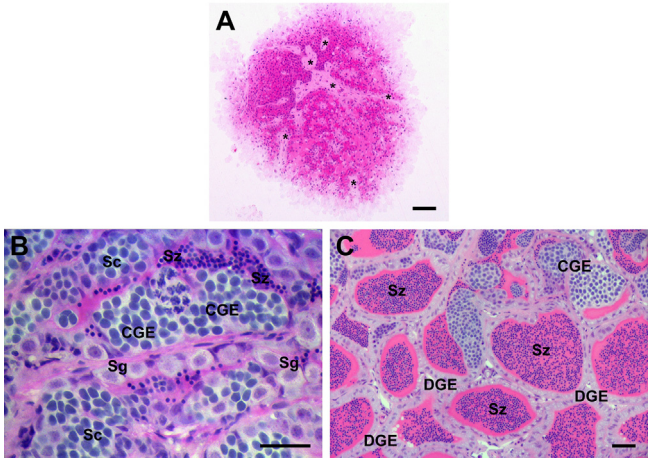

Fig. 3. (A) *A. altiparanae* pituitary gland morphology after 72 h of incubation. Asterisks (*) show the neurohypophysis. **(B)** Testicular histology of males used in the pituitary culture explant standardization experiment. Males were at the developing phase of the reproductive cycle. Note that the germinal epithelium is continuous and formed by cysts at different stages of spermatogenesis (Sg: spermatogonia, Sc: spermatocytes; Sz: spermatozoa; CGE: continuous germinal epithelium). **(C)** Testicular histology of males used in the pituitary explants treated with GnRH or/and GnIH. Males were at the mid-germinal epithelium subphase of the spawning capable phase. Note the presence of CGE and discontinuous germinal epithelium (DGE) in the testis, and Sz in the testicular lumen. Bars: A e B: 50 μm, C: 25 μm.

3.2. In vitro effects of cGnRH2 and zGnIH-3 on fshb and lhb expression

Once we have determined the culture conditions, we used the same period of incubation (12 h) for all hormonal treatments. All males used were at the spawning capable phase (mid-GE subphase) of the reproductive cycle. In this phase, males have already undergone gonadal development and are physiologically prepared to reproduce. Histological analysis of testes showed all stages of spermatogenesis, GE continuous or discontinuous, and a large amount of spermatozoa in the testicular lumen (Fig. 3C).

Pituitary glands were incubated with cGnRH2 or/and zGnIH-3 for 12 h to evaluate the medium-term effects of these hypothalamic neuropeptides on gonadotropin expression. Our results showed that pituitaries treated with 10 nM cGnRH2 did not exhibit changes in *fshb* (Fig. 4A) or *lhb* (Fig. 4B) gene expression compared to the control. On the other hand, higher doses of cGnRH2 (100 nM and 1000 nM) significantly stimulated both *fshb* (Fig. 4A) and *lhb* (Fig. 4B) mRNAs in relation to the control group. Interestingly, pituitary glands treated with doses of 10, 100 and 1000 nM zGnIH-3 did not show any changes on basal *fshb* (Fig. 5A) or *lhb* (Fig. 5B) mRNA levels. However in the pituitaries co-treated with cGnRH2 and zGnIH-3, the cGnRH2-induced gonadotropin expression was decreased in the presence of zGnIH-3 for both *fshb* (Fig. 6A) and *lhb* (Fig. 6B) mRNAs.

3.3. In vitro effects of cGnRH2 and zGnIH-3 on gnrh2 and gnrh3 expression

To evaluate the effects of cGnRH2 and zGnIH-3 on *gnrh2* and *gnrh3* mRNA levels, brain slices were treated with cGnRH2 (100 nM), zGnIH-3 (100 nM) and cGnRH2 + zGnIH-3 (100 nM) for 12 h (Fig. 7). There were no significant differences in the expression levels of *gnrh2* between cGnRH2 treatment and control group (Fig. 7A). On the other hand, zGnIH-3 alone or combined with cGnRH2 increased *gnrh2* mRNA levels in relation to the control (Fig. 7A). The relative expression of *gnrh3* did not changed among the groups (Fig. 7B). Fig. 8 summarizes our main results regarding cGnRH2 and zGnIH-3 effects on *A. altiparanae*.

4. Discussion

This study aimed to evaluate the *in vitro* effects of cGnRH2 and zGnIH-3 on male *A. altiparanae* gonadotropin and GnRH expression using pituitary explants and brain slices. The first step we performed was to determine whether the gonadotropin expression could be affected under basal culture conditions in order to evaluate the viability of the pituitary explant technique for this species. The integrity of the pituitary gland tissue was also evaluated after the period of incubation. Altogether, these results allowed us to employ the pituitary explant

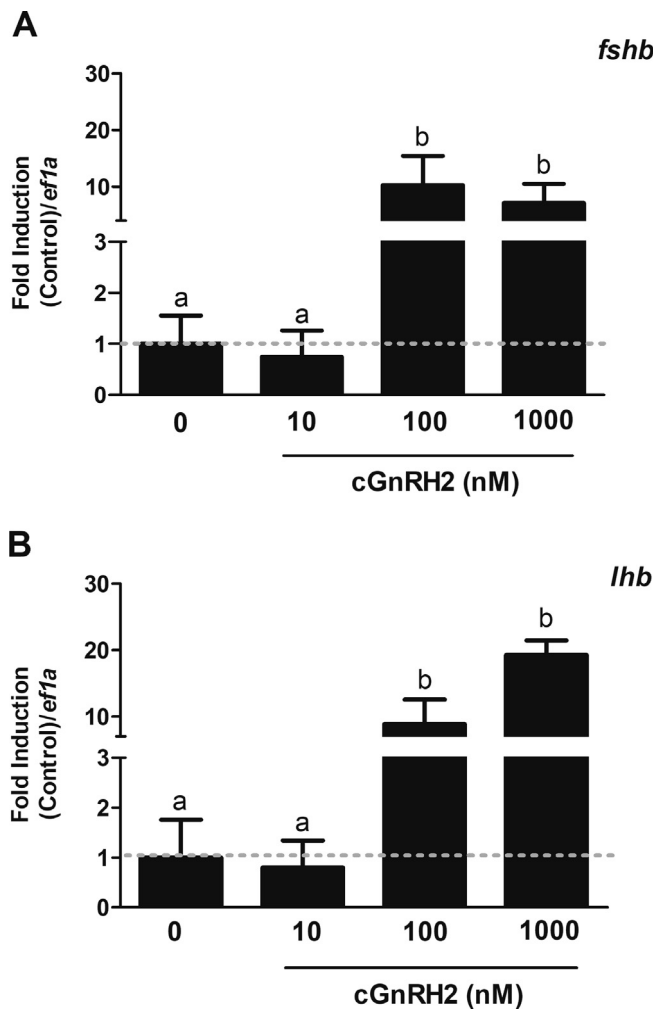

Fig. 4. Medium-term effects of cGnRH2 on *A. altiparanae fshb* and *lhb* expression. All males were at the spawning capable phase of the reproductive cycle. Pituitaries were incubated with different concentrations of cGnRH2 (10, 100, and 1000 nM) to evaluate the relative mRNA levels of *fshb* **(A)** and *lhb* **(B)** after 12 h of culture. Bars represent mean ± standard error (SE) of the relative mRNA levels normalized with *elongation factor 1α* (*ef1a*) levels and expressed as fold induction of the control group. Different letters denote statistically significant differences among the groups (ANOVA followed by Student-Newman-Keuls method; $p < 0.05$).

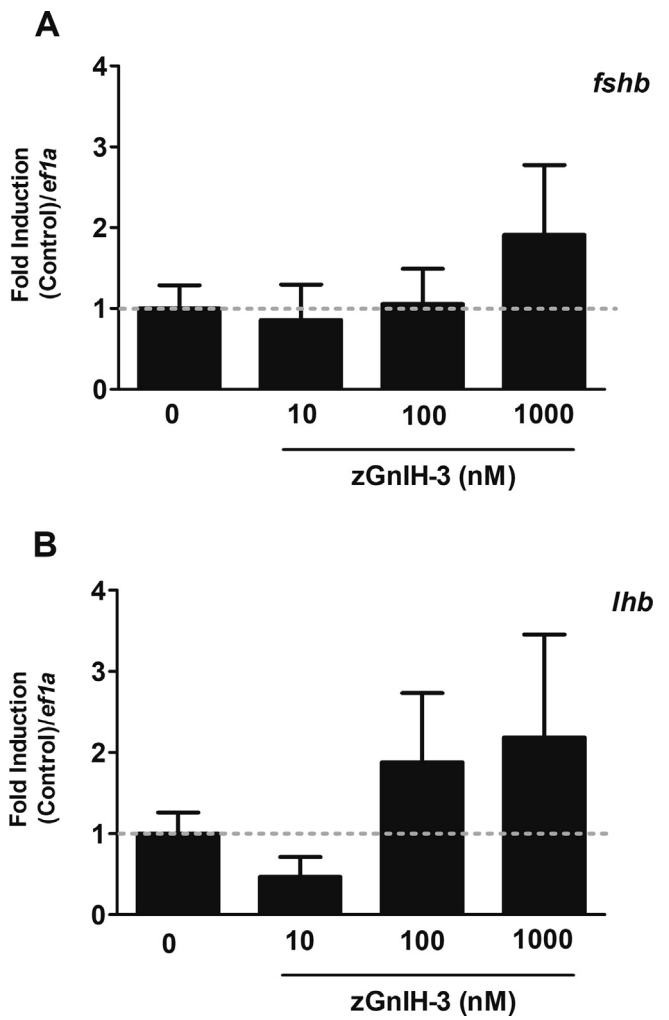

Fig. 5. Medium-term effects of zGnIH-3 (zf LPXRFa-3) on *A. altiparanae fshb* and *lhb* expression. All males were at spawning capable phase of the reproductive cycle. Pituitaries were incubated with different concentrations of zGnIH-3 (zf LPXRFa-3) (10, 100, and 1000 nM) to evaluate the relative mRNA levels of *fshb* **(A)** and *lhb* **(B)** after 12 h of culture. Bars represent mean ± standard error (SE) of the relative mRNA levels normalized *elongation factor 1α* (*ef1a*) levels and expressed as fold induction of the control group. Different letters denote statistically significant differences among the groups (ANOVA followed by Student-Newman-Keuls method; $p < 0.05$).

system and also to determine the period of incubation for the hormonal treatments used in this study.

Immediately before the pituitary glands were placed in the culture medium, we showed that *lhb* levels were much higher than *fshb* for both sexes. The varying patterns of gonadotropin expression, synthesis, and secretion are derived from interspecific differences, physiological condition and reproductive stage (Mateos et al., 2002). In this context, studies on juveniles of *Morone saxatilis* have shown that *lhb* expression was higher than *fshb* (Hassin et al., 1995). In pre-gametogenic salmonids, however, *fshb* expression has been found to be much higher than that of *lhb* (Gomez et al., 1999; Weil et al., 1995).

Interestingly, when evaluating female gonadotropin expression under basal culture conditions, there is a remarkable decrease of *lhb* mRNA levels, while *fshb* remained stable in comparison with time zero. This result suggests that *lhb* is more dependent of hypothalamic and/or gonadal inputs than *fshb*. It has been shown that gonadotropic cell stimulation depends not only on GnRH stimulation via the hypothalamus, but also on stimuli produced by the gonads by means of positive or negative feedback loops (Yaron and Levavi-Sivan, 2011). With respect to the latter one, Mateos et al. (2002) found that estradiol (E2), testosterone (T) and dihydrotestosterone (DHT) contribute to an increase in *lhb* expression in the pituitary, indicating positive feedback exerted on *lhb* mRNA levels during the resting phase of *Dicentrarchus labrax* gonads. E2 and T stimulatory effects on *lhb* mRNA levels have been also described in *Oncorhynchus tschawytscha* (Trinh et al., 1986; Xiong et al., 1994), *Oncorhynchus kisutch* (Dickey and Swanson, 1998), *Carassius auratus* (Huggard et al., 1996), and *Anguilla anguilla L.* pituitary cell cultures (Huang et al., 1997). Future studies involving the administration of different concentrations of steroid hormones and subsequent analysis of the gonadotropin expression will likely aid in the understanding of the effects of androgens and estrogens on *A. altiparanae* pituitaries.

