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The effects of sGnRHa implants on *Piaractus mesopotamicus* female breeders. An approach addressed to aquaculture

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Abstract In this study, we evaluated the potential use of sGnRHa slow-release implants (150 μg – Ovaplant®) for promoting an increased number of suitable *Piaractus mesopotamicus* females for induced spawning in a predictable period. To that, *fshb*, *lhb*, and *gh* gene expression, ovarian stereological evaluation and gonadal steroid concentrations were compared between treated and control females (n = 18) at 21, 35, and 115 days after sGnRHa implantation (DAI). The major differences between groups were observed at 35 DAI, when *lhb* and *fshb* were, respectively, up and downregulated for treated females compared to the controls, and the frequency of treated females in an advanced maturation class was higher (60%) than the controls (20%). Especially for promoting a significant change in the gonadotrophins gene expression levels, we demonstrated for the first time that sGnRHa slow-release implants has potential to be used to manipulate the reproductive cycle increasing the number of suitable *Piaractus mesopotamicus* females for induced spawning in a predictable period. However, a definitive protocol still depends on more studies involving a greater number of treatments and replicates.

Keywords Pacu · Synchronization · Ovarian maturation · sGnRHa · Implants

Introduction

Pacu (*Piaractus mesopotamicus*) is a neotropical omnivorous characiform fish (Moro et al. 2013), which was the sixth most produced fish species in Brazil with 14.600T in 2014 (IBGE 2015). The

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consolidation of this species in national and international aquaculture is due to favorable zootechnical characteristics, such as its rustic trait and fast growth under a variety of conditions (Gelman et al. 2004). However, despite the hormonal induction technique that has been used for decades, irregularities and deficiencies are still very common in the fry production of this species, especially in relation to unpredictable spawning success due to unsuccessful ovulation (Criscuolo-Urbinati et al. 2012).

When kept in captivity, ovarian and testicular maturation takes place, reaching the more advanced stages of maturation between October and January (Lima et al. 1991; Romagosa et al. 1993). This fish is a total spawner species with group-synchronous oocyte development; hormonal stimulation is required for spawning in captivity (Criscuolo-Urbinati et al. 2012; Kuradomi et al. 2016). The most common method used for induced spawning is the application of crude carp pituitary extract (CPE) [two doses (0.6 and 5.4 mg/kg with a 24-h interval between the doses)], but higher ovulation rates are achieved if a dose of synthetic prostaglandin F (2 mL Ciosin® containing 0.25 mg/mL cloprostenol) is given at the time of the second CPE dose (Criscuolo-Urbinati et al. 2012). Concerning the use of synthetic products, in the 1980s, Carolsfeld and collaborators (1988a) showed that [D-Ala' des-Gly"] LHRH ethylamide analogue (100 μg/kg in single dose) provoked final maturation and ovulation in this species. However, the use of LHRHa or other synthetic products was not continued due to inconsistent results obtained in later studies with pacu (Paulino et al. 2011) as well as for other South American reophilic species (Carneiro and Mikos, 2008; Acuña and Rangel 2009; Paulino et al. 2011; Pereira et al. 2017).

Among the many causes of unpredictable spawning (see review by Zohar and Mylonas 2001; Mylonas et al. 2010), we draw attention to the existence of intense heterogeneity in the distribution of ovarian maturation classes during the breeding season for pacu. First, this situation requires excessive management to obtain breeders suitable for induced spawning (in advanced maturation) among many other reproductive classes, which often impairs fish welfare, inhibiting reproduction (Campbell et al. 1992, 1994; Brooks et al. 1997; Bobe and Labbe 2010; Schreck 2010). Furthermore, this heterogeneity decreases the accuracy of selecting suitable females due to the imprecise methods employed by pacu fish farmers. Oftentimes, females considered suitable, with the softest bulging abdomens and hemorrhagic papillae, do not ovulate when subjected to conventional hormonal treatments, possibly because external assessments are not 100% reliable in selecting suitable females for spawning (Zohar 1989). Thus, the ability to ensure a higher frequency of suitable females at a predictable time is important for avoiding excessive handling and enabling a predictable spawning in this species.

In this regard, GnRH implants might be useful in producing a core group to trigger mass spawning (Carolsfeld et al. 1988b). Therefore, in combination with other hormones or alone, GnRHa slow-release implants have been applied successfully in some species of teleosts, such as *Chanos chanos* (Lee et al. 1986), *Mugil cephalus* (Aizen et al. 2005), and *Pagrus major* (Kumakura et al. 2003). In this context, these implants were used to anticipate the spawning period in milkfish, *C. chanos* (Lee et al. 1986), to intensify the vitellogenic process in gray mullet, *M. cephalus* (Aizen et al. 2005), and to induