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The use of mGnRHa provokes ovulation but not viable embryos in *Leporinus macrocephalus*

T. S. B. Pereira[1] · C. N. P. Boscolo[1] · R. G. Moreira[2] · S. R. Batlouni[1]

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Abstract In rheophilic tropical fish, the use of carp pituitary extract (CPE) is still the main protocol since the use of gonadotropin-releasing hormone (GnRH) induces ovulation, but usually does not provide viable embryos. Thus, in order to generate knowledge to support the establishment of a successful protocol using GnRH, we compared the ovulatory process and reproductive performance of *Leporinus macrocephalus* using CPE (0.5 and 5.0 mg kg^{-1}) and mGnRHa (7 μg kg^{-1}) associated with metoclopramide. Females of both treatments ovulated, but the mGnRHa treatment did not provide viable embryos, which was associated with a more potent ovulation, lower latency period and less intense hydration of eggs, suggesting a possible treatment overstimulation. This is the first report associating a less intense egg hydration process to a failure in obtaining viable eggs in induced fish spawning. Considering that the mGnRHa doses applied here were quite low, it is possible that *L. macrocephalus* as well as other rheophilic tropical species present a higher sensibility to mGnRHa and/or metoclopramide (or derived substances) and future approaches must consider using even lower doses of these substances as well as testing different types of GnRH and dopamine inhibitors.

Keywords Final maturation · Gonadal steroids · Hormonal treatment · Ovulation · Spawning performance

Introduction

Rheophilic fish are the most important native fish for commercial purposes in South American countries (MPA 2013; FAO 2014). For these species, different from many marine (Rosenfeld et al. 2012; Fernández-Palacios et al. 2015) and temperate (Szabó et al.

✉ S. R. Batlouni
batlouni@caunesp.unesp.br

[1] Centro de Aquicultura, Universidade Estadual Paulista (UNESP), Campus de Jaboticabal, Jaboticabal, SP, Brazil

[2] Instituto de Biociências, Universidade de São Paulo (USP), Campus de São Paulo, São Paulo, SP, Brazil

2002; Genz et al. 2014) fresh water species, the most commonly used technique for obtaining viable embryos in captivity is still the application of carp pituitary extract (CPE) (Caneppele et al. 2015; De Souza et al. 2015; Ittzés et al. 2015; Viveiros et al. 2015) because this, at a certain extent, has been practically the only effective technique for this purpose in this species (Leonardo et al. 2004; Felizardo et al. 2012; Santos et al. 2013). On the other hand, the main problem related to the use of CPE is a constant uncertainty and unpredictability of a successful ovulation (Criscuolo-Urbinati et al. 2012; Hainfellner et al. 2012a, b).

In these species, even when associating GnRH with dopaminergic inhibitors, results are still inadequate because although females ovulate, the embryo viability rates are frequently very low or close to zero (Acuña and Rangel 2009; Paulino et al. 2011). Thus, in order to generate knowledge about the failure in obtaining viable embryos and to support the establishment of successful protocol using GnRH in tropical rheophilic fish, in this study, we aimed to compare the evolution of the final maturation process and ovulation using CPE and mammalian analogue gonadotropin-releasing hormone (mGnRHa). In order to check the possible toxic effects provoked by both treatments upon egg development, we evaluated the reproductive performance between treatments (latency period, ovulation rate, fecundity, fertility and hatching) and the intensity of the egg hydration process. In both treatments, we also compared the intensity of final maturation and ovulation by means of stereological evaluation and plasma concentration of gonadal steroid levels. We used *L. microcephalus*, a large sized total spawning rheophilic fish, commercially relevant and known to be a good model for studies concerning the reproductive biology of rheophilic species (Muñoz et al. 2011).

Materials and methods

Ethical note

This study was conducted in agreement with the precepts of the National Council for the Control of Animal Experimentation (CONCEA) and was approved by the Animal Ethics and Welfare Committee from UNESP, Jaboticabal, SP, Brazil, under permission number 015279/10.

Maintenance of fish

Males and females (at a sex ratio of 1:1) were maintained at *Centro de Aquicultura da Unesp* (CAUNESP), located in Jaboticabal, São Paulo, Brazil (21°15′17″S, 48°19′20″W), in 200 m^3 earthen ponds with a stocking density of 1.5 fish per m^3 and with a constant water flow between 15 and 20 L min^{-1}. Fish were manually fed a pelleted balanced commercial diet (moisture content (max) 10.0 %; crude protein (min) 32.0 %; ethereal extract (min) 10.0 %; fibrous matter (max) 7.0 %; ash (max) 10.0 %; calcium (max) 1.2 %; phosphorus (min) 1.2 %) corresponding to 3.0 % of total body weight twice a day. Water parameters were measured weekly at 9 a.m., using a YSI model 55 oximeter and a YSI model 63 multiparameter sonde (Yellow Spring Instruments, Yellow Springs, OH, USA) to determine pH, conductivity levels (mS cm^{-1}), dissolved oxygen (mg L^{-1}) and temperature (°C). The N-ammonia concentration (μ L^{-1}) was determined every two weeks according to the method described by Solorzano (1969).

Experimental protocols

Nineteen mature females with a mean biomass of 3.17 ± 1.0 (mean ± SEM) kg were randomly selected for the induced breeding experiments. At the time of spawning, broodstock fish were transported to the laboratory for acclimatization and maintained in 500 L tanks at 25 °C. Females were randomly submitted to two different hormonal treatments routinely used in fish farms: (1) crude carp pituitary extract (CPE) (Fish braz®)—two doses (0.5 and 5.0 mg kg^{-1}, 12 h interval, diluted in 0.5 mL saline—0.9 % and (2) mammalian analogue gonadotropin-releasing hormone in liquid form (mGnRHa) (conceptal®/Intervet)—single dose (7 μg kg^{-1}, diluted in 10 ml sterile buffered diluent, associated with a dopamine inhibitor (10 mg kg^{-1} metoclopramide, diluted in 0.5 ml saline—0.9 %) (Table 1). Selection of the doses of both hormonal inducers was based on previous studies with other rheophilic South American species which showed efficacy in the reproductive process (Criscuolo-Urbinati et al. 2012; Ittzés et al. 2015).

Reproductive performance

The latency period was defined as the time between the first or single injection and fish ovulation according to the methodology applied by Heyrati et al. (2006). The spawning rate (the percentage of spawning females) was determined using the following formula: total number of spawned females/total number of induced females × 100. The total number of oocytes released from each female (absolute fecundity) was estimated. To analyze this data, after ovulation and just before fertilization, the total mass of eggs released by each female was recorded. Sub-samples (∼1 g) of the egg mass were used to extrapolate the total egg numbers. After weighing, the oocytes of each female were fertilized using a pool of semen from eight males from the same broodstock. To avoid the effects of factors unrelated to the influence of the females during the artificial breeding process, the same pool of semen was used for the mGnRHa and CPE treatments. Approximately 0.5 mL semen was used to fertilize 50 g of oocytes. The sperm concentration in this species ranges approximately from 4.8 to 6.9 × 10^9 cells mL^{-1} (Moraes et al. 2004; Muñoz et al. 2011). The average number of oocytes present in a gram of spawn is approximately 1800. Therefore, the sperm/egg ratio used in this study was estimated to be from 2.6 to 3.8 × 10^4 sperm per egg.

Soon after fertilization, eggs from each female were distributed into five 200 L incubators. Then, 150 g of eggs were placed in each incubator with a constant water flow of 5 L min^{-1}, at

Table 1 Details of experimental groups, dosages, methods of application, number of fish and mean biomass in each group

Treatment	Dosage		Interval	No. of injected females	Mean biomass
	1st	2nd			
CPE	0.5	5.0 mg kg^{-1}	12 h	10	3.40 ± 1.12
mGnRHa[a]	–	7.0 μg kg^{-1}	Single dose	9	2.94 ± 1.98

CPE carp pituitary extract, *mGnRHa* gonadotropin-releasing hormone in liquid form

[a] Associated with a dopamine inhibitor (10 mg kg^{-1} metoclopramide, diluted in 0.5 ml saline—0.9 %). Female mean biomass is expressed as mean SEM

25 °C and dissolved oxygen concentration average values equal to 5.72 ± 0.84 mg L^{-1}. To determine the fertilization success of the eggs, 8–12 h post-fertilization (hpf) (after the blastopore closure stage), 100 eggs from each female were randomly sampled and counted, and those which were normally dividing were scored. Four counts were performed to determine the mean fertilization success. After 17 hpf, overall hatching success was determined by counting the number of hatched eggs/number of fertilized eggs × 100. Four counts were performed to determine the mean hatching success.

For the evaluation of the hydration process, a pool of eggs from each treatment was collected at different time intervals (0, 5, 10, 40, 120 and 600 s) after fertilization. For this purpose, 72 recipients (200 mL) (4 treatments × 3 replicates) were filled with 10 g of eggs and 100 mL of water. The time was recorded from the moment the mixture of oocyte and semen came in contact with water, starting the process of hydration. Eggs were collected ($n = 30$ per time intervals), and the average diameter was determined in each unit using a stereomicroscope (LEICA MZ8) coupled to automatic equipment micrograph (Leica DFC 280), with measurement software Image-Pro Plus Version 4.1.0.0.

All treated females which spawned were euthanized after the spawn, with an overdose of anesthesia (2 g ethylaminobenzoate: 150 mL alcohol:20 L water) for the collection of ovaries. The gonadosomatic index (GSI) was determined through the following formula: (the total weight of oocytes released by each female + the weight of the respective remaining ovary/total body weight) × 100.

Ovarian histology

Oocytes subtypes found in the ovaries have been previously described for comparatively evaluating the characteristics of the ovaries after spawning in both treatments. The evolution of the meiotic process differed between the two treatments, but oocytes subtypes were the same; therefore, we used the CPE treatment to describe the oocytes subtypes found. For the characterization of oocytes subtypes, we used only oocytes showing the nucleus in transversal sections, which were classified according to criteria suggested by Felizardo et al. (2012) and Coward and Bromage (1998). The morphological changes in the oocyte cytoplasm, position and aspect of the germinal vesicle were considered and characterized during the process of spawning induction in both treatments. Eight morphological stages (seven pre-ovulatory stages and ovulated eggs) (Table 1) were described using an Olympus BX41 microscope system (4× magnification) with an Olympus DP11 imaging apparatus (with measurements taken using Image-Pro Plus Version 4.1.0.0 software).

Histomorphometric analyses

For histologic evaluation (volume density), four randomly collected spawned females for each treatment were euthanized with a lethal dose of benzocaine (2 g L^{-1}) at the time of ovulation. The cranial, medial and tail regions of the ovary tissues were fixed in Bouin solution for routine histologic procedures, embedded in Historesin for histologic preparation and stained with hematoxylin-floxin. Volume density was determined using light microscopy and a 320-intersection grid. Three fields from each region of the ovary (anterior, medial, and cranial; total of nine fields) were randomly selected, with a total of 2880 points scored for each animal at magnification 4×. For this analysis, the methodology applied by Pereira et al. (2013, 2016) was used with some modifications. Points were classified as one of the following: previtellogenic oocyte (PV), cortical alveoli oocyte (CA), immature oocytes with incomplete vitellogenesis and cytoplasm not fully filled with

yolk (IV), mature vitellogenic oocytes with cytoplasm filled entirely by yolk and central nucleus (CNV), mature vitellogenic oocytes with cytoplasm filled entirely by yolk and migrated nucleus (MNV), mature vitellogenic oocytes with cytoplasm filled entirely by yolk and showing germinal vesicle break down (GVBD) and atretic oocyte (AT). The appearance of post-ovulatory follicles (POF) and interstitial tissue (IT) was also characterized. Artifacts were rarely observed and were not considered in the total number of points used to obtain the percentages.

Blood sampling and steroids assays

Blood was collected at the moment of each hormonal dose and at the time of ovulation ($n = 8$). Animals were anesthetized with benzocaine (9 mg L^{-1}) for blood sampling. Blood was collected by puncturing the caudal vein with heparinized syringes (Liquemine, Roche, Rio de Janeiro, RJ, Brazil) and needles. Blood was centrifuged at 1300*g* for 10 min. The plasma was separated into aliquots and frozen at −80 °C for the subsequent 17β-estradiol (E^2) and 17α-hydroxyprogesterone (17α-OHP) assays. The plasma steroid level was measured by enzyme-linked immunosorbent assay (ELISA) (E^2 and 17α-OHP: Interteck, Virginia, USA). Plasma samples were run in duplicate with an acceptable limit of ≤20.0 for the intra-assay coefficients of variation (Brown et al. 2004). Absorbance measurements were collected using a microplate reader (Molecular Devices, CA, USA).

Data analysis

Data normality was verified using the Cramer-von Mises test. Homoscedasticity was checked with the F_{max} test. Student *t* test was used to analyze all parameters of reproductive performance, except for the percentage of spawning, which was analyzed through the Chi-square test (χ^2). The volume density was analyzed by comparing different treatments with a one-way analysis of variance (ANOVA). In order to analyze the hydration of eggs and gonadal steroids, two-way ANOVA for repeated measures was used. The Tukey's test was used as a post hoc analysis. A threshold of $p \leq 0.05$ was set to infer statistical significance. All statistical analyses were based on Zar (1999).

Results

The average values of physical and chemical water parameters were: pH (7.75 ± 0.5), electrical conductivity (70.33 ± 17.95 mS cm^{-1}), concentration of dissolved oxygen (5.64 ± 1.42 mg L^{-1}), water temperature (25.8 ± 3.16 °C) and concentration of total ammonia (67.47 ± 50.86 μg L^{-1}).

Reproductive performance

The latency period ranged from 12.00 (mGNRHa) to 19.35 h (CPE) (Student *t* test, $p < 0.0001$, Table 2). The percentage of spawning, absolute fecundity and GSI values were similar in mGnRHa and CPE treatments (Table 2). Concerning the reproductive performance, the fertilization and hatching success of CPE (63.57 and 59.07 % respectively) were markedly higher than those of mGnRHa (6.25 and 0 % respectively). The

Table 2 Percentage average values (±SE) of the reproductive performance of female *L. macrocephalus* undergoing hormonal induction

Treatments	Latency period (h)	Percentage of spawning (%)	Absolute fecundity (oocyte/fish)	GSI (%)	Fertility (%)	Hatching (%)
CPE	19.35 ± 0.44^{a}	71.43 ± 18.44^{a}	73.521 ± 12.23^{a}	13.34 ± 0.78^{a}	63.57 ± 16.45^{a}	59.07 ± 15.25^{a}
mGnRHa	12.00 ± 0.00^{b}	100.00 ± 0.00^{a}	90.335 ± 12.23^{a}	12.80 ± 1.97^{a}	6.25 ± 2.46^{b}	0^{b}

CPE carp pituitary extract, *mGnRHa* mammalian analogue gonadotropin-releasing hormone in liquid form. Different letters indicate differences between treatments ($p < 0.05$)

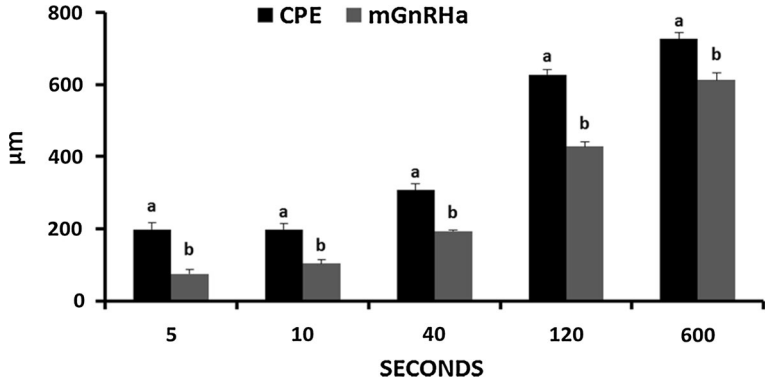


Fig. 1 Percentage average values (±SE) of the diameter of oocytes collected during the hydration process at different times after fertilization ($n = 4$ per treatment). *Different letters* indicate significant difference between treatments ($p < 0.05$). *CPE* carp pituitary extract, *mGnRHa* mammalian analogue gonadotropin-releasing hormone in liquid form

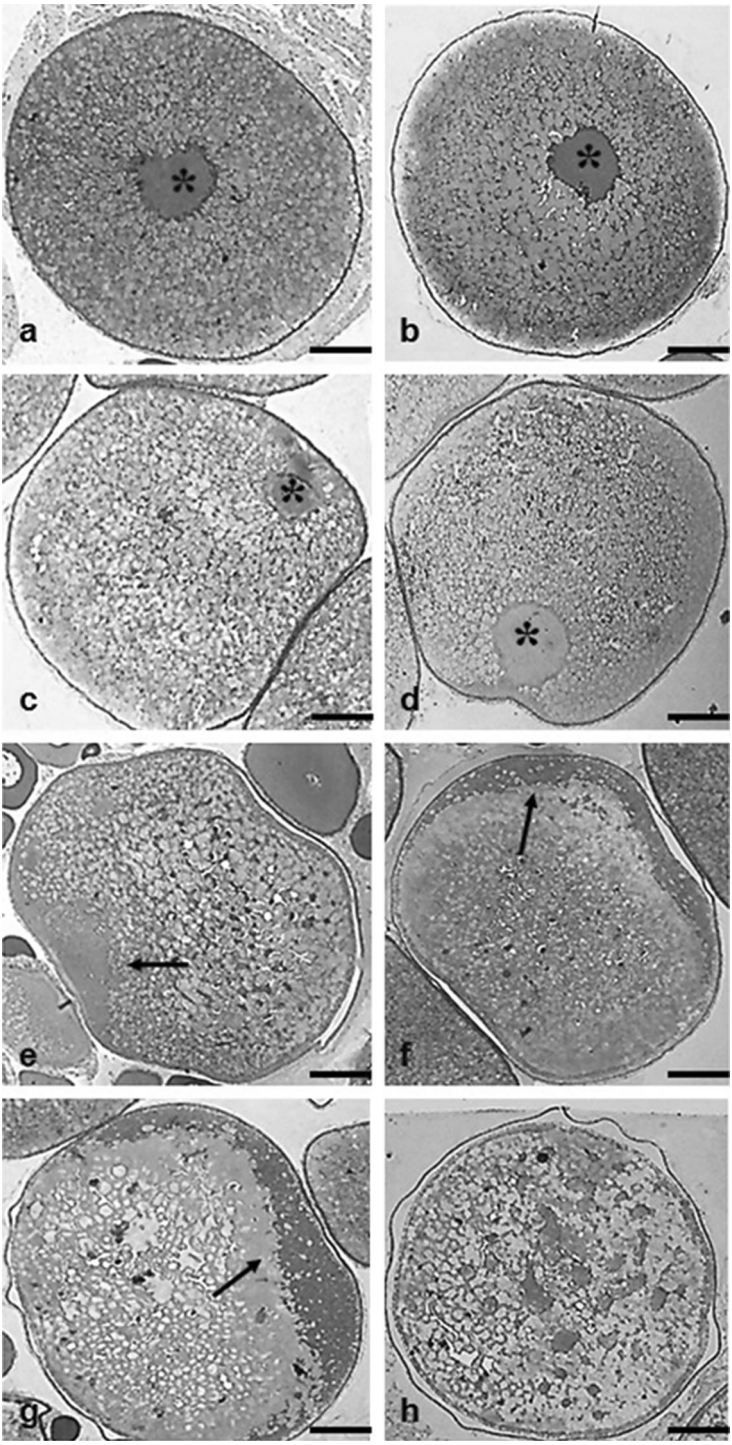
average diameter of the eggs was higher in CPE compared to mGnRHa in all periods post-fertilization evaluated (two-way repeated measures ANOVA, $p < 0.0001$, Fig. 1).