Temporal gene expression analysis of male pituitary explants showed that relative mRNA levels of *fshb* and *lhb* remained stable over the period with tendency of decrease after 72 h of culture. In addition, the morphological analyses indicated that the *in vitro* conditions did not change the structure or integrity of the pituitary gland. Moreover, previous studies with goldfish primary pituitary cell culture used 12 h of incubation and showed stimulatory/inhibitory actions of GnIH

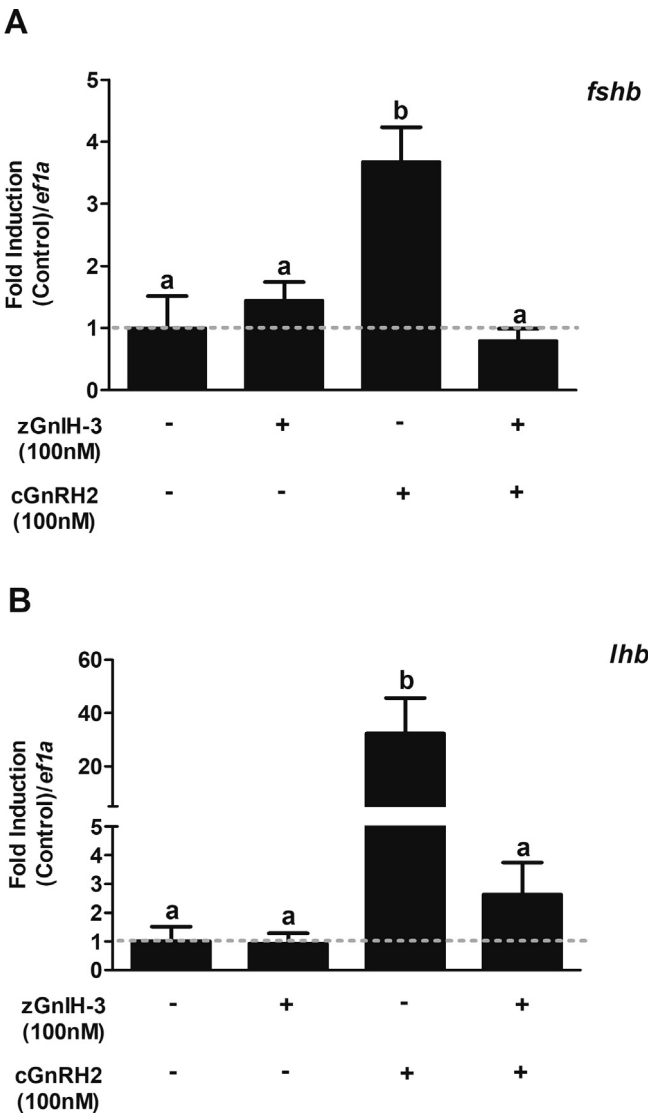

Fig. 6. Co-treatment effects of cGnRH2 and zGnIH-3 (zf LPXRFa-3) on *A. altiparanae fshb* and *lhb* expression. All males were at spawning capable phase of the reproductive cycle. Pituitaries were incubated with 100 nM cGnRH2 in the presence or absence of 100 nM zGnIH-3 to evaluate the relative mRNA levels of *fshb* **(A)** and *lhb* **(B)** after 12 h of culture. Bars represent mean ± standard error (SE) of the relative mRNA levels normalized with *elongation factor 1α* (*ef1a*) levels and expressed as fold induction of the control group. Different letters denote statistically significant differences among the groups (ANOVA followed by Student-Newman-Keuls method; $p < 0.05$).

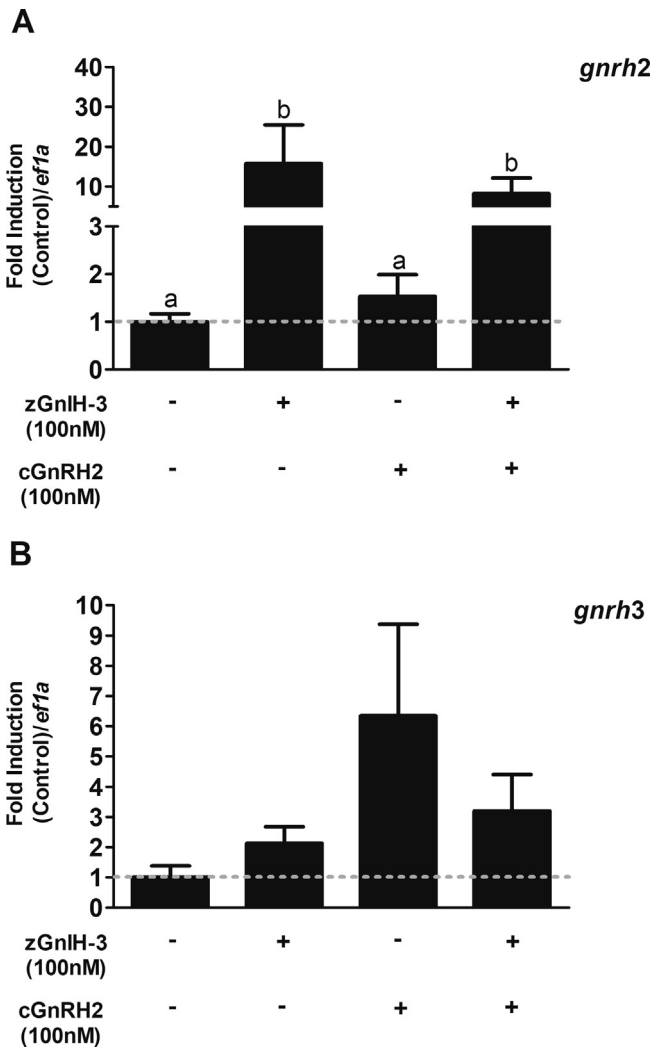

Fig. 7. Medium-term effects of zGnIH-3 and cGnRH2 on *A. altiparanae gnrh2* and *gnrh3* mRNA levels. All males were at spawning capable phase of the reproductive cycle. Brain slices were incubated with 100 nM zGnIH-3 (zf LPXRFa-3) and 100 nM cGnRH2 either separately or combined to evaluate the relative mRNA levels of *gnrh2* **(A)** and *gnrh3* **(B)** after 12 h of culture. Bars represent mean ± standard error (SE) of the relative mRNA levels normalized with *elongation factor 1α* (*ef1a*) levels and expressed as fold induction of the control group. Different letters denote statistically significant differences among the groups (ANOVA followed by Student-Newman-Keuls method; $p < 0.05$).

peptide (Moussavi et al., 2012, 2013). Based on that, we decided to use a medium-term exposure (12 h) to evaluate the acute effects of cGnRH2 and zGnIH-3 in *A. altiparanae* pituitaries.

It has been shown in many teleosts that GnRH regulates gonadotropin expression according to developmental and reproductive stages (Martyniuk et al., 2009). In the current study, when cultivated at higher concentrations (100 nM and 1000 nM), cGnRH2 significantly increased the expression of *A. altiparanae fshb* and *lhb* after 12 h of culture. Similar results were observed in primary culture of pituitary cells from salmon *Oncorhynchus kisutch*, where GnRH increased *fshb* and *lhb* mRNA levels (Dickey and Swanson, 2000). This effect was also seen for protein levels using organotypic cultures prepared from hypothalamic-pituitary slices of tilapia; GnRH induced increases in Lh and Fsh secretion (Levavi-Sivan et al., 2005). On the other hand, GnRH3 reduced *lhb* mRNA levels in coho salmon pituitary cells *in vitro* (Luckenbach et al., 2010). Kumakura et al. (2003, 2004) showed that GnRH has no effect on the *fshb* subunit mRNA levels in *Pagrus major*. Our results indicate that lower doses of cGnRH2 (such as 10 nM) are insufficient to stimulate expression of *fshb* and *lhb* in *A. altiparanae* male pituitaries, but that, at higher concentrations, cGnRH2 most likely stimulates the production of gonadotropins. Future experiments to quantify the amount of Fsh and Lh released in the culture media by the pituitary explants are likely to provide more information on this topic.

