GnRHa implants and temperature modulate Lambari -do - rabo - amarelo, *Astyanax altiparanae* (Characiformes: Characidae) induced reproduction out of the reproductive season.

Lambari reproduction modulation in winter

Gabriela Brambila-Souza [1], Constantinos Chrysanthos Mylonas [2], Paulo Henrique de Mello [1], Rafael Yutaka Kuradomi [3], Sergio Ricardo Batlouni [1], Carlos Eduardo Tolussi [4], Renata Guimarães Moreira [5*].

*1 - UNESP- Universidade Estadual Paulista - Centro de Aquicultura (*CAUNESP*), Jaboticabal, SP, Brazil.*
2 - Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Center for Marine Research, Heraklion Crete, Greece.
3 – Universidade Federal do Amazonas, Manaus, AM, Brazil.
4 – Universidade Anhembi Morumbi, São Paulo, SP, Brazil.
5 - Instituto de Biociências –Universidade de São Paulo (USP), SP, Brazil.
*Corresponding author: renatagm@ib.usp.br

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Ethics approval statement

The experimental protocol was approved by the Ethics Committee on Animal Use, CEUA, Institute of Biosciences, University of São Paulo, protocol 228/2015. 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).

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Abstract

We evaluated the effect of gonadotropin in controlled-release implant together with temperature manipulation in the reproduction of *Astyanax altiparanae* (lambari) during the non-breeding season. Three group of adult females were maintained at 20°C and three at 28°C. For each temperature, one group was treated with 4 μg GnRHa g^{-1}, other with 8 μg GnRHa g^{-1} and other was sham implanted as a control during 4 weeks. Afterwards, 4 females/experimental group were induced to spawn with human chorionic gonadotropin, while 4 females/group were injected with saline. GnRHa implants increased *fshβ* and *lhβ* gene expression in females maintained at 28˚C. The *vtgA* gene expression increased in the high GnRHa dose at 20˚C, while this higher GnRHa dose associated with the higher temperature increased Gonadosomatic Index. The hCG injection increased plasma Mature Induction Steroid levels and trigger ovulation, except in control at 28°C. The control females at 20°C spawned but with low reproductive performance, confirming that this is not a limiting temperature for reproduction. The administration of GnRHa as implant increased gonadotropins gene expression at 28ºC, temperature at which GSI also increased. Qualitatively, the implants improved reproduction at 20°C, providing offspring out of the reproductive season, improving lambari production throughout the year.

Keywords: gonadotropins; neotropical fish; ovaries; steroids; vitellogenin

1. Introduction

The characid lambari-do-rabo-amarelo (lambari) *Astyanax altiparanae*, has a great potential for aquaculture in South America, due to its easy management, acceptance of artificial food, sexual dimorphism, short growth cycle, reaching commercial size in about three months (Gonçalves et al., 2014; Porto-Foresti, 2010). *A. altiparanae* have been used as a model to study reproductive biology (Abreu et al., 2021; Cassel et al., 2017a,b; Chehade et al., 2020; Costa et al., 2014; De Paiva Camargo et al., 2017; Gomes et al., 2013; Jesus et al., 2017), however, few studies focused on applying physiological tools to enhance the production of this fish species (Brambila-Souza et al., 2019).

Lambari has the first maturity at 4 months (Porto-Forest et al., 2010), exhibits a rapid ovarian development and after a single spawning, the volume of complete vitellogenic oocytes decreases by 94% and the females are mature again about a week later (Lira et al., 2018), even though spawning in the wild is observed only during the summer. Vitellogenesis and spermiogenesis apparently occurs throughout the year in captivity (Jesus et al., 2017), but with a low gamete production. This gamete production, even if it is low, allows this fish to respond to artificially hormonal induced reproduction, outside the reproductive period, in the winter (Brambila-Souza et al., 2019; Evangelista et al., 2019). This is an important characteristic for captive rearing and commercial aquaculture, allowing juvenile production throughout the year (Duncan et al., 2013), decreasing the off-season for the producers.

Fish reproduction out of the reproductive period can be improved by the manipulation of the environmental factors and/or by hormonal therapies (Mylonas et al., 2010; Zohar and Mylonas, 2001). Reproduction in teleosts is modulated by environmental cues, such as temperature and photoperiod which stimulate the hypothalamus-pituitary-gonads (H-P-G) axis, through the release of gonadotropin-releasing hormone (GnRH), gonadotropin-inhibitory hormone (GnIH) and dopamine. These neurohormones modulate the synthesis and release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) by the adenohypophysis. In females, FSH acts on the ovarian follicular cells, stimulating the synthesis of 17β-estradiol, which acts on the liver stimulating the

synthesis of vitellogenin (vtg). The increase in 17β-estradiol (E2) plasma levels triggers a negative feedback in FSH synthesis and increases LH secretion, that stimulates progestogens synthesis responsible for oocytes final maturation and ovulation (Alix et al., 2020; Lubzens et al., 2010; Schulz et al., 2010; Zohar et al., 2010).

One hormone used to induce fish spawning is the gonadotropin releasing hormone agonist (GnRHa) which present more advantages because of its lower species-specificity and targeting a higher level of pituitary-gonads axis, stimulating the fish to produce and release the endogenous gonadotropins (Mylonas et al., 2010). For over 30 years, GnRHa can be administered as injections (liquid injection), or as controlled-release implants of cholesterol or ethylene-vinyl acetate (EVAc) or injectable biodegradable microspheres (Barbaro et al., 2002; Mylonas et al., 2010; Mylonas et al., 2007; Mylonas and Zohar, 2001; Weil and Crim, 1983, Zohar, 1988, 1996).

Considering the aquaculture importance of lambari, the potential of this species to maintain vitellogenic follicles throughout the year in captivity and the fact that vitellogenesis is a rapid process in this species, in the present study we examined the potential of sustained treatment with GnRHa via implants together with temperature manipulation, on stimulating vitellogenesis. Since this species does not spawn spontaneously in captivity, spawning induction can be implemented using the established methods used during the natural reproductive season after a period of GnRHa uninterrupted release. In a previous study it was observed that the increase in water temperature (27°C), associated with GnRHa injections generated positive effects on spawning parameters of lambari females (Brambila-Souza et al., 2019). However, this study used weekly injections, a method that involves animal handling and GnRHa levels are not maintained constant as a slow release system. Therefore, in this study we examined the effects of different concentrations of GnRHa, delivered as implants, associated with thermal manipulation on the reproductive physiology of lambari outside the reproductive season. We also evaluated if spawning, fertilization and larval rearing were viable under winter temperatures.