Ovarian histology

This description of the stages found was the same to both treatments. The oocytes described herein were considered for stereological analysis. At the first maturation stage, the germinal vesicle was situated in the center of the oocyte (Fig. 2a), and the proteic yolk globules were small, presenting a similar shape and being homogeneously distributed in oocytes CNV. After the first hormonal dose, an onset of germinal vesicle migration toward the micropyle (future animal pole) was detected (Fig. 2b). After the complete migration of the germinal vesicle, we observed the beginning of a yolk globule fusion in oocytes MNV (Fig. 2c, d), which gave transparency to the oocytes (data not shown). Subsequently, the nuclear envelope ruptured (germinal vesicle breakdown), releasing the nuclear content and causing a loss of the regular spherical cell shape (Fig. 2e). Shortly thereafter, we observed an increased dispersion of nuclear content throughout the periphery of the oocyte GVBD (Fig. 2f, g). The ovulation process took place just after the completion of the final oocyte maturation. At this stage, we observed an increase in the fusion and hydrolysis of yolk globules and a discrete hydration of oocytes (Fig. 2h). The entire process and the characteristics of each stage are shown in Table 3.

Volume density of oocytes of ovaries collected at the time of ovulation

The volume density of PV (one-way ANOVA, $p = 0.1634$), CA ($p = 0.6393$), IV ($p = 0.2983$), CNV ($p = 0.0630$), MNV ($p = 0.3547$), AT ($p = 0.6505$) and IT ($p = 0.5633$,) was similar among treatments as shown in Fig. 3. The volume densities of GVBD ($p = 0.0150$) were higher for CPE (22.01 %) compared to mGnRHa (7.39 %). On the other hand, the volume density of POF ($p < 0.0001$, Fig. 3) was higher in the mGnRHa (26.69 %) compared to the CPE (14.98 %) treatment.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◀**Fig. 2** Cross-sections of different *Leporinus macrocephalus* ovaries during spawning induction. **a** Stage I (before hormonal induction): central nucleus (*asterisk*); **b** Stage II (after the first dose): migration of the nucleus (*asterisk*); **c** Stage III (after the first dose): germinal vesicle partly shifted to the periphery of the oocyte (*asterisk*); **d** Stage IV (after the first dose): germinal vesicle completely migrated to the periphery of the oocyte (*asterisk*); **e** Stage V (after the second dose): initial disruption of the nuclear envelope (*arrow*); **f** Stage VI (after the second dose): nuclear envelope breakdown and initial nuclear envelope rupture (*arrow*); **g** Stage VII (after the second dose of hormone): rupture of the nuclear envelope and release of nuclear material (*arrow*); **h** Stage VIII (after the second dose): final oocyte maturation completed and ovulated oocyte. *Bars* 100 μm

Table 3 Histologic description of the morphological changes in *L. macrocephalus* oocytes during induced spawning