The gonadotropin-inhibitory hormone, GnIH, is known by its inhibitory roles on the reproductive processes of birds and mammals (Kriegsfeld et al., 2006; Tsutsui et al., 2000; Ubuka et al., 2006, 2012). GnIH acts on GnRH neurons modulating the GnRH-induced gonadotropin release (Smith et al., 2012; Ubuka et al., 2012). Nevertheless, the effects of GnIH orthologs in fish seems to be conflicting, because GnIH can either exert stimulatory or inhibitory roles on gonadotropin expression and release (Muñoz-Cueto et al., 2017; Ubuka and Parhar, 2018). In our study, zGnIH-3 (LPXRFa-3 or GnIH-3) showed no effects on the basal expression of male *A. altiparanae fshb* or *lhb* mRNA levels. However, the same peptide reduced the plasma levels of Lh in goldfish

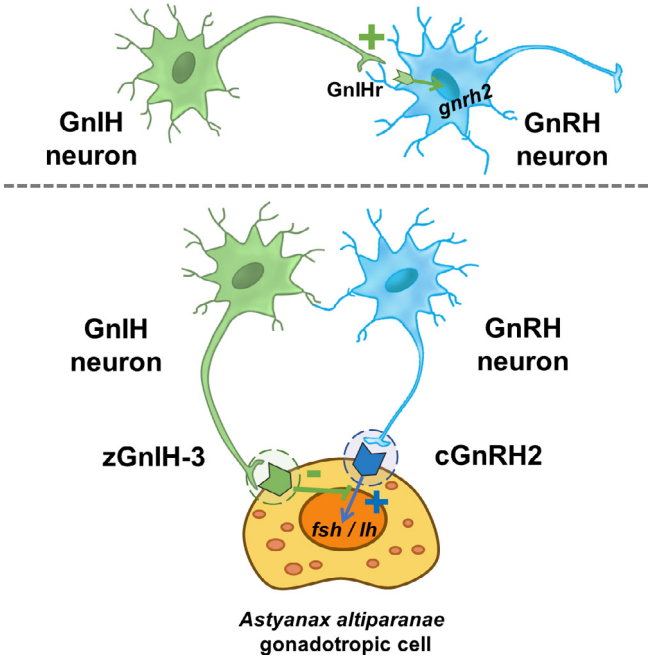

Fig. 8. Schematic representation summarizing the effects of cGnRH2 and zGnIH-3 in the *A. altiparanae* pituitary explants and brain slices. In the brain slices, zGnIH-3 stimulates *gnrh2* expression in GnRH2 neurons (upper part). cGnRH2 stimulates gonadotropin expression, increasing both *fshb* and *lhb* mRNA levels in the gonadotropic cells. zGnIH-3 attenuates the cGnRH2-induced gonadotropin expression, decreasing both *fshb* and *lhb* mRNA levels. Stimulatory effects are represented by + and ↓; inhibitory action is denoted by − and ⊥. GnIH receptor is indicated as GnIHr.

(Zhang et al., 2010). Interestingly, when evaluating the co-treatment of cGnRH2 with zGnIH-3, we showed that zGnIH-3 decreased the cGnRH2-induced expression of *A. altiparanae fshb* and *lhb*. Similar results were found in goldfish primary pituitary cell culture; where goldfish GnIH-2 (gGnIH-2) suppressed the LHRH (Luteinizing hormone-releasing hormone)-induced gonadotropin expression (Qi et al., 2013). In accordance with these results, Moussavi et al (2013) showed that co-administration with gGnIH-3 attenuated the stimulatory effects of salmon GnRH on *fshb* and *lhb* mRNA levels in mid and late gonadal recrudescence of goldfish. Taken together, the results presented in this study indicate that zGnIH-3 does not modulate basal *fshb* and *lhb* expression levels, but it attenuated the cGnRH2 stimulatory effects on gonadotropin expression of *A. altiparanae* males (Fig. 8).

