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Thermal manipulation and GnRHa therapy applied to the reproduction of lambari-do-rabo-amarelo, *Astyanax altiparanae* females (Characiformes: Characidae) during the non-breeding season

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ABSTRACT

Lambari-do-rabo-amarelo *Astyanax altiparanae* in the wild reproduce during spring and summer, but females undergo vitellogenesis throughout the year, including the non-spawning winter period when water temperatures are low. The present study investigated the physiological role of temperature modulation on the hypothalamus-pituitary-gonads axis of lambari during winter, as well as the effects of gonadotropin releasing hormone agonist (GnRHa) therapy. Captive females were exposed to two different temperatures (20 °C and 27 °C) and were injected weekly with GnRHa for 21 days during winter (Control, CTR; Low dose; LD and high dose of GnRHa, HD). At the end of the 21-days period gonadosomatic index (GSI), oocyte stage of development and theoretical fecundity were evaluated, together with plasma levels of 17β-estradiol (E_2). Gene expression of the two pituitary gonadotropins follicle-stimulating hormone (*fshβ*) and luteinizing hormone (*lhβ*), as well as hepatic vitellogenin-A (*vtgA*) expression were also analyzed. At the end of the experimental period, females from the six different experimental conditions were induced to spawn using human chorionic gonadotropin (hCG). Spawning performance parameters and plasma levels of the maturation inducing steroid (MIS) were analyzed. Gene expression of *fshβ* did not change with temperature manipulation, but females exposed to 27 °C and supplemented with a HD of GnRHa exhibited an increased *fshβ* gene expression, associated with higher E_2 levels. The higher water temperature alone was able to increase E_2 levels. At both water temperatures GnRHa injections induced a decrease in E_2 levels. GnRHa injected females had a lower *vtgA* gene expression levels at 20 °C. Even with differences in the gene expression of gonadotropins among the various temperature/GnRHa treatments, GSI and oocyte diameter did not change, but GnRHa enhanced the number of vitellogenic oocytes at 20 °C. The reproductive performance of lambari induced to spawn with hCG was better after the combined treatment with GnRHa and summer temperature.

1. Introduction

Reproduction in vertebrates, including teleosts, is modulated by environmental cues, such as temperature and photoperiod (Zohar et al., 2010). These environmental cues stimulate the hypothalamus-pituitary-gonad (H-P-G) axis, through the release of gonadotropin releasing hormone (GnRH), gonadotropin inhibitory hormone (GnIH), dopamine and kisspeptin, among other factors (Zohar et al., 2010). These neurohormones modulate the synthesis and release by the adenohypophysis of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), the two gonadotropins (Levavi-Sivan et al., 2010), and these gonadotropins control the synthesis of gonadal steroids by the ovaries and testes (Lubzens et al., 2010; Schulz et al., 2010).

In captivity, reproduction can fail in some fish species due to physiological and/or environmental factors. Migratory teleosts kept in captivity exhibit difficulties in completing their reproductive cycle, especially the stages of oocyte maturation (OM) and spawning (Agostinho et al., 2007a; Agostinho et al., 2007b; Miranda and Ribeiro,

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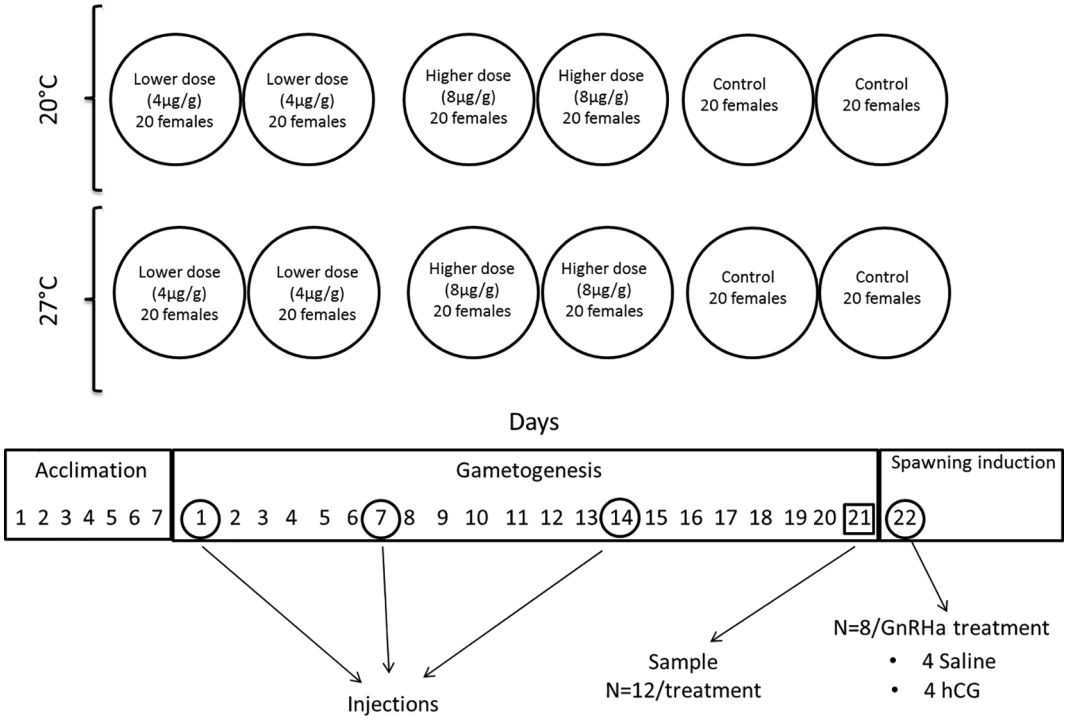

Fig. 1. Lambari *Astyanax altiparanae* induction of gametogenesis and spawning experimental design. Injections of GnRHa in lower (4 µg/g) and higher dose (8 µg/g), divided in 3 weekly doses, at 20 °C or 27 °C. At the end of the period, fish from each experimental condition were treated with hCG or saline (control), to induce spawning. Females were maintained in the same temperature throughout the experimental period (acclimation, gametogenesis period, and induction of spawning).

1997; Resende et al., 1995). Zohar and Mylonas (2001) suggested three different types of reproductive dysfunctions in teleost females in captivity. One of these dysfunctions includes the absence of OM and ovulation. This is the failure that is observed often in tropical potamodromous fish (*i.e.* freshwater fish that migrate within the freshwater environment for spawning). To improve reproduction in captivity, environmental conditions can be manipulated and/or exogenous hormone stimulating the H-P-G can be administered (Mylonas et al., 2010). These practices are normally effective within the reproductive period, which occurs during spring and summer for most tropical freshwater migratory species (Caneppele et al., 2009; Hainfellner et al., 2012; Honji et al., 2009, 2011; Moreira et al., 2015; Jesus et al., 2017).

The lambari-do-rabo-amarelo (lambari) *Astyanax altiparanae* (Garutti and Britski, 1997) is a potamodromous species that reproduces during spring/summer (Jesus et al., 2017), presents sexual dimorphism, high fecundity, fast growth, and reach sexual maturation at the age of four months in captivity (Gonçalves et al., 2014; Porto-Foresti et al., 2010). The species has an interest for the aquaculture industry in South America, due to canned fish production, protein meal for animal feed or live bait for sport fishing (Porto-Foresti et al., 2010). Another characteristic of lambari making it a good aquaculture species, is that it may respond successfully to artificially induced reproduction in captivity during the winter, a season that was initially described as non-breeding for fish in the wild (Evangelista et al., 2015). This characteristic allows the production of this fish throughout the year, reducing the interharvest interval (Duncan et al., 2013). However, even with the existence of predominantly vitellogenic oocytes in the ovaries throughout the year, the gonadosomatic index (GSI) of females is lower during autumn/winter (Jesus et al., 2017) when compared to spring/summer, suggesting an incomplete development of oocytes in this period. Despite the evidence showing the plasticity of the vitellogenic process in lambari, the physiological role of temperature modulation of the H-P-G axis in the winter has not been studied yet.

Considering the above, thermal manipulation and exogenous hormonal therapies were used to modulate the function of the H-P-G axis in lambari, in order to improve the reproductive potential of this species outside the reproductive season. We tested the hypothesis that vitellogenesis in lambari may be induced during the winter non-breeding season, if the fish are exposed briefly to summer temperatures, and that the treatment with GnRH agonist (GnRHa) may stimulate the H-P-G axis, thus improving vitellogenesis and reproductive performance.

2. Materials and methods

2.1. Experimental design

Two hundred and forty lambari adult female (approximately 1-year-old) reared in the Centro de Aquicultura da Universidade do Estado de São Paulo (CAUNESP), Jaboticabal, São Paulo, Brazil (21°15′17″ S 48°19′20″ W) were kept under natural temperature and photoperiod, and were fed with commercial feed once a day. The animals were randomly divided in 12 tanks of 750 L (20 animals/tank) and 6 experimental groups were established (in duplicate). Six tanks were kept at 20 °C (winter average water temperature in the fish farm) and six at 27 °C (summer average water temperature in the fish farm) for a period of 5 weeks (acclimation, gametogenesis and spawning induction). After a period of acclimation of 7 days, fish in four tanks (two at 20 °C and two at 27 °C) were treated with an intraperitoneal injection of saline (0.7% NaCl) (control, CTR); the animals in another four tanks (two at 20 °C and two 27 °C) were treated intraperitoneally with GnRHa [des-Gly10, D-Ala6] (Sigma-Aldrich, Saint Louis, MO, USA) at 1.33 µg/g body weight (lower dose, LD); and the fish in the last four tanks (two at 20 °C and two at 27 °C) were treated with GnRHa injection at a higher dose of 2.66 µg/g (higher dose, HD). The injections were repeated every seven days (1, 7 and 14 days) during the experimental period, with a total of three injections, resulting in a total dose of 4 (LD) and 8 µg/g (HD). The doses were calculated based on information from the literature, in experiments with other teleost species (Kagawa et al., 2009; Kanemaru et al., 2012).

At the end of the gametogenesis experiment (21 days), six females were sampled from each tank (Fig. 1). At the day 22, four females from each experimental group (two/tank) were induced to spawn with an

injection of 5000 IU/kg of human chorionic gonadotropin (hCG, ovidrel, Merck) and the same number of animals (four females/group) were injected with saline as controls (Spawning experiment – Fig. 1). All four females from each of the six gametogenesis experimental groups (two females from each replicated tank) were placed in the same 2.5-L spawning box with 8 males (6 spawning boxes with females injected with hCG and 6 injected with saline). Males were induced with 3 mg/kg of carp pituitary extract at the same temperature that was used during the gametogenesis experiment period (*i.e.* 20 or 27 °C) and were allowed to spawn spontaneously. After spawning (6 h) a sample of blood was collected to measure progestogen 17α-20β-dihydroxy-4-pregnen-3-one (maturation inducing steroid, MIS) levels, fertilization, embryonic development and hatching of eggs from each spawning box were monitored, and larvae were followed until opening of the mouth.

The experimental protocol was approved by the Ethics Committee opinion n°228/2015, USP Institute of Biosciences. All procedures involving animals were conducted in accordance to the "Guidelines for the treatment of animals in behavioral research and teaching" (Anonymous, 1998), the Ethical justification for the use and treatment of fishes in research: an update (Metcalfe and Craig, 2011) and the "Directive 2010/63/EU of the European parliament and the council of 22 September 2010 on the protection of animals used for scientific purposes" (EU, 2010).

2.2. Sampling procedure

Sampled fish were anesthetized in 0.1 g/L of benzocaine, and blood samples were collected by puncturing the caudal vein with disposable heparinized syringes and needles (Hepamax® S5000UI). Blood samples were centrifuged at 655.1g for 5 min, and plasma was frozen at −80 °C until processing. After blood collection, length (cm) and body mass (g) were recorded. Females were subsequently euthanized by sectioning the spinal cord at the level of the operculum (Ethics Committee on Animal Use, CEUA, Institute of Biosciences, University of São Paulo, protocol 228/2015). The ovaries were removed and weighed to calculate gonadosomatic index (GSI = [gonads mass/body mass] × 100), and portions of the ovaries were stored to calculate fecundity and measure oocyte diameter, and for histological evaluation of stage of maturation. The pituitary was collected and frozen at −80 °C for analyses of gene expression of fshβ and lhβ. The liver was also frozen at −80 °C for analysis of gene expression of vitellogenin (VTGA).