2. Material and methods

124 2.1. Experimental design

125 The animals used in this experiment were produced in the Centro de
126 Aquicultura da Universidade do Estado de São Paulo (CAUNESP), Jaboticabal,
127 São Paulo, Brazil (21°15′17″ S 48°19′20″ W) and were 1-year-old at the time of
128 the experiment. Two hundred and forty lambari adult females (10±1.8g and
129 9±0.4 cm) were divided randomly among 12 tanks of 750 L (20 animals/tank)
130 divided in 6 experimental groups in duplicates (there were no statistical
131 differences in the initial and final weights and lengths of the animals in each
132 experimental unit P>0.05). Three groups were maintained at 20°C and three at
133 28°C, for a total period of 5 weeks. After 7 days (period of acclimation), for each
134 temperature one group was treated with implants at a dose of 4 µg GnRHa g^{-1}
135 body weight, one group with 8 µg GnRHa μg^{-1} and one group was sham
136 implanted as a control. The control fish were implanted with microchips of the
137 same size as the implants, just to simulate the same incision procedure. The
138 doses were based on experiments that induced sexual maturation in other
139 teleost species (Kagawa et al., 2009; Kanemaru et al., 2012). The EVAc
140 GnRHa implants (Mylonas and Zohar, 2001) were loaded with D-Ala^6, Pro^9, NEt
141 GnRHa (Bachem Bioscience, King of Prussia, PA) and takes about 21 days for
142 total hormone release (Mylonas and Zohar, 2001). The implants, as well as the
143 microchips in the control group, were implanted with a syringe fitted with an 18-
144 gauge needle, in the basis of the pectoral fin. The experimental design was
145 based on the one used by Brambila-Souza et al., (2019) (Fig.1).

146

147 2.2. Sampling procedure

148 At the end of the first experimental period (21 days, under natural
149 photoperiod), hereafter named as gametogenesis experiment, 6 females were
150 sampled from each tank for the following analyses: gonadotropin and *vtg* gene
151 expression, plasma E2 concentration, gonadosomatic index (GSI), oocyte
152 diameter and relative fecundity. To ensure that spawning and larval rearing
153 would be viable at 20°C at day 22, four females from each experimental group
154 (2 females/tank), (from the experimental unit, but not sampled in the
155 gametogenesis experiment) were induced to spawn with an injection of 5000 IU
156 $kg^{-1}$of human chorionic gonadotropin (hCG, ovidrel, Merck) and the same

number of animals (4 females/group) were injected with saline (NaCl 0.9%) as control (spawning experiment). The 4 females from each treatment (gametogenesis experiment) were placed in a 10L spawning tank with 8 males (6 spawning tanks with females injected with hCG and 6 injected with saline). These spawning tanks consisted in plastic boxes with running water, where females and males (4 females: 8 males) were allowed to spawn in a semi-natural procedure (fish spawn spontaneously in the tank). Males were taken from the same initial ponds of females at CAUNESP 24 hours before induction and stored at the same temperatures used during the gametogenesis experiment (*i.e.* 20 or 28°C), and were induced with 3 mg kg^{-1} of carp pituitary extract (Yasui et al., 2014). Six hours after the first visualization of eggs in the collection boxes a sample of blood was collected to analyze plasma levels of 17α-20β-dihydroxy-4- pregnen-3-one (maturation inducing steroid, MIS). Fertilization (%), hatching (%) and embryonic development from each spawning tank were monitored, and larvae was maintained until the mouth opening.

Fish were anesthetized with 0.1 g/L of benzocaine, and blood samples were collected by puncturing the caudal vein with disposable heparinized needles and syringes (Hepamax® 5000 IU). Blood samples were centrifuged at 655.1*g* (5 minutes), and plasma was maintained at −80°C. Afterwards, length (cm) and body mass (g) were recorded and females were euthanized by sectioning the spinal cord at the level of the operculum. The ovaries were removed and weighed to calculate gonadosomatic index (GSI = [gonads mass/body mass-gonad mass] × 100). Portions of the ovaries were stored to calculate fecundity and measure the oocyte diameter average. The pituitary gland and liver were collected in RNA*later*® (Invitrogen) and maintained at −80°C for genes expression analyses of β-subunits of follicle-stimulating hormone and luteinizing hormone (*fshβ and lhβ*) and vitellogenin A (*vtgA*) respectively.

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

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. The RNA purity and concentration were checked by agarose gel electrophoresis and Nanodrop™ Spectrophotometer (ND-1000) ensured that the OD260/280 of all samples ranged between 1.7 and 2.0. Genomic DNA was removed with DNase using the DNA-free Turbo kit (Life Technologies®). The purified RNA was stored and maintained at −80°C. For cDNA synthesis, a commercial kit (SuperScript™ II Reverse Transcriptase) was used according to the manufacturer's protocol with the samples at a RNA concentration of 10ng/µl for pituitary and 200ng/µl for liver.

The real-time PCR primers for *fshβ* (Forward: GTCCTGATGATTCTGCTGCT; Reverse: GCATTCCTCGCTCTCCAC) (KJ544557/ r^2 0.999) and *lhβ* (Forward: TGCCCAAAATGCCTAGTGTTTC; Reverse: TCTTGTACACCGGATCCTTGGT) (KJ544556 / r^2 0.999) genes of lambari have been already designed by Jesus et al., (2017) and the primer for *vtgA* gene (Forward: GCCTCTGCGTTGTTGATCTT; Reverse: AAACTCTGACCCTGCTGGAA) was designed by Tolussi et al., (2018) for the congeneric species *Astyanax fasciatus* and used successfully in lambari (r^2 0.997) (Brambila-Souza et al., 2019). The real-time PCR reaction was performed in duplicate using 12.5 µl of Power Sybr Green PCR Master Mix (Applied Biosystems), 0.5 µM primers (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). As an endogenous reference, the *β-actin* (Forward: CCCAGTCCTTCTCAC; Reverse: ACCAGAAGCGTACAG) (r^2 0.998) gene was used to calculate the relative expression of *fsh*β, *lh*β and *vtgA*. The efficiency of the primers used in the assay varied from 98.0 to 104.9%. Gene expression was calculated according to the value of Ct, which was normalized by ΔΔct through the endogenous gene. The control group in each temperature was used as Delta Ct calibrator (Livak and Schmittgen, 2001).

2.4. Plasma steroid analysis

Plasma E2 levels were measured in pre-spawning females by an enzyme linked immunosorbent assay (ELISA), using commercial kits (IBL®, International/Hamburg, Germany). 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%). Plasma MIS was quantified in females after spawning using a commercial ELISA (Cayman Chemical®) according to manufacturer's protocol. CV was 19.3% while the inter-assay CV was 12.3%. All analyses and plasma dilutions were carried out according to the method validated for this species (Brambila-Souza et al., 2019) and absorbance was measured using a microplate reader (Spectra Max 250 Molecular Devices) at 405 nm.

2.5. Oocyte diameter and relative fecundity

Pre-weighed ovary samples were fixed with Gilson solution (Simpson, 1951) for 30 days to detach the oocytes and then samples were stored in 70% ethanol. Oocytes were sampled with a 2 ml Stempel pipette (Hensen Stempel Pipette ™ 1806 series; Wildlife Supply Company, Florida, USA), counted and measured in triplicate using the computer imaging system (Leica DM1000 light microscope, Leica DFC295 photographic camera, Leica Application Suite Professional, LASV3.6). The oocytes with diameter larger than 601 µm were considered vitellogenic for the fecundity estimative (Brambila-Souza et al., 2019). Relative fecundity (RF) was expressed in relation to body mass (grams) after measuring and counting the oocytes (Vazzoler, 1996).

2.6. Reproductive parameters

To ensure that the larvae would survive at low temperatures, in each group, the fertilization success (Fertilization = number of fertilized eggs × 100/total number of eggs) was calculated. To calculate embryo development and larval survival, fertilized eggs were placed individually in 96-well microtiter plates (in duplicate), covered with a plastic lid and maintained under the spawning temperature of the collected eggs until hatching, following the

protocol of Panini et al., (2001). Embryonic and early larval survival was evaluated with a stereoscope once a day. The number of live embryos was recorded 1 day after egg collection. Viable larvae, as well as uptake of the yolk sac were also registered. Embryo survival was calculated as the number of eggs with live embryos/total number of fertilized eggs placed 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 the mouth opening/number of hatched larvae. Estimated larvae number = total eggs -% of unfertilized eggs -% of eggs not hatched -% of deaths. Accumulated Thermal Units (ATU) of spawning was calculated as the sum of the water temperature per hour, from the hormonal application until spawning (Weingartner and Filho, 2005).

2.7. Statistical analysis

Statistical analysis was performed using IBM SPSS Statistic 22. Data were expressed as the mean ± SEM (standard error of the mean). For each dependent variable (GSI, relative fecundity, oocyte diameter, gene expression levels of *fshβ*, *lhβ, vtgA* and plasma level of E2) we used a General Linear Model (GLM) having the predictor variables treatment (control, GnRH 4ug/g and GnRH 8 ug/g) and temperature (20 and 28ºC). When the effect of one or both predictor variables was obtained, the Bonferroni post hoc test was performed with the interactions. MIS levels were also compared with GLM having the predictor variables treatment (control, GnRH 4ug/g and GnRH 8 ug/g from the gametogenesis period), temperature (20 and 28^0C) and spawning substance (hCG and saline) followed by Bonferroni post hoc tests and their interactions. In all analyses, the level of significance was 95% ($P<0.05$).

3. Results

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

Implants of GnRHa and thermal manipulations resulted in interaction in gonadotropin gene expression during the gametogenesis period, with the high GnRHa dose increasing *fsh*β ($P= 0.027$) and *lh*β ($P= 0.009$) gene expression in

females maintained at 28°C (Fig. 2A, 2B). Regarding gene expression of *vtgA* there was also interaction during the gametogenesis period (P=0.0001), with the higher dose of GnRHa increasing the expression of this gene, but in females maintained at 20°C, and without alteration in the animals maintained at 28°C (Fig.2C).

3.2. Plasma steroid analysis

Thermal manipulation and GnRHa treatment did not show interaction in plasma E2 levels during the gametogenesis period (P=0.097), but animals maintained at the higher temperature without GnRHa administration, showed an increase in the plasma concentration of this steroid (P=0.011) (Fig. 3).

Animals of all saline-induced experimental groups did not show statistical differences in MIS levels and maintained low levels of this hormone when compared to females induced with hCG (Fig. 4). All groups, except control maintained at 28°C, had success in spawning after hCG injection and exhibited higher levels of MIS, while all animals injected with saline did not spawn. As there was an increase in the dose of GnRHa, there was also a significant increase in the plasma concentration of MIS in females induced to ovulation with hCG. There was a interaction between the GnRHa treatment, temperature and hCG injection with the higher MIS concentration in females maintained at 28°C previously injected with the higher GnRH dose (P= 0.0001, Fig.4).

3.3. Gonadosomatic index, oocyte diameter and relative fecundity

Thermal manipulation and GnRHa treatments showed interaction in GSI values (P=0.019). Females maintained at 20°C showed increased GSI in the group treated with the lower dose of GnRHa compared with females treated with the higher GnRHa dose (P=0.011) and females maintained at 28°C showed higher GSI compared with females maintained at 20°C in the higher GnRHa dose (P=0.012) (Fig. 5). There was no interaction of GnRHa treatment and temperature in oocyte diameter (P=0.784) and relative fecundity (P=0.836) (Fig. 5).

318

319 3.4. Reproductive parameters

320 Animals induced with hCG spawned (single spawning) except for the
321 control group maintained at 28°C. Spawning time after hCG induction varied
322 according to the temperature (from 6 to 16 hours). The largest spawning in
323 absolute numbers occurred in females previously implanted with the higher
324 dose of GnRHa and maintained at 20°C. These females also presented
325 qualitatively the highest fertilization and hatching, thus generating a higher
326 estimated number of larvae. Animals of the control group maintained at 20°C
327 (gametogenesis experiment) spawned, but with low reproductive parameters,
328 thus generating a lower estimated number of larvae than the other groups
329 (Table 1). The animals induced with saline, regardless of the treatment in the
330 gametogenesis experiment, did not spawn.

331

332 **4. Discussion**

333

334 This study showed that GnRH implants improved lambari reproduction at
335 20°C, providing eggs and larvae out of the reproductive season. Some studies
336 carried out with *A. altiparanae* under natural temperature have been showed
337 that females actually maintain vitellogenic oocytes in the ovary throughout the
338 year, but GSI clearly decreases during the winter, with values near to 5.0%,
339 followed by a clear trend to reduction in E2 plasma levels (about 75 pg/ml) to
340 half of the summer values (about 150 pg/ml) (Jesus et al., 2017). Our data show
341 that regarding the thermal treatment, the maintenance of control fish at 28°C,
342 without GnRHa supplementation, triggered an increase in E2 levels to values
343 about 380 pg/ml, but did not improve the parameters of the vitellogenic process,
344 such as GSI, fecundity or oocyte diameter. However, when the higher
345 temperature (28°C) was combined with higher doses of GnRHa implants the
346 gene expression of both gonadotropins increased. Additionally, the implant of
347 the high dose of GnRHa triggered an increase in GSI in females to values near
348 10.0% when maintained at 28°C, compared with 20°C, suggesting that the
349 combination of both factors (*i.e.*, higher temperature and GnRHa dose)

accelerated vitellogenesis in *A. altiparanae* during the winter. The increase in E2 plasma levels at the higher temperature (about 350 pg/ml) was also obtained by Brambila-Souza et al., (2019) in this same species, however, the supplementation of GnRHa carried out by these authors, using weekly injections, even triggering the increase in the gene expression of gonadotropins, was not able to increase GSI , that was maintained below 10%. These differences suggest that the process of ovarian maturation was more efficient when *A. altiparanae* females were uninterruptedly exposed to GnRHa delivered as implants.

The effects of GnRHa implants in gene expression of *vtgA* did not follow the same pattern of gonadotropins. An increase in the expression of this gene in fish implanted with the higher dose of GnRHa was observed, but only in females maintained at 20°C, suggesting that at lower temperatures even with the same E2 plasma levels, there was a compensation in *vtgA* gene expression in these animals, but even though GSI was lower in females implanted with the high GnRHa dose at 20°C. The increase in *vtgA* gene expression in lower temperature was also observed in *A. altiparanae*, but without any supplementation of GnRHa (Brambila-Souza et al., 2019), suggesting that the lower temperature is a trigger to induce *vtgA* synthesis in this species. The increase in ovarian mass is mainly due to the uptake of vitellogenin in response to plasma E2 levels (Lubzens et al., 2010) and temperature can modulate this process (Anderson et al., 2017). Due to the relationship between the process of vitellogenesis and the increase of ovarian volume and mass, GSI value is considered as an important parameter to evaluate the maturation status of fish (Flores et al., 2019). These parameters evaluated together suggest that the higher GnRHa dose stimulates pituitary gonadotropin gene expression at 28°C, but the action at ovarian level (E2 synthesis) does not necessarily follows the same profile. Additionally, although it is suggested a compensation in *vtgA* gene expression at 20°C using GnRHa at the higher dose, the uptake probably did not follow the synthesis, as GSI reached the lowest level in this experimental group. However, as stated, the rate of vitellogenin synthesis and uptake by the oocyte is not necessarily correlated, as it is a process receptor mediated (Reading et al., 2017).

The *lhβ* gene expression data corroborate those of *fshβ*, which increased in fish implanted with GnRHa in 8µg/g but only at the higher water temperature, maintaining lower expression levels at 20°C. This profile suggests that at winter temperature the implant did not stimulate pituitary gland responses, however, even with a lower *lh*β expression, if hCG was supplemented, the females produced MIS and ovulated. The reproduction parameters measured showed that females treated with GnRHa and injected with hCG spawned even at 20°C, while females of the control group at 28°C, even injected with hCG did not spawn, and this failure to spawn can be attributed to the deficiency of MIS production and not to insufficient *lhβ* expression, as MIS levels is these females were similar with the levels of the females from the control group. In contrast, animals from the control group, but maintained at 20°C showed higher plasma concentration of MIS when compared to animals injected with saline, and spawned, probably due to some physiological trigger not evaluated in this study, which could be related to stress axis (brain-pituitary-interrenal) which is also related with reproduction (Milla et al., 2009). Previous studies showed that *A. altiparanae* exposed to high densities and high water renewal spawn spontaneously without hormonal induction, during the spring and summer period (Chehade et al., 2015) and shows an increase in plasma cortisol concentration, exhibiting the so-called stress induced spawning (Parreira, 2015).

It is important to highlight that the induction of females to spawning was carried out just to be sure that the larvae from all treatments would survive at 20°C, otherwise it would not make sense to stimulate vitellogenesis at this temperature. Thus, it was not a goal of this study to evaluate the influence of temperature in sperm parameters, but we cannot exclude the fact that males were also maintained, even for a short time, at different temperatures, which could affect the reproductive process. As previously reported, in all treatments in which females successfully spawned, they received hCG injections and the concentration of MIS was higher than saline injected females, that did not spawn. The higher GnRHa dose combined with the higher temperature, triggered higher MIS plasma concentration in hCG treated females (about 35 ng/ml), however, these data did not corroborate with the spawning data,

considering that the best reproductive parameters (in absolute numbers) were obtained in animals treated with the higher dose of GnRHa, but maintained at 20°C (about 15 ng/ml). This profile reinforces the evidences of previous studies that after the production of MIS many other physiological processes (not evaluated in the present study) can influence the effective ovulation (Nagahama and Yamashita 2008).

Astyanax altiparanae females implanted with GnRHa before spawning induction, regardless of the temperature or dose, showed MIS plasma levels that varied from about 10 to 35 ng/ml, while without GnRH supplementation, MIS plasma levels after spawning induction were about 1 to 2.5 ng/ml (Abreu et al., 2021). During the spring-summer period, when *A. altiparanae* is normally induced to spawning in captivity, some reproductive data, such as egg size, plasma levels of MIS, and time of spawning and hatching, remained similar to the data obtained in our study (Abreu et al., 2021; Evangelista et al., 2019; Nascimento et al., 2018).

Repeated GnRHa injections in lambari enhanced the number of vitellogenic oocytes (33.226 oocytes) at lower temperatures (20°C) (Brambila-Souza et al., 2019), what was not achieved with the implants, and this difference can be due to the level of GnRH provided by these protocols. However, when GnRHa was administered as implants, in an uninterrupted way, pituitary gonadotropins gene expression increased but only at the higher temperature, as well as GSI. Therefore, this study suggests that using a sustained treatment of GnRHa through controlled-release implants during gametogenesis, the pituitary can be more responsive under summer temperature, but at 20°C females undergo oocyte maturation, ovulation and spawning even outside the reproductive season. Additionally, the spawning induction, even considering only the absolute values of the reproductive parameters, showed that spawning, fertilization and larval rearing was also viable under winter temperatures, exhibiting the plasticity of the species under hormonal manipulation, which means a new frontier of production of this species, providing a year-round good quality seed.

5. Conclusions

Lambari females induced or not with GnRHa implants during the non-breeding season completed final oocyte maturation and ovulation after hCG administration. However, uninterrupted GnRHa at 8µg/g release as implants increased pituitary gonadotropins gene expression at 28°C and *vtgA* at 20°C. The data confirmed that 20ºC is not a limiting temperature for reproduction, as *fshβ* and *lhβ* gene expression was lower, but present at this temperature. These results can enable producers to build new reproductive protocols as hormonal therapies allied with environmental manipulations to obtain good quality seed all over the year, improving the commercial aquaculture of lambari.

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6. References

Abreu M.R., Silva, L.M.J., Figueiredo-Ariki D.G., Sato R.T., Kuradomi R.Y., Batlouni S.R. (2021) Reproductive performance of lambari (*Astyanax altiparanae*) in a seminatural system using different protocols. Aquaculture Research, 52 (2) 1–13. DOI: 10.1111/are.14905.

Alix M., Kjesbu O.S., Anderson, K.C. (2020) From gametogenesis to spawning: How climate-driven warming affects teleost reproductive biology. Journal of Fish Biology, 97, 607-632. DOI: 10.1111/jfb.14439.

Anderson, K., Pankhurst, N., King, H., Elizur, A. 2017. Effects of GnRHa treatment during vitellogenesis on the reproductive physiology of thermally challenged female Atlantic salmon (*Salmo salar*). PeerJ, 5, e38985. DOI:10.7717/peerj.3898.

Anonymous, 1998. Guidelines for the treatment of animals in behavioural research and teaching. Animal Behaviour, 55, 251-257. DOI: 10.1016/j.anbehav.2011.10.031.

Barbaro, A., Franceson, A., Bertotto, D., Bozzato, G., Di Maria, I., Patarnello, P., Furlan, F., Colombo, L., 2002. More effective induction of spawning with long-acting GnRH agonist in the shi drum, *Umbrina cirrosa* L. (*Sciaenidae, Teleostei*), a valuable candidate for Mediterranean mariculture. Journal of Applied Ichthyology. 18, 192–199. DOI: 10.1046/j.1439-0426.2002.00341.x.

Brambila-Souza, G., Mylonas, C.C., Mello, P.H., Kuradomi, R.Y., Batlouni, S.R., Tolussi, C.E., Moreira, R.G. 2019. Thermal manipulation and GnRHa therapy applied to the reproduction of lambari-do-rabo-amarelo, *Astyanax altiparanae* females (Characiformes: Characidae) during the non-breeding season. General and Comparative Endocrinology, 279:120-128. DOI: 10.1016/j.ygcen.2019.02.016.

Cassel, M., de Paiva Camargo, M., Jesus, L.W.O., Borella, M.I. 2017a. Involution processes of follicular atresia and post-ovulatory complex in a characid fish ovary: a study of apoptosis and autophagy pathways. Journal of Molecular Histology, 48: 243-257. DOI: 10.1007/s10735-017-9723-6.

Cassel, M., Chehade, C., Souza, G.B., Caneppele, D., Romagosa, E., Borella, M. I. 2017b. Ovarian development and the reproductive profile of *Astyanax altiparanae* (Teleostei, Characidae) over one year: Applications in fish farming. Theriogenology, 98: 1-15. DOI: 10.1016/j.theriogenology.2017.04.044.

Chehade, C., Cassel, M., Borella, M.I. 2015. Induced reproduction in a migratory teleost species by water level drawdown. Neotropical Ichthyology,13 (1): 205–212. DOI: 10.1590/1982-0224-20140028.

Chehade, C., Amaral, F.G., Branco, G.S., Cassel, M., De Jesus, L.W.O., Costa, F.G., Bordin, S.A., Moreira, R.G., Borella, M. I. 2020. Molecular Characterization of Different PreproGnRHs in *Astyanax altiparanae* (Characiformes): Effects of GnRH on female reproduction. Molecular Reproduction and Development, v. 87:10. DOI:10.1002/mrd.23351.

Costa, F.G., Adolfi, M.C., Gomes, C.C., Jesus, L.W.O., Batlouni, S.R., Borella, M.I. 2014. Testes of *Astyanax altiparanae*: The Sertoli cell functions in a semi cystic spermatogenesis. Micron, 61: 20-27. DOI: 10.1016/j.micron.2014.02.004.

De Paiva Camargo, M., Cassel, M., Jesus, L. W. O., Nóbrega, R.H., Borella, M.I. 2017. Characterization of undifferentiated spermatogonia and the spermatogonial niche in the lambari fish *Astyanax altiparanae*. Theriogenology, 96: 97-102. DOI: 10.1016/j.theriogenology.2017.03.027.

Duncan, N.J., Sonesson, A.K., Chavanne, H. 2013. Principles of finfish broodstock management in aquaculture: control of reproduction and genetic improvement. In: Allan, G., Burnell, G. Advances in aquaculture hatchery technology. Cambridge: Woodhead Publishing, p.23-75.

Evangelista, M.M., Pereira, M.P., Sussel, F.R., Romagosa, E. 2019. Environmental manipulation on *Astyanax altiparanae* out-of-season spawning. Latin American Journal of Aquatic Research, 47(2): 292-302. DOI: 10.3856/vol47-issue2-fulltext-9.

Flores, A., Wiff, R., Ganias, K., Marshall, C.T., 2019. Accuracy of gonadosomatic index in maturity classification and estimation of maturity ogive. Fisheries Research, 210: 50–62. DOI: 10.1016/j.fishres.2018.10.009.

Gomes, A.D., Tolussi, C.E., Ribeiro, C.S., Honji, R.M., Moreira, R.G. 2015. The role of ovarian steroids in reproductive plasticity in *Hoplias malabaricus* (Teleostei: Characiformes: Erythrinidae) in tropical reservoirs with different degrees of pollution. General and Comparative Endocrinology, 222: 1-10. DOI: 10.1016/j.ygcen.2014.10.008.

Gomes, C.C., Costa, F.G., Borella, M.I., 2013. Distribution of GnRH in the brain of the freshwater teleost *Astyanax altiparanae* (Garutti & Britski, 2000). Micron, 52 (53): 33-38. DOI: 10.1016/j.micron.2013.07.008.

Gonçalves, L.U., Parisi, G., Bonelli, A., Sussel, F.R., Viegas, E.M.M. 2014. The fatty acid compositions of total, neutral and polar lipids in wild and farmed lambari *Astyanax altiparanae*) (Garutti & Britski, 2000) broodstock. Aquaculture Research, 45 (2): 195–203. DOI: 10.1111/j.1365-2109.2012.03215.x.

542 Jesus, L.W.O., Bogerd, J., Vieceli, F.M., Branco, G.S., Camargo, M.P., Cassel,
543 M., Moreira, R.G., Yan, C.Y.I., Borella, M.I. 2017. Gonadotropin subunits of
544 the characiform *Astyanax altiparanae*: Molecular characterization,
545 spatiotemporal expression and their possible role on female reproductive
546 dysfunction in captivity. General and Comparative Endocrinology, 15 (246):
547 150-163. DOI: 10.1016/j.ygcen.2016.12.004.

548 Kagawa, H., Kasuga, Y., Adachi, J., Nishi, A., Hashimoto, H., Imaizumi, H., Kaji,
549 S. 2009. Effects of continuous administration of human chorionic
550 gonadotropin, salmon pituitary extract, and gonadotropin-releasing hormone
551 using osmotic pumps on induction of sexual maturation in male Japanese
552 eel, *Anguilla japonica*. Aquaculture, 296: 117–122. DOI:
553 10.1016/j.aquaculture.2009.07.023.

554 Kanemaru, T., Nakamura, M., Murata, R., Kuroki, K., Horie, H., Uchida, K.,
555 Senthilkumaran, B., Kagawa, K. 2012. Induction of sexual maturation of the
556 female honeycomb grouper, *Epinephelus merra*, in the non-breeding season
557 by modulating environmental factors with GnRH analogue implantation.
558 Aquaculture, 358: 85-91. DOI: 10.1016/j.aquaculture.2012.06.026.

559 Lira, L.V.G., Kuradomi, R.Y., Souza, T.G., Hainfellner, P., Batlouni, S.R. 2018.
560 *Astyanax altiparanae* ovarian maturation after spawning in water recycling
561 systems. Boletim do Instituto de Pesca, 44: 4. DOI: 10.20950/1678-
562 2305.2018.44.4.207.

563 Livak, K.J., Schmittgen, T.D., 2001. Analysis of Relative Gene Expression Data
564 Using Real-Time Quantitative PCR and the 22DDCT. Method Methods, 25,
565 402–408. DOI: 10.1006/meth.2001.1262.

566 Lubzens, E., Young, G., Bobe, J., Cerdà, J. 2010. Oogenesis in teleosts: how fish
567 eggs are formed. General and Comparative Endocrinology, 165: 367–389.
568 DOI: 10.1016/j.ygcen. 2009.05.022.

569 Luckenbach, J.A., Lliev, D.B., Goetz, F.W., Swanson, P., 2008. Identification of
570 differentially expressed ovarian genes during primary and secondary oocyte
571 growth in coho salmon, *Oncorhynchus kisutch*. Reproductive Biology and
572 Endocrinology, 6: 2–16. DOI: 10.1186/1477-7827-6-2.

573 Metcalfe, J.D., Craig, J.F., 2011. Ethical justification for the use and treatment of
574 fishes in research: an update. Journal of Fish Biology, 78, 393-394.
575 DOI:10.1111/j.1095-8649.2010.02900.x.

576 Milla, S., Wang, N., Mandiki, S.N.M., Kestemont, P., 2009. Corticosteroids:
577 Friends or foes of teleost fish reproduction? Ecotoxicology, 20:305–319. DOI:
578 10.1007/s10646-010-0588-7.

579 Mylonas, C.C., Bridges, C.R., Gordin, H., Belmonte Ríos, A., García, A., De la
580 Gándara, F., Fauvel, C., Suquet, M., Medina, A., Papadaki, M., Heinisch, G.,
581 De Metrio, G., Corriero, A., Vassallo-Agius, R., Guzmán, J.M., Mañanos, E.,
582 Zohar, Y., 2007. Preparation and administration of gonadotropin-releasing
583 hormone agonist (GnRHa) implants for the artificial control of reproductive
584 maturation in captive-reared Atlantic bluefin tuna (*Thunnus thynnus thynnus*).
585 Reviews in Fisheries Science & Aquaculture, 15: 183–210. DOI:
586 10.1080/10641260701484572.

587 Mylonas, C.C., Fostier, A., Zanuy, S. 2010. Broodstock management and
588 hormonal manipulations of fish reproduction. General and Comparative
589 Endocrinology, 165: 516–534. DOI: 0.1016/j.ygcen.2009.03.007.

590 Nagahama, Y., Yamashita, M., 2008. Regulation of oocyte maturation in fish. Dev
591 Growth Differ 50 Suppl 1, S195–219. DOI: 10.1111/j.1440-169X.2008.01019.x

592 Nakamura, I., Kusakabe, M., Young, G. 2003. Regulation of steroidogenic
593 enzyme mRNAs in rainbow trout (*Oncorhynchus mykiss*) ovarian follicles in
594 vitro. Fish Physiology and Biochemistry, 28: 355–356. DOI:
595 10.1023/B:FISH.0000030586.74085.0f.

596 Nascimento N.F., Lázaro T.M., Alcântara N.R., Rocha, Senhorini J.A., Santos
597 S.C.A., Nakaghi L.S.O., Yasui G.S. (2018) In vivo storage of oocytes leads to
598 lower survival, increased abnormalities and may affect the ploidy status in the
599 yellowtail tetra *Astyanax altiparanae*. Zygote, 26: 471–475. DOI:
600 10.1017/S0967199418000527.

601 Panini, E., Mylonas, C.C., Zanuy, S., Carrillo, M., Ramos, J., Bruce, M., 2001.
602 Incubation of embryos and larvae of marine fish using microtiter plates.
603 Aquaculture International, 9: 189–196. DOI: 10.1023/A:1014261830098.

Parreira, W.S.P. Corticosteroids as inductor of ovulation in *Astyanax altiparanae* (Characiformes: Characidae) during artificial reproduction. 84f. Dissertação (Mestrado) em Ciências - Instituto de Biociências da Universidade de São Paulo. Departamento de Fisiologia, USP, 2015.

Porto-Foresti, F., Castilho-Almeida, R.B., Senhorini, J.A., Foresti, F. 2010. Biologia e criação do lambari-do-rabo-amarelo (*Astyanax altiparanae*). In: Baldisseroto, B. and Gomes, L.C. Espécies Nativas Para Piscicultura No Brasil. B. G. Baldisserotto, L.C. Santa Maria RS Brasil, Editora UFSM. p.101-115.

Reading, B.J., Sullivan, C.V. & Schilling, J., 2017. Vitellogenesis in Fishes. Elsevier Inc. DOI.org/10.1016/S0044-8486(01)00772-4.

Rurangwa, E., Kime, D.E., Ollevier, F., Nash, J.P., 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 234, 1-28. DOI: 10.1016/j.aquaculture.2003.12.006.

Silva, J.V., Andrade, D.V., Okano, W.Y., 1996. Desenvolvimento sexual e crescimento de lambaris – tambiú, *Astyanax bimaculatus* Linnaeus, 1758, submetidos a diferentes tipos de alimentação. Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 48(1): 47-54.

Simpson, A.C., 1951. The fecundity of the plaice. Fish Investigation, 5: 1–27.

Schulz, R.W., França, L.R., Lareyre, J.J., LeGac, L., 2010. Spermatogenesis in fish. General and Comparative Endocrinology, 165, 390–411. DOI:10.1016/j.ygcen.2009.02.013.