Stages	Histologic appearance
I	Homogeneous distribution of proteic yolk globules and germinal vesicle situated in the center of the cell
II	Yolk globules remain intact, but germinal vesicle migration starts
III	Beginning of yolk globule fusion and the completion of germinal vesicle migration toward the micropyle region
IV	More intense fusion of yolk globules; germinal vesicle migration ends and reaches the periphery of the oocyte
V	Shift from a regular to an irregular oocyte shape, beginning of nuclear envelope breakdown and release of nuclear material
VI	Nuclear content is spread along the periphery of the oocyte
VII	Nuclear material is released and remains together at the cell periphery
VIII	Oocytes are released from the ovary stroma and reach the ovary ducts to be released. Oocytes are ovulated, presenting slight hydration with heterogeneously distributed yolk globules, which may or may not be fused

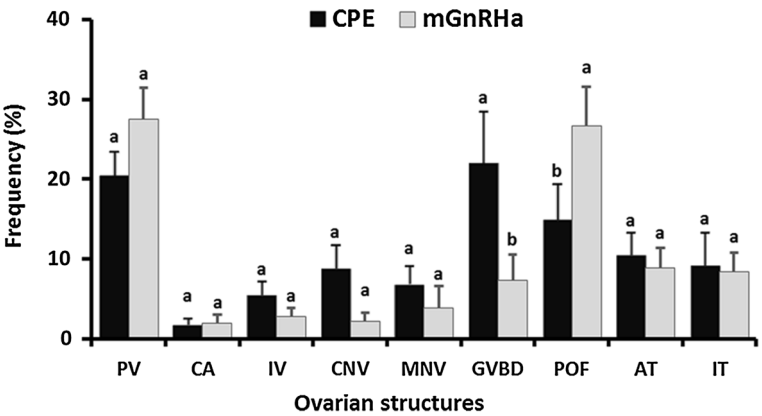

Fig. 3 Percentage average values (±SE) of the volume density of different types of ovulated ovarian oocytes collected at the time of ovulation ($n = 4$ per treatment). *Different letters* indicate significant difference between treatments ($p < 0.05$). Oocytes: *PV* previtellogenic, *CA* cortical alveoli, *IV* immature vitellogenic, *CNV* vitellogenic oocytes with central nucleus, *MNV* mature vitellogenic oocytes with migrated nucleus, *GVBD* mature vitellogenic oocyte with germinal vesicle breakdown, *POF* post-ovulatory follicle, *AT* atretic and *TI* interstitial tissue

Table 4 Average values (±SE) of plasma levels of estradiol (E^2) and 17α-hydroxyprogesterone (17α-OHP) at different times during hormonal induction ($n = 8$ per treatment)

Treatments	Period		
	First dose (pg mL^{-1})	Second dose (pg mL^{-1})	Ovulation (pg mL^{-1})
E_2			
CPE	266.38 ± 26.03^{aB}	344.30 ± 82.31^{bB}	623.40 ± 135.22^{aA}
mGnRHa	249.03 ± 30.35^{aB}	–	429.46 ± 77.72^{bA}
17α-OHP			
CPE	51.37 ± 5.99^{aB}	52.87 ± 5.22^{bB}	110.85 ± 15.82^{aA}
mGnRHa	59.00 ± 4.05^{aB}	–	88.25 ± 4.37^{bA}

CPE Carp pituitary extract, *mGnRHa* mammalian analogue Gonadotropin-releasing hormone in liquid form

Different letters indicate differences between treatments ($p < 0.05$). Different lowercase letters indicate differences between different treatments for the same periods and different capital letters indicate differences between the same treatment in different periods ($p < 0.05$)

Gonadal steroids

Plasma E_2 levels were similar between treatments before hormonal induction (two-way repeated measures ANOVA, $p = 0.9996$, Table 4); however, at ovulation, mGnRHa (429.46 pg mL^{-1}) levels were lower than CPE (623.40 pg mL^{-1}) ($p = 0.0477$). As we evaluated the plasma levels of E_2 during different sampling times within the same treatment, we observed that in the CPE-induced group there was an increase between the first dose (266.38 pg mL^{-1}) and ovulation (623.40 pg mL^{-1}) ($p = 0.0091$). Similarly, we observed an increase in E_2 levels between the time of the first dose (249.03 pg mL^{-1}) and ovulation (429.46 pg mL^{-1}) ($p = 0.0161$, Table 4) in the mGnRHa-induced group.

Plasma levels of 17α-OHP of CPE- and mGnRHa-induced fish were similar during the first dose ($p = 0.7130$), but lower in the latter (88.25 pg mL^{-1}) compared to the former (110.85 pg mL^{-1}) ($p = 0.0077$, Table 4) at the time of ovulation. As we evaluated plasma levels of 17α-OHP during different sampling times within the same treatment, we observed that, in both treatments, levels reached a peak at the time of ovulation.