2.3. Gene expression of gonadotropins (*fshβ and lhβ*) and vitellogenin (*vtg*)

2.3.1. RNA extraction

There are no commercial kits available for the determination of FSH and LH in lambari, or even in phylogenetically related species. Therefore, the most accurate method to evaluate the concentrations of these hormones was the gene expression of the beta (β) subunit, specific for each gonadotropin. The extraction of total RNA from individual pituitaries was carried out using the kit RNAqueous™ (Micro RNA Isolation Kit) according to the manufacturer's protocol. Total liver RNA was extracted using the Trizol reagent (Sigma®) according to the manufacturer's protocol. For quantification and analysis of the integrity of the material the Abs260/Abs280 ratio was evaluated in the Nanodrop™ Spectrophotometer (ND-1000) and only samples that presented this relation between 1.7 and 2.2 were used. The purified RNA was stored and maintained at −80 °C.

2.3.2. Synthesis of cDNA

For cDNA synthesis, a commercial kit (SuperScript™ II Reverse Transcriptase) was used. In 0.2 ml microtubes, 12 µl of each sample was added at a RNA concentration of 10 ng/µl for pituitary and 200 ng/µl for liver. In each tube, 1 µl of Randon hexamer (50 ng/µl) was added and placed in the thermocycler for 10 min at 70 °C. The samples were placed on ice for a thermal shock (4 °C), and 5 µl buffer, 2.5 µl DTT and 1.25 µl dNTPs were added and placed again in the thermocycler at 25 °C for 10 min. A new heat shock was given at 4 °C, and 0.6 µl of the enzyme was added and finally the samples were placed in the thermocycler at 42 °C for 50 min and at 70 °C for 10 min, and then stored at −20 °C.

2.3.3. Real-time PCR

The real-time PCR primers for *fshβ* and *lhβ* genes of lambari have been already designed by Jesus et al. (2017) and the primer for *vitellogenin* gene was designed by Tolussi et al. (2018) for the congeneric species *Astyanax fasciatus* and tested successfully in lambari. The real-time PCR reaction was performed using 12.5 µl of Power Sybr Green PCR Master Mix (Applied Biosystems), 0.5 µM primer (forward and reverse) and 2 µl cDNA. The reaction was incubated for 2 min at 50 °C, 10 min at 95 °C followed by forty cycles of 15 sec at 95 °C and 40 sec at 60 °C. At the end of these cycles an analysis of the melting curve was performed to test the specificity of the reaction that was made in duplicate in the Step One Real Time-PCR System (Applied Biosystems). The β-actin gene was used as an endogenous reference for the analysis of the relative expression of *fshβ, lhβ* and *vtgA*. Gene expression was calculated according to the value of Ct, which was normalized by ΔΔct through the endogenous gene and made log10 (Livak and Schmittgen, 2001). The CTR group was used as the reference group at each temperature.

2.4. Plasma steroids

Plasma 17β-estradiol (E_2) levels were quantified by an enzyme-linked immunosorbent assay (ELISA), using commercial kits (IBL®, International/Hamburg, Germany). Analyzes were performed according to the manufacturer's instructions. Samples were tested for dilution accuracy, and as concentrations were above the standard curve, a 1: 2 dilution was required using EIA Buffer (Cayman Chemical®) as diluent. The limit of detection of the test is 9.7 pg/ml. Absorbance was measured using a microplate reader (Spectra Max 250 Molecular Devices) at 450 nm. Samples were assayed in duplicate according to the manufacturer's instructions. Intra-assay coefficient (CV) (18.0%) was calculated considering the duplicates of the samples in the same plate, while the inter-assay CV was calculated considering samples analyzed in different plates (17.5%). Both values were below 20% as recommended by Sink et al. (2008).

MIS was quantified in plasma after spawning also using a commercial ELISA (Cayman Chemical®) according to manufacturer's protocol. The best dilution varied according to the experimental group, *i.e.* treated or not with hCG, at 1:70 and 1:30 respectively, and the dilutions were also carried out with ELISA buffer (Cayman Chemical®). The detection limit of the test was 15 pg/ml and the absorbance was measured using a microplate reader (Spectra Max 250 Molecular Devices) at 405 nm. Intra-assay coefficient (CV) (19.3%) was calculated considering the duplicates of the samples in the same plate, while the inter-assay CV was calculated within the samples analyzed in different plates (12.3%).

2.5. Morphological analyses

2.5.1. Histological analyses

To evaluate gonadal maturation stage using histological processing, samples from the middle third of the ovaries were fixed with Bouin's solution for 24 h. Samples were then transferred to ethanol 70% and were dehydrated in increasing concentrations of ethanol and xylene (dimethylbenzene) before being embedded in Paraplast® according to routine histological procedures. Sections of 5 µm thick were obtained using a microtome (Leica RM2255) equipped with disposable blades, and were stained with hematoxylin and eosin (Behmer et al., 1976). After staining, the sections were photographed using a computer image system (Leica DM1000 light microscope, Leica DFC295 photographic camera, Leica Application Suite Professional, LASV3.6). The stage of maturation of the ovary was classified as primary growth (only perinuclear oocytes), secondary growth (early vitellogenesis with cortical alveoli oocytes), tertiary grown (vitellogenesis with lipid droplets

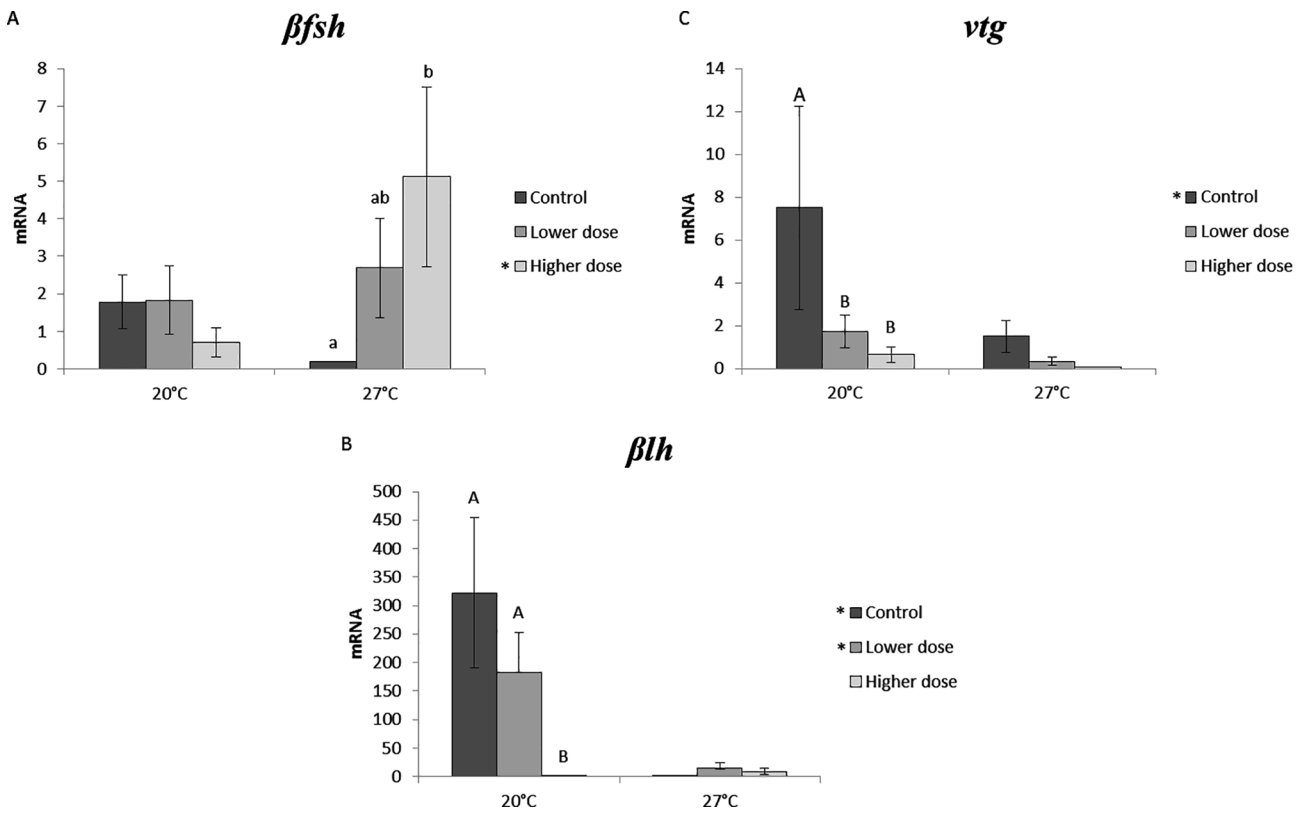

Fig. 2. *fshβ* (A), *lhβ* (B) and *vtg* (C) gene expression in lambari *Astyanax altiparanae* females after 21 days of induction with GnRH injections. Means with different symbols were significantly different ($P < 0.05$) between temperatures in the same GnRHa treatment. Capital letters indicate differences between GnRHa treatments at 20 °C and lowercase at 27 °C.

forming yolk granules), final growth (nucleus migration) or ripe (ovulated oocyte and post-ovulatory follicle) (Rocha and Rocha, 2006) according to the developmental stage of the oocyte based on the predominant cell types according to West (1990) and using a qualitative analysis of histological slides, documenting the presence/absence (+/−) and frequent/predominant presence (++/+++) of cell types (Gomes et al., 2015).

2.5.2. Oocyte diameter and relative fecundity

Fecundity was evaluated in females at the tertiary growth stage (Murua et al., 2003) and not used in the spawning induction experiment. Pre-weighed ovary samples were fixed with Gilson solution (Simpson, 1951) for 30 days to dissolve the ovarian tissue. Samples were then stored in 70% ethanol. The oocytes contained in a 2 ml subsamples (in triplicate) obtained with a Stempel pipette (Hensen Stempel Pipette™ 1806 series; Wildlife Supply Company, Florida, USA) were counted and measured using the same microscope/computer system described above. The counted oocytes were then allocated into 10 classes of 100 μm increments in diameter (0–100 μm, 101–200 μm up to 1000 μm) to determine the percentage of oocytes in each diameter class. The oocytes with diameter greater than 601 μm were considered vitellogenic for the fecundity calculations. After measuring and counting the oocytes, relative fecundity (RF) was expressed in relation to body mass (Vazzoler, 1996).

2.6. Reproductive parameters

In each experimental group, the fertilization success (Fertilization = number of fertilized eggs × 100/total number of eggs) was calculated from data obtained from the tank. To calculate embryo development and larval survival, fertilized eggs were placed individually in 96-well microtiter plates (in duplicate), covered with a plastic cap and kept under their spawning temperature until hatching. Embryonic and early larval development was evaluated with a stereomicroscope once a day, with the number of live embryos being recorded 24 h after egg collection. Hatched and viable larvae, as well as uptake of the yolk sac were also documented. Embryo survival was calculated as the number of eggs with live embryos/total number of fertilized eggs loaded in the microtiter plates. Hatching was calculated as the number of hatched eggs/number of live embryos on day 1. Larval survival was calculated as the number of live larvae on the day of mouth opening/number of hatched eggs. Accumulated Thermal Units (ATU) of spawning and hatching were calculated.

2.7. Statistical analyses

Data were expressed as the mean ± SEM (standard error of the mean). The GSI, RF, oocyte diameter, plasma concentration of E_2, gene expression of pituitary gonadotropins and vitellogenin were compared with a General Linear Model (GLM) analysis followed by Bonferroni *post hoc* tests with the factors "Treatment" (GnRHa treatment during the gametogenesis period) and "Temperature", and their interactions. The MIS levels were compared with GLM analysis followed by Bonferroni *post hoc* tests with the factors "GnRHa" treatment (gametogenesis period), "hCG" (spawning induction) and "Temperature" and their interactions. In all analyses, the level of significance was 95% ($P < 0.05$). Statistical analyzes were performed using IBM SPSS Statistic 22.

3. Results

3.1. Gene expression of pituitary hormones (*fshβ* and *lhβ*) and vitellogenin (*vtgA*)

Thermal manipulation and GnRHa injections had a significant interaction on *fshβ* gene expression during the gametogenesis period ($P = 0.01$), with increasing GnRHa levels resulting in higher *fshβ* gene

expression in females exposed to 27 °C (Fig. 2a).