Tolussi, C.E., Gomes, A.D.O., Kumar, A., Ribeiro, C.S., Lo Nostro, F.L., Bain, P.A., Souza, G.B., Cuña, R., Honji, R.M., Moreira, R.G. 2018. Environmental pollution affects molecular and biochemical responses during gonadal maturation of *Astyanax fasciatus* (Teleostei: Characiformes: Characidae). Ecotoxicology and Environmental Safety, 14: 926–934. DOI: 10.1016/j.ecoenv.2017.09.056.

Vazzoler, A.E.A.M., 1996. Biologia da reprodução de peixes teleósteos: Teoria e Prática, first ed. Nupelia, Maringá.

Weil, C., Crim, L.W., 1983. Administration of LHRH analogues in various ways: effect on the advancement of spermiation in prespawning landlocked salmon, *Salmo salar*. Aquaculture, 35, 103–115. DOI 10.1016/0044-8486(83)90078-9.

Weingartner, M., E. Zaniboni-Filho. 2005. Dourado. Pp. 257-281. In: Baldisseroto, B. & L. C. Gomes (Eds.). Espécies nativas para piscicultura no Brasil. Santa Maria, Editora da UFSM, 470p.

Yasui, G.S.; Senhorini, J.A.; Shimoda, E.; Pereira-Santos, M.; Nakaghi, L.S.O.; Fujimoto, T.; Arias-Rodriguez L.; Silva L.A. 2015. Improvement of gamete quality and its short-term storage: an approach for biotechnology in laboratory fish. Animal 9 (3): 464-470. DOI:10.1017/S1751731114002511.

Young, G., Todo, T., Kusakabe, M., Kobayashi, T., Nagahama, Y. 2002. 3β-hydroxysteroid dehydrogenase gene: sites of expression in trout gonads and cAMP-dependent regulation. In: Norberg, B., Kjesbu, O.S., Taranger, G.L., Andersson, E., Stefansson, S.O. (Eds.), Reproductive Physiology of Fish. Institute of Marine Research, Bergen, p. 202.

Zohar, Y., 1988. Gonadotropin releasing hormone in spawning induction in teleosts: basic and applied considerations. In: Zohar, Y., Breton, B. (Eds.), Reproduction in Fish: Basic and Applied Aspects in Endocrinology and Genetics. INRA Press, Paris, p. 47–62.

Zohar, Y., 1996. New approaches for the manipulation of ovulation and spawning in farmed fish. Bulletin of National Research Institute of Aquaculture, 2: 43–48.

Zohar Y., Mylonas, C.C. 2001. Endocrine manipulations of spawning in cultured fish: from hormones to genes. Aquaculture, 197: 99–136. DOI 10.1016/j. ygcen.2009.04.017.

Zohar, Y., Muñoz-Cueto, J., Elizur, A.; Kah,O., 2010 O. Neuroendocrinology of reproduction in teleost fish. General and Comparative Endocrinology, 165, 438-455. DOI:10.1016/j.ygcen.2009.04.017.

Tables:

Table 1: Qualitative reproductive parameters of lambari females induced to spawn with hCG after 21 days whith GnRHa implants treatment.

Treatment	Temperature	Total number of eggs (per 4 females)	Fertilization (%)	Hatching (%)	Accumulated Thermal Units (ATU)	Mortality up to mouth opening (%)	Estimated absolute larvae number
Control	20°C	3.564	28	62	324	3	609,9
	28°C	NS	NS	NS	NS	NS	NS
4µg/g	20°C	7.718	53	72	270	2	2939,6
	28°C	8.340	27	72	168	2	1620,1
8µg/g	20°C	12.662	55	77	280	1	5352,6
	28°C	4.884	38	77	243,6	1	1429,4

NS – not spawned

Figure captions:

Figure 1: *Astyanax altiparanae* experimental design of gametogenesis and spawning induction. Implants of GnRHa (4 µg/g and 8 µg/g), at 20°C and 28°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)

Figure 2: Gene expression of *fshβ* (A), *lhβ* (B) and *vtgA* (C) in lambari females after 21 days of GnRHa treatment (Mean ± SEM). $^{*\#}$Different symbols indicate a significant difference between temperatures in the same GnRHa treatment. ^{ab}Different letter superscripts indicate differences between GnRHa treatments within the same temperature ($P<0.05$).

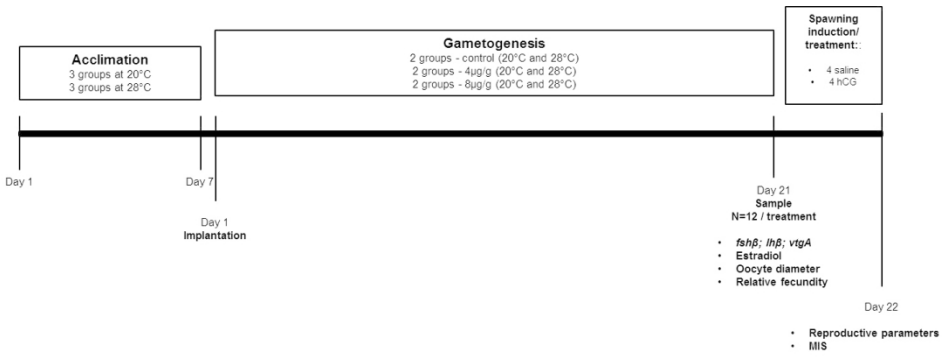
687 Figure 3: Plasma E2 concentration in lambari females after 21 days of GnRHa
688 treatment (Mean ± SEM). *#Different symbols indicate a significant difference
689 between temperatures in the same GnRHa treatment (P<0.05).

690

691 Figure 4: Plasma concentration of 17α-20β-dihydroxy-4-pregnen-3-one (MIS)
692 (Mean ± SEM) in lambari females after 21 days of GnRHa treatment and
693 spawned. Light bars represent animals that did not spawn and dark bars
694 animals that spawned. Brackets mean statistical differences (P<0.05) between
695 hCG and saline in the same temperature and the same GnRH treatment during
696 gametogenesis. Brackets with symbols mean differences between temperatures
697 within the same GnRH treatment during gametogenesis, induced with hCG, and
698 different letters mean differences between GnRHa treatments during the
699 gametogenesis, in the same temperature (P<0.05).

700

701 Figure 5: Gonadosomatic index (GSI) (A), relative fecundity (B), and oocyte
702 diameter (C), in lambari females after 21 days of GnRHa treatment (Mean ±
703 SEM). *#Different symbols indicate a significant difference between
704 temperatures in the same GnRHa treatment. [ab]Different letters superscripts
705 indicate differences between GnRHa treatments within the same temperature
706 (P<0.05).