To determine whether zGnIH-3 might be acting at the central level to modulate the neuroendocrine system of *A. altiparanae* males, we evaluated *gnrh2* and *gnrh3* mRNA levels in brain slices treated either with zGnIH-3 or cGnRH-2 or both combined. To date, most of the reported effects of different GnIH orthologs on brain neuroendocrine systems are inhibitory and seems to be dependent of the route of administration (Muñoz-Cueto et al., 2017; Ubuka and Parhar, 2018). For example, intracerebroventricular injection of sea bass GnIH-2 (sbGnIH-2) reduced *gnrh2* expression levels, while the intramuscular administration of the same peptide increased *gnrh2* mRNA levels in male European sea bass (Paullada-Salmerón et al., 2016). In female goldfish, *gnrh3* mRNA levels are decreased by gGnIH-2 and gGnIH-3 (Qi et al., 2013), similarly to the inhibitory effects of zGnIH-3 on *gnrh3* expression in zebrafish brain slices (Spicer et al., 2017). In this work, zGnIH-3 increased *gnhr2* mRNA levels in male brain slices and did not affect *gnrh3* expression. Interestingly, increased levels of *gnrh2* were also seen in the co-treatment of both peptides. Since cGnRH2 alone did not modulate *gnrh2* levels, the increase of *gnrh2* transcripts in the co-treatment are attributed to zGnIH-3. The stimulatory effects of GnIH orthologs on the expression of different GnRH forms were reported in other species; GnIH-2 increased *gnrh3* mRNA in female grouper (*Epinephelus coioides*) (Wang et al., 2015); LPXRFa-2 stimulated GnRH1 and GnRH3 content in the lamprey brain (Osugi et al., 2012); and sbGnIH-2 elevated *gnrh2* transcripts in European sea bass (Paullada-Salmerón et al., 2016). In birds, GnIH plays a role in the socio-sexual behavior through GnRH2 neurons, which express GnIH receptor (Ubuka et al., 2016). Therefore, the GnIH-induced *gnrh2* expression in *A. altiparanae* (this study) and European sea bass (Paullada-Salmerón et al., 2016) could suggest that GnIH might also be involved in regulation of socio-sexual behavior. Interestingly, a recent review from Ubuka and Parhar (2018) suggested that the stimulatory effects of GnIH on GnRH neurons may be explained by (1) GnIH stimulation of neuroestrogens which are released by neurons that terminate on GnRH neurons that express estrogen receptor; (2) heteromerization of GnIH and GnRH receptors in GnRH neurons and gonadotropes, modifying ligand binding and signaling transduction mechanisms; and (3) internalization of GnIH receptor by high concentration or chronic administration of zGnIH-3 (Ubuka and Parhar, 2018). The above mentioned mechanisms could explain our observations regarding the stimulatory effect of GnIH on *gnrh2* mRNA in *A. altiparanae* males.

Overall, this work described two regulatory roles of zGnIH-3 in *A. altiparanae* males (Fig. 8); zGnIH-3 inhibits the cGnRH2-induced gonadotropin expression at the pituitary level, while zGnIH-3 increased *gnrh2* mRNA levels in the male brain slices. Therefore, we conclude that zGnIH-3 exerts a regulatory role over the *A. altiparanae* male reproductive axis by modulating the gonadotropic cell and GnRH neuronal functions.

This study provided basic information on endocrine regulation of *A. altiparanae* males with respect to GnRH and GnIH. The obtained results will expand our knowledge on *A. altiparanae* reproduction and improve the reproductive management of this economically important freshwater species. Moreover, these results will help to clarify the role of GnIH in regulating *A. altiparanae* reproduction.

5. Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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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.2018.08.006.

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