Similarly, thermal manipulation and GnRHa injections also had a significant interaction on *lhβ* gene expression during the gametogenesis period (P = 0.01), but the effect was opposite to *fshβ* (Fig. 2b). Females exposed to 27 °C had significantly lower *lhβ* gene expression, while GnRHa therapy at the higher dose reduced *lhβ* gene expression significantly in females maintained at 20 °C.

Thermal manipulation and GnRHa injections had no significant interaction on *vtgA* gene expression during the gametogenesis period (P = 0.10), but females exposed at 20 °C had significantly lower *vtgA* gene expression when with GnRHa therapy (Fig. 2c).

3.2. Plasma steroids

Thermal manipulation and GnRHa injections had significant interaction on plasma E_2 levels during the gametogenesis period (P = 0.04), with increasing GnRHa levels resulting in lower E_2 in females exposed to 20 °C but not at 27 °C (Fig. 3).

Animals from all experimental groups induced with saline did not show statistical differences in MIS levels, and maintained low levels of this hormone when compared with females induced with hCG (Fig. 4). All animals except from the CTR at 27 °C spawned successfully after injection with hCG and exhibited higher MIS levels than the saline group, while all animals injected with saline did not spawn. Animals from the CTR group kept at 27 °C during the gametogenesis experiment, did not exhibit any increase in MIS after treatment with hCG, and the MIS levels were significantly lower than the levels of the hCG-injected fish in the CTR group at 20 °C (P = 0.150). The MIS levels of this group were also significantly lower from the other hCG treated animals at 27 °C (P = 0.015 for LD and P = 0.001 for HD) (Fig. 4).

3.3. Morphological analyses

3.3.1. Histological analyses

All animals of all groups exhibited the same proportion of perinucleolar (PN) and vitellogenic (V) oocytes in the ovaries that were classified as tertiary growth (TG) (Table 1).

3.3.2. Gonadosomatic index, oocyte diameter and potential relative fecundity

Both mean GSI (Fig. 5a) and oocyte diameters (Fig. 5b) remained the same throughout the experiment in all GnRHa treatments regardless of temperature exposure. However, RF was higher in the fish given the HD of GnRHa when fish were reared at 20 °C, while at 27 °C fish that were not treated with GnRHa had the highest value (see Figs. 5c and 6).

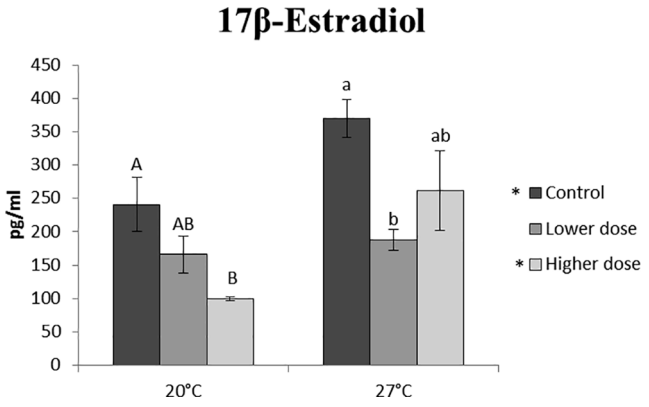

Fig. 3. Mean (± SEM or SD) plasma 17β-estradiol concentration in lambari *Astyanax altiparanae* females after 21 days of induction with GnRHa injections. Symbols mean statistical differences (P < 0.05) between temperatures in the same treatment. Capital letters differences between treatments at 20 °C and lowercase at 27 °C.

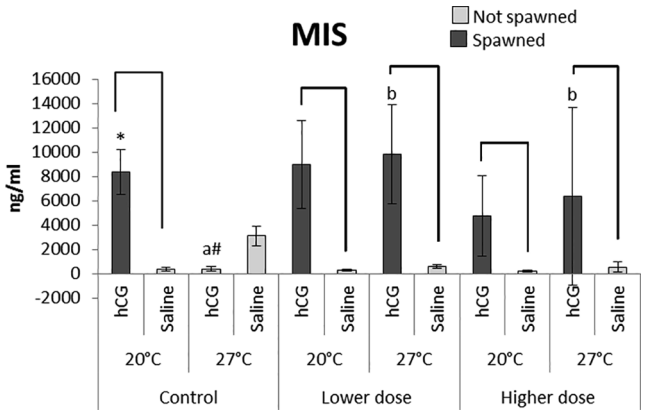

Fig. 4. Mean (± SEM or SD) plasma concentration of 17α-20β-dihydroxy-4-pregnen-3-one MIS in lambari *Astyanax altiparanae* females after 21 days of GnRHa injection at three doses (Control, Low and High). Light bars mean animals that did not spawn and dark bars animals that spawned. Brackets mean statistical differences (P < 0.05) between hCG and saline; symbols mean differences between temperatures within the same treatment and letters mean differences between treatments of GnRH.

3.4. Reproductive parameters

Females induced with hCG spawned in all groups except CTR at 27 °C, while the females treated with saline did not spawn. The highest number of oocytes was observed in the LD group at 27 °C, but fertilization and hatching success remained at 50%. An estimation of number of larvae was done according to the amount of eggs and their respective survival at different developmental stages. The highest estimated number of larvae was found in the HD group at 27 °C. The CTR females at 20 °C spawned a few eggs with low fertilization and hatching success. After hatching, there was no mortality of the larvae in the plates until mouth opening (Table 2).

The interaction, F values, and degrees of freedom of GLM analysis followed by Bonferroni post hoc tests with the factors are available as Table 3.

4. Discussion

Lambari makes short migrations within its freshwater environment during the reproductive period (Carolsfeld et al., 2003), and exhibit an asynchronous ovarian development with multiple spawns within the summer spawning season. However, when kept in captivity females do not spawn without hormonal or environmental manipulations (Chehade et al., 2015; Jesus et al., 2017). Previous studies with lambari (Jesus et al., 2017) and the congener *A. fasciatus* (Tolussi et al., 2018) demonstrated that females maintain vitellogenic oocytes in their ovaries throughout the year, both in captivity and in the wild; and apparently they are able to respond to artificial induction of maturation and spawning throughout the year. In the present study we demonstrated that thermal manipulation during the winter, together with exogenous GnRHa treatment, are necessary to optimize gamete production and the availability of larvae.