Discussion

In this study, we observed that the failure in obtaining *L. macrocephalus* viable embryos with the mGnRHa treatment was associated with a shorter latency period, a more potent ovulatory process, and less intense egg hydration, suggesting a possible overstimulation with toxic effects by mGnRHa and/or metoclopramide (and derived substances) upon oocytes and eggs.

Even though the absolute fecundity was statistically similar between treatments, a more potent ovulatory process observed in the mGnRHa treatment was evidenced by the higher volume density of POF and by the three times reduced volume density of GVBD oocytes retained in the post-spawning ovaries of this treatment in comparison to that of CPE. The percentage of GVBD in post-spawning ovaries of the former treatment (8 %) was also reduced comparing to that of post-spawning ovaries of two other tropical rheophilic fish, *Brycon amazonicus*, (~20 %) (Hainfellner et al. 2012a) and *Piaractus mesopotamicus* (35–60 %) (Criscuolo-Urbinati et al. 2012).

It has been previously shown that overstimulation with the use of high doses of mGnRHa (50–150 μg kg^{-1}) (Mateos et al. 2002; Mañanos et al. 2002; Rosenfeld et al. 2012) is one of the factors which can potentially affect the quality of egg, promoting an overstimulation of the pituitary and causing an excessive elevation in the secretion level of LH. In this study, we did not evaluate LH levels, but considering the gonadal steroid levels evaluated, we have no indirect evidence of excessive levels of LH. On the other hand, considering that the doses used here for the mGnRHa treatment (7 μg kg^{-1} + 10 mg kg^{-1} metoclopramide) were much lower than doses reported as hyperstimulatory (Mateos et al. 2002; Mañanos et al. 2002; Rosenfeld et al. 2012), it is possible that *L. macrocephalus* present a greater sensibility to stimulation provoked by the mGnRHa treatment.

Such possibility is corroborated by previous studies, in which the use of mGnRHa has provoked ovulation, but not viable embryos in other rheophilic species, such as, *Rhamdia quelen* (two doses: 2 and 20 μg kg^{-1} + 10 mg kg^{-1} metoclopramide) (Carneiro and Mikos 2008), *Colossoma macropomum* (single dose: 10 μg kg^{-1} + 10 mg kg^{-1} domperidone) (Acuña and Rangel 2009) and *Piaractus mespotamicus*, *Brycon orbygnianus* and *Prochilodus lineatus* (priming dose: 0.5 μg kg^{-1} and two doses: 1 and 3 μg kg^{-1}) (Paulino et al. 2011).

Another aspect to be considered is the overstimulation caused by specific formulations of mGnRHa. In this study and others with South American migratory fish, the failure in obtaining viable embryos has been specifically associated with the use of mGnRHa (Carneiro and Mikos 2008; Acuña and Rangel 2009; Paulino et al. 2011). On the other hand, more recent investigations concerning South American migratory species, such as *Rhamdia quelen* (Ittzés et al. 2015) and *Prochilodus lineatus* (Viveiros et al. 2015), have shown that the induction of spawning using salmon GnRH [Des-Gly10, D-Arg6, Trp7, Leu 8] enable high ovulation rates and viable embryos. Thus, it is possible that the GnRH formulation used in this study (mammalian analogue gonadotropin-releasing hormone) is associated with hyperstimulation and toxicity, causing a failure when obtaining viable embryos.

This hypothesis is supported by previous reports showing that different GnRH formulations can produce different results. According to Vazirzadeh et al. (2011), an injection of sGnRHa is more potent in spawning induction in wild carp (*Cyprinus carpio carpio*) compared to a single injection or implanting of mGnRHa. Also, Levavi-Sivan et al. (2004) has showed that an injection of mGnRHa was more potent than sGnRHa in spawning induction of *Bidyanus bidyanus*, resulting in a longer lasting increase in plasma LH and reduced fertility. Therefore, future protocols for standardization of GnRH-based synthetic products in *L. macrocephalus* must include not only different levels, but also different types of GnRH. Furthermore, the type and concentration of dopamine inhibitors (metoclopramide, domperidone and pimozide) should also be standardized.

In the hormonal induction procedures, it is known that LH increases plasma concentration by exogenous stimulation of mGnRHa (Mylonas and Zohar 2001; Levavi-Sivan et al. 2004; Rosenfeld et al. 2012). LH, in turn, provokes a peak of 17α,20β-Dihydroxy-4-pregnen-3-one (17α, 20β-DHP) named "maturation inducing substance" (MIS) which promotes germinal vesicle breakdown and the ovulation in teleosts (Nagahama and Yamashita 2008; Lubzens et al. 2010; Ogiwara et al. 2013; Hagiwara et al. 2014). In the present study, although we have not evaluated the plasma concentrations of MIS, we have observed that levels of 17α-OHP (main MIS precursor) in the CPE treatment (with viable embryos) were higher than those in the mGnRHa treatment (without viable embryos) at the time of ovulation. Therefore, we observed neither clear evidence of overstimulation in the

mGnRHa treatment by means of gonadal steroid evaluation, nor indirect evidence of overstimulation by high levels of LH.