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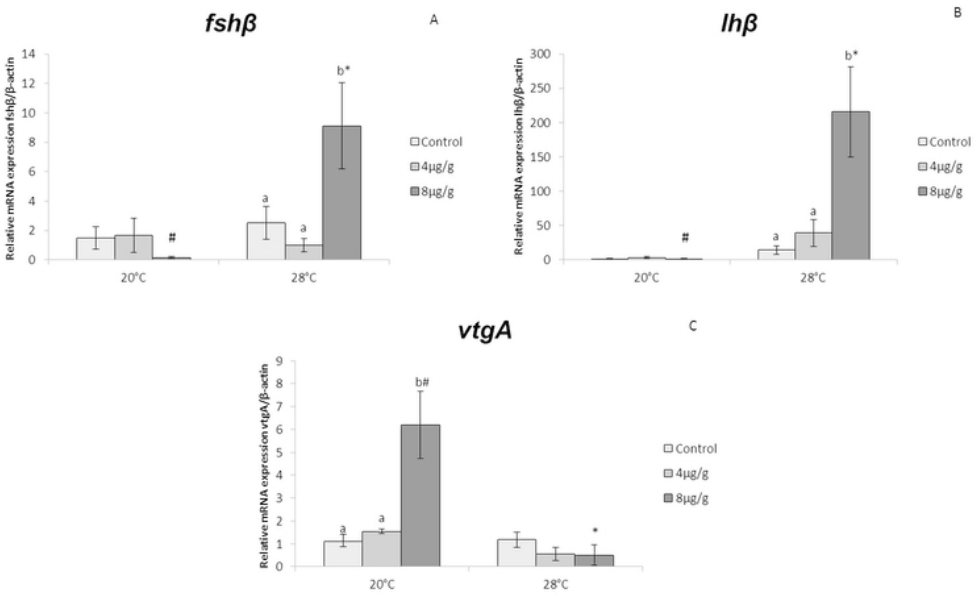

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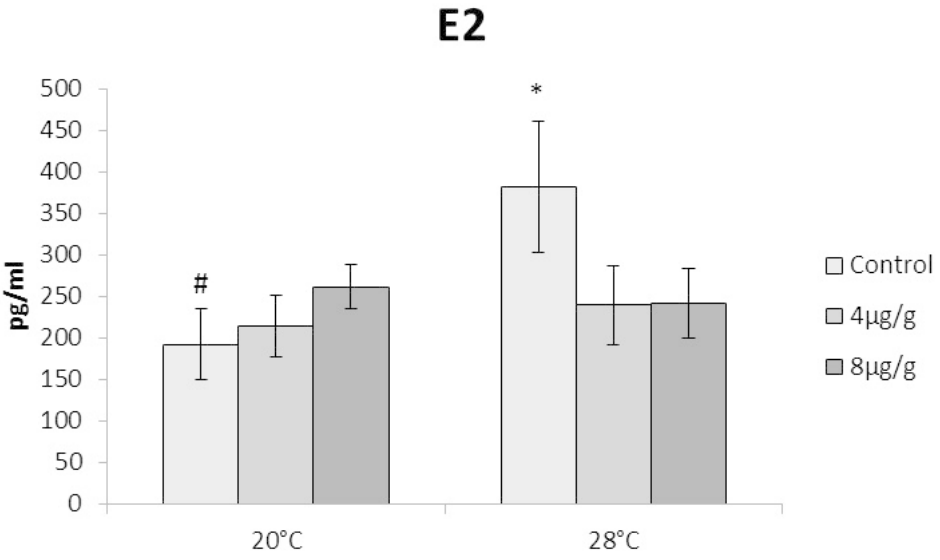
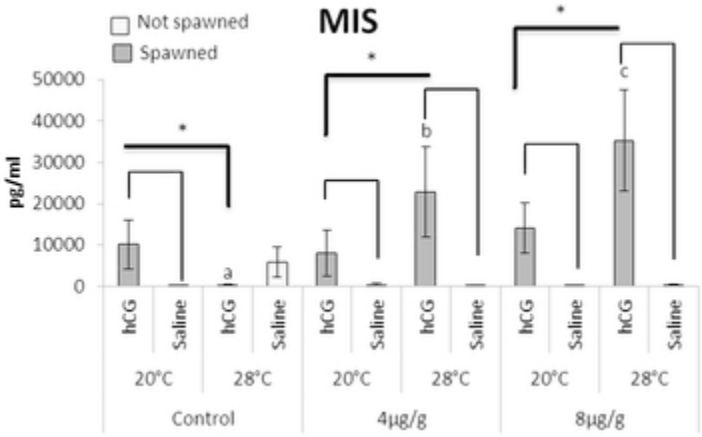


Figure 3: Plasma E2 concentration in lambari females after 21 days of GnRHa treatment (Mean ± SEM). *#Different symbols indicate a significant difference between temperatures in the same GnRHa treatment ($P<0.05$).

31x19mm (600 x 600 DPI)



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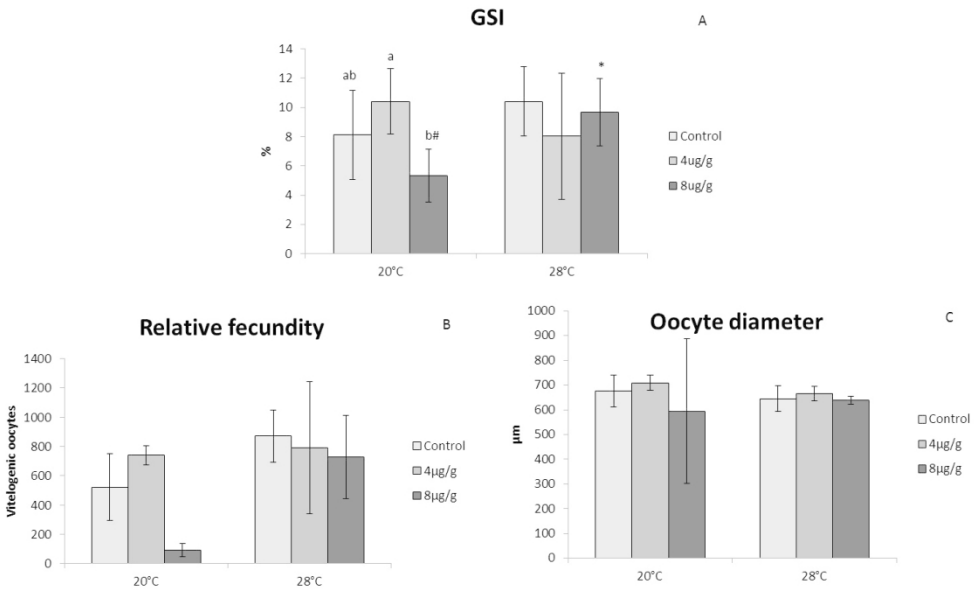

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64x38mm (600 x 600 DPI)