The role of FSH is well known in fish reproduction, mainly in salmonids, stimulating the proliferation of oogonia and the production of E_2, which in turn regulates vitellogenesis by the liver and oocyte growth (Levavi-Sivan et al., 2010; Lubzens et al., 2010). Gene expression of *fshβ* in female lambari did not change with the exposure to summer spawning temperatures alone. But when the females were exposed to summer temperatures during the winter and were also treated with GnRHa, *fshβ* gene expression increased, resulting also in higher plasma E_2 levels. However, in the absence of GnRHa injections, the increase in temperature alone was able to increase E_2 levels, indicating that other factors than plasma FSH may be also regulating the production of E_2, as

Table 1
Classification of ovarian maturation stage based on phases of oocyte development of lambari exposed to thermal manipulation and GnRHa injections.

Treatment	Temperature	Phase of Oocyte Development					POF	Maturation Stage
		PN	CA	V	Nuclear migration	A		
CTR	20 °C	+ +	–	+ +	–	–	–	TG
	27 °C	+ +	+	+ +	–	–	–	TG
LD	20 °C	+ +	–	+ +	–	–	–	TG
	27 °C	+ +	+	+ + +	–	–	–	TG
HD	20 °C	+ +	+	+ +	–	–	–	TG
	27 °C	+ +	+	+ +	–	–	–	TG

CTR: saline control; LD: GnRHa lower dose; HD: GnRHa higher dose; PN: perinucleolar; CA: cortical alveoli; V: vitellogenic; A: atretic; POF: post-ovulatory follicle; + present; + + frequent; + + + predominant; − absent; TG: tertiary growth.

LH that is released at ovulation and can also stimulate E_2 release (Mylonas and Zohar, 2001; Reading et al., 2017). In striped bass (*Morone saxatilis*) after GnRHa injections, LH and E_2 (as well as T) increased and once GnRHa was cleared from the circulation LH decreased significantly and plasma E_2 and T returned to pre-treatment levels.

At both temperatures, injections of GnRHa were accompanied by a decrease in E_2 levels, perhaps due to a previous negative feedback response, since oocytes were already vitellogenic and at this time it would be no longer necessary to produce E_2 but progestogens. The *fshβ* in this species was evaluated throughout the reproductive cycle in females kept in captivity, being higher in summer and decreasing from fall to winter (Jesus et al., 2017), demonstrating the important role of environmental cues in the production of this hormone.

Ovarian E_2 stimulates VTG synthesis by the liver in oviparous teleosts, and *vtgA* expression was higher in lambari kept at 20 °C when compared with 27 °C. The increase in VTG sequestered by the oocytes can alter hepatic VTG synthesis, suppressing E_2 production (Reading et al., 2017) and temperature seems to be affecting this process. When females were treated with GnRHa, perhaps because vitellogenesis was already in progress, the effects were observed just at the lower temperature, when the decrease of E_2 in the HD GnRHa group reflected lower *vtgA* gene expression, suggesting somehow that GnRHa compensates the effect of lower temperature. The maintenance of the animals at 27 °C was probably enough for the oocytes to reach the ovulation diameter, so the supplementation with GnRHa, did not increase vtgA gene expression.

During the gametogenesis phase of the reproductive cycle in fish, increased ovarian mass is mainly achieved due to the incorporation of VTG produced in the liver, in response to increased plasma E_2 levels (Lubzens et al., 2010). Although these differences found in *fshβ*, *vtgA* and E_2 levels, GSI, oocyte diameter and gonadal histology did not change in the different experimental groups, but temperature modulates the number of vitellogenic oocytes. When females were maintained at lower temperature, a higher dose of GnRHa compensated RF. On the other hand, at 27 °C, RF was lower in animals treated with GnRHa, probably due to some physiological variable that was not