Still concerning the gonadal steroid profile, a successful spawning in fish has been associated with a "steroidogenic shift" process characterized by a respective gradual reduction and abrupt rise of 17β-estradiol (E_2) and 17α,20β-Dihydroxy-4-pregnen-3-one during ovulation (Levavi-Zermonsky and Yaron 1986; Rahmann et al. 2001; Nagahama and Yamashita 2008). However, in the present study, in both treatments, ovulation was associated with an elevation of the 17α-OHP (MIS precursor) at the time of ovulation, but not with a reduction in E_2 levels. Thus, although we have not directly dosed MIS, we observed that a successful ovulation in this species is associated with a 17α-OHP peak at the time of ovulation, but not with a concomitant reduction in E_2 in both treatments. Plasma E_2 levels, contrary to previous reports (Miura et al. 2007; Aizen et al. 2012; Elisio et al. 2014), increased in the CPE and mGnRHa treatments at the time of ovulation. The increase in E_2 concentration in hormone treatments has been attributed to the incidence of previtellogenic oocytes in the ovary, because immature vitellogenic and previtellogenic oocytes may respond to high levels of gonadotropins (FSH and LH) present in CPE and produced in the mGNRHa treatments (Levavi-Zermonsky and Yaron 1986; Weil et al. 1986; Drori et al. 1994; Aizen et al. 2012). The increase in 17α-OHP was expected, since this is the sex steroid precursor of 17α, 20β-DHP, which contributes to germinal vesicle breakdown and the ovulation process in teleosts (Nagahama and Yamashita 2008). Thus, the endocrine control of ovulation in this species may be different from species in which a steroidogenic shift has been reported and this aspect must be deeply investigated for this and other rheophilic species in order to produce better results in their captivity reproduction.

If on the one hand concentration of steroid hormones do not explain failures obtained with the mGNRHa treatment, on the other hand, failures in obtaining viable embryos in the mGnRHa group were directly associated with the oocyte less intense hydration process. The process of egg hydration is associated with the ability of the egg in absorbing water by osmosis due to the presence of molecular channels (King et al. 2004; Fabra et al. 2005) and increased osmotic pressure (Fabra et al. 2006). We did not find in the literature previous specific reports associating a less intense egg hydration with a specific hormonal treatment and/or egg loss; however, considering that, during the final maturation, the disorganization of yolk globules increases the colloid osmotic pressure thus inducing the water inlet and hydrating oocytes and eggs (Oshiro and Hibiya 1981; Thorsen and Fyhn 1996; Matsubara and Koya 1997; Finn et al. 2002), it is likely that a less intense hydration of the eggs may be a consequence of oocyte and egg cell machinery malfunctioning, and this may be related to toxic effects.

Since semen used in both treatments was the same and the deficit hydration process obtained through the mGnRHa treatment occurred immediately after ovulation (from the first analysis period), we assume here that possible causative agents of a less intense egg hydration were transferred from broodstock to the oocytes, probably within the ovaries, supporting the hypothesis of toxicity by mGnRHa or metoclopramide (or derived substances) present in the mGnRHa treatment.

In conclusion, the use of mGnRHa associated with metoclopramide is more powerful as an inducer of ovulation then CPE (considering the doses and intervals applied here), but the former does not provide viable embryos, which is in turn associated with a reduced latency period, a more intense ovulation and a less intense egg hydration process. All these results indicate that there may have been a hyperstimulation of the ovaries and a consequent toxicity of eggs in the mGnRHa treatment. The successful ovulation in both

treatments was associated with increased plasma concentrations of E_2 and 17OHP in both treatments at the time of ovulation, but lacking a "steroidogenic shift" process in this species. This is the first study on rheophilic fish in which low doses of mGnRHa applied must have caused a hyperstimulation and/or toxicity to oocytes and eggs. According to the literature, such sensibility may be due to mGnRHa or metoclopramide doses and formulations. The bad quality of eggs obtained from the mGnRHa treatment is strongly supported by a failure in the egg hydration process, fertility and hatching rates, which are close to zero. Further studies are needed to determine the type and concentrations of GnRH (especially less than 7 μg kg^{-1}) and dopamine inhibitors to be used in *L. macrocephalus*.

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