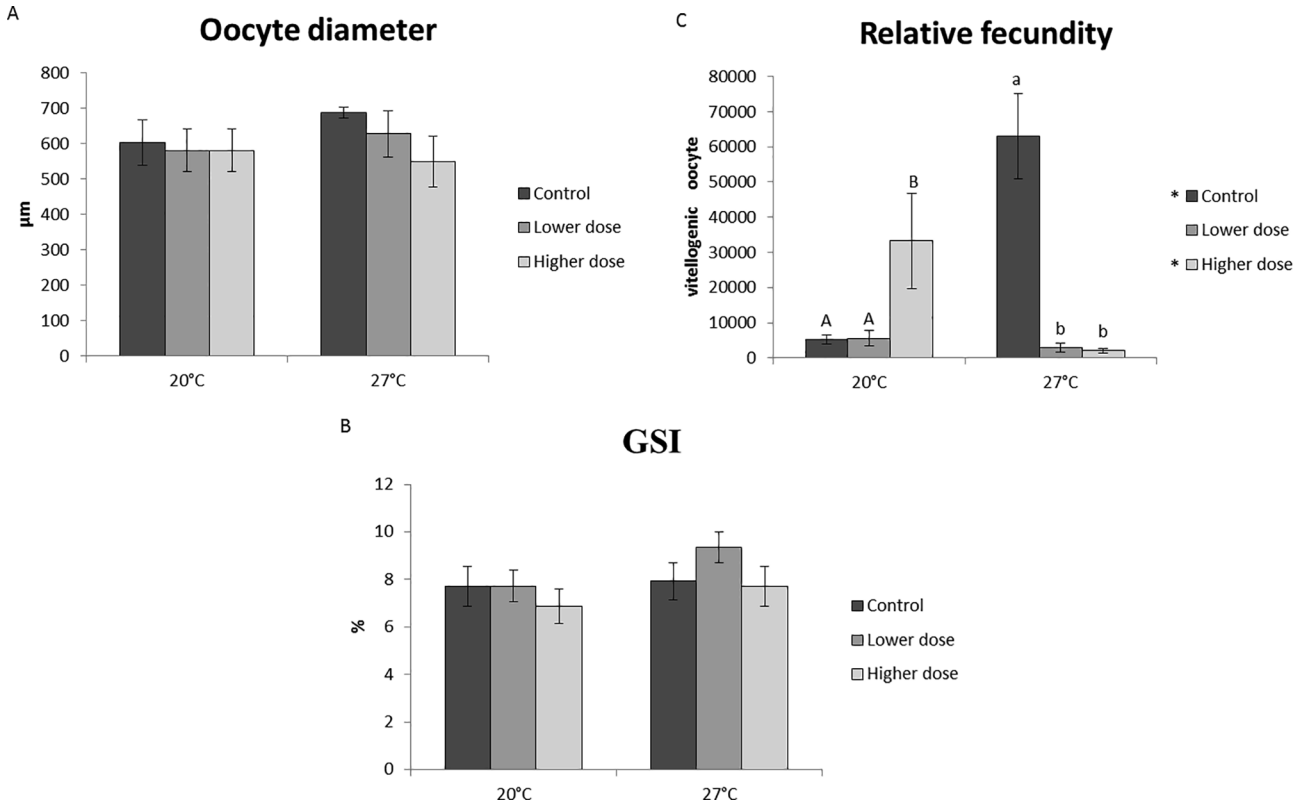

Fig. 5. Mean (± SEM or SD) oocyte diameter (A), Gonadosomatic index (GSI) (B) and relative fecundity (C) in lambari *Astyanax altiparanae* females after 21 days of GnRHa injections. Symbols mean statistical differences ($P < 0.05$) between temperatures in the same treatment. Capital letters differences between treatments at 20 °C and lowercase at 27 °C.

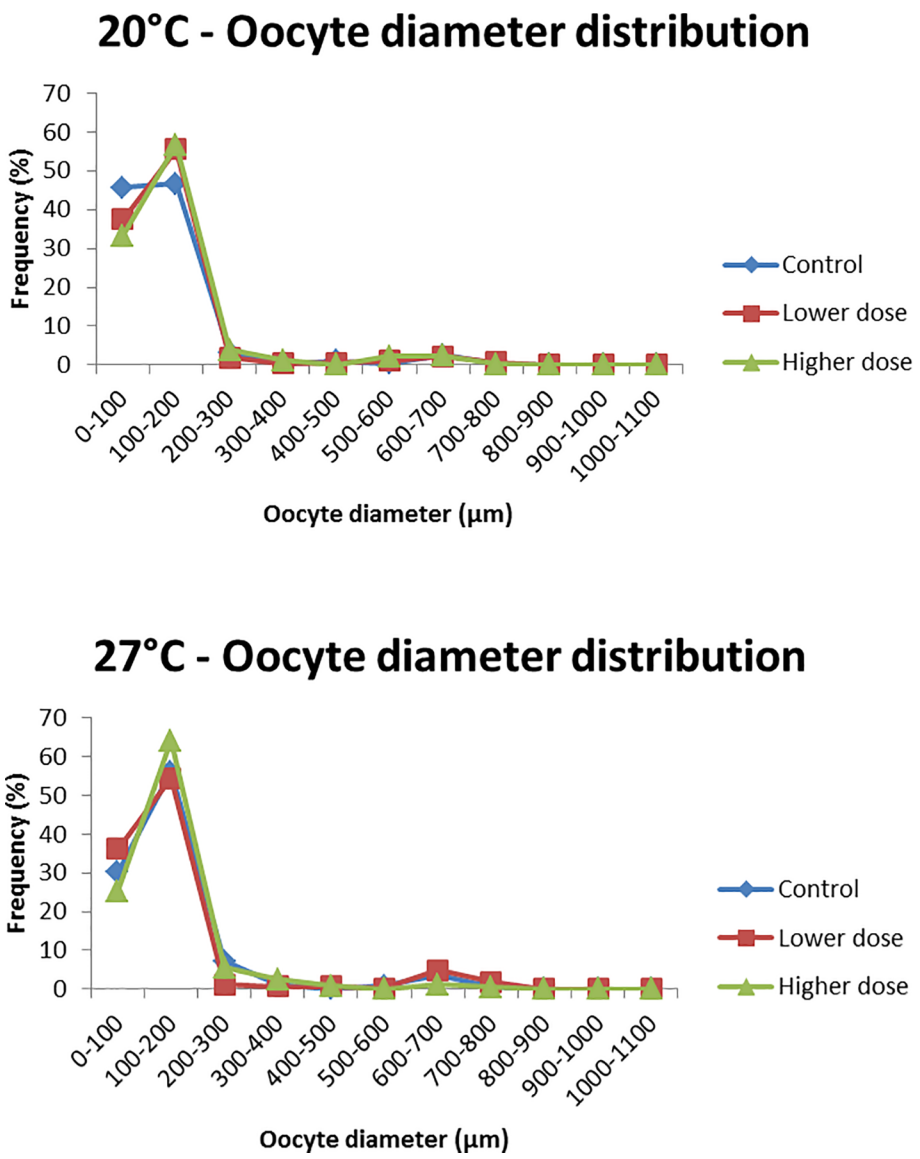

Fig. 6. Distribution frequency of oocyte diameters (mean) of *Astyanax altiparanae* females after 21 days of GnRHa injections. Different line colors indicate different treatments at the same temperature.

evaluated in this study, however this profile did not interfere with the spawning performance.

It is well known in salmonids and other teleost species that when oocytes complete vitellogenesis, there is a decrease in FSH levels in response to E_2 negative feedback at the level of the pituitary, and a positive feedback in the synthesis of LH together with the action of GnRH from the hypothalamus (Levavi-Sivan et al., 2010; Lubzens et al., 2010). Pituitary LH stimulates theca cells to produce 17α-hydroxyprogesterone (17αOHP), which is transported to the follicular cells and is converted to 17α-20β-dihydroxy-4-pregnen-3-one (17α20βDHP), the MIS that induces oocyte maturation in most teleosts (Levavi-Sivan et al., 2010; Lubzens et al., 2010). In lambari, *lhβ* gene expression was higher at 20 °C, suggesting the lower temperature compensation, except when GnRHa was injected at the HD, when probably this compensation was not necessary due to the higher GnRH stimulation. However, when females from all these groups were

Table 2
Spawning performance parameters in lambari females induced to mature and spawn with hCG after thermal manipulation and GnRHa injections during the gametogenesis period.

Group	Temperature	Number of eggs (per 4 females)	Fertilization (%)	Hatching (%)	Accumulated Thermal Units (ATU)	Interval in hours	Estimated number of larvae
CTR	20 °C	4.010	42	10	330	16.5	168
	27 °C	NS	NS	NS	NS	NS	NS
LD	20 °C	8.340	70	47	340	17.0	2743
	27 °C	18.360	50	50	140	5.1	4590
HD	20 °C	7.960	92	73	340	17.0	5345
	27 °C	9.648	88	83	280	10.3	7046

CTR: saline control; LD: GnRHa lower dose; HD: GnRHa higher dose; NS: not spawned.

Table 3
F values and degrees of freedom with statistical analyses of General Linear Model analysis followed by Bonferroni post hoc tests with the factors "Treatment" and "Temperature" and the interactions.

Tests of Between-Subjects Effects

Dependent Variable: *βfsh*			Dependent Variable: *vitellogenin*		
Source	F	Sig.	Source	F	Sig.
Corrected Model	3.844	0.006	Corrected Model	4.411	0.002
Intercept	28.555	0.000	Intercept	13.228	0.001
Treatment	2.003	0.148	Treatment	5.605	0.006
Temperature	2.562	0.117	Temperature	6.033	0.017
Treatment* Temperature	5.199	0.010	Treatment*Temperature	2.406	0.100
a. R Squared = 0.314 (Adjusted R Squared = 0.232)			a. R Squared = 0.290 (Adjusted R Squared = 0.224)		
Dependent Variable: *βlh*			Dependent Variable: 17β-Estradiol		
Source	F	Sig.	Source	F	Sig.
Corrected Model	8.314	0.000	Corrected Model	4.465	0.000
Intercept	17.606	0.000	Intercept	18497.251	0.000
Treatment	4.684	0.015	Treatment	18.119	0.000
Temperature	14.496	0.000	Temperature	5.007	0.029
Treatment* Temperature	4.981	0.011	Treatment* Temperature	4.376	0.041
a. R Squared = 0.497 (Adjusted R Squared = 0.438)			a. R Squared = 0.454 (Adjusted R Squared = 0.353)		
Dependent Variable: MIS			Dependent Variable: GSI		
Source	F	Sig.	Source	F	Sig.
Corrected Model	14.258	0.000	Corrected Model	1.135	0.351
Intercept	103.393	0.000	Intercept	654.477	0.000
GnRH	8.949	0.001	Treatment	1.373	0.261
hCG	73.783	0.000	Temperature	2.082	0.154
temperature	10.700	0.002	Treatment*Temperature	0.425	0.656
GnRH * hCG	16.190	0.000			
GnRH * temperature	5.688	0.007			
hCG * temperature	4.534	0.041			
GnRH * hCG * temperature	12.659	0.000			
a. R Squared = 0.822 (Adjusted R Squared = 0.764)			a. R Squared = 0.079 (Adjusted R Squared = 0.009)		
Dependent Variable: Oocyte diameter			Dependent Variable: Relative fecundity		
Source	F	Sig.	Source	F	Sig.
Corrected Model	3.110	0.002	Corrected Model	2.916	0.004
Intercept	552.439	0.000	Intercept	32.907	0.000
Treatment	2.806	0.105	Treatment	0.005	0.945
Temperature	0.671	0.519	Temperature	0.333	0.566
Treatment* Temperature	0.590	0.561	Treatment* Temperature	2.019	0.161
a. R Squared = 0.356 (Adjusted R Squared = 0.241)			a. R Squared = 0.364 (Adjusted R Squared = 0.239)		

induced to spawn with hCG at the end of the GnRHa exposure period, the MIS levels after spawning were not different in females exposed to the different experimental conditions. Only the females of the CTR at 27 °C treatment did not spawn in response to hCG treatment, which was reflected in lower plasma MIS levels, even if gene expression of *lhβ* during the gametogenesis period was similar to the other groups. The absence of spawning in the animals of control groups was expected, however the spawning of the animals of the control group at 20 °C can be related to thermal stress, a physiological process not assessed in this study. It is well known that stress can have a positive effect in ovulation in teleosts (Milla et al., 2009; Schreck, 2010) and in *A. altiparanae* triggers ovulation and spawning (Chehade et al., 2015).

The LH stimulates the shift in the ovarian follicle steroidogenic pathway from the production of E_2 to the production of MIS, which binds to oocyte membrane receptors activating the synthesis of maturation promoting factors that activate the germinal vesicle breakdown (Nagahama and Yamashita, 2008). The results of *lhβ* combined with the MIS profile in lambari in the present study suggest that at lower temperatures increased *lhβ* gene expression allows the production of MIS at levels that OM, ovulation and spawning may occur. When GnRHa was injected at HD, there was no temperature effect on *lhβ* gene expression.

Temperature manipulation and GnRHa treatment during the gametogenesis period influenced the spawning performance of lambari, with females injected with HD of GnRHa and maintained at 27 °C exhibiting higher hatching success and estimated larvae production. This result was corroborated by Evangelista et al. (2015), who manipulated temperature and photoperiod during spawning induction of lambari during the winter. Our data demonstrate that previous supplementation of GnRHa during a period of 3 weeks optimizes the spawning performance, compared with the effects of temperature manipulation.

5. Conclusion

The study confirms that lambari maintain large numbers of vitellogenic oocytes during the winter "non-spawning" season, and that these oocytes can be induced to mature and produce viable eggs and larvae after hormonally induced spawning. Thermal manipulation with summer temperatures alone increased the number of vitellogenic oocytes and ensured good reproductive performance, while weekly injections of GnRHa had a similar effect when administered at winter temperatures and the females exhibited better reproductive indices. Even with significant differences in the gene expression of gonadotropins among different thermal manipulations/GnRHa treatment

combinations, mean GSI and oocyte diameter did not change, but GnRH increased the number of vitellogenic oocytes in lower temperature. During vitellogenesis GnRHa administration seems to be able to overcome the effects of lower temperature, but the reproductive performance of lambari is better with the combination of GnRHa injection and exposure to high temperatures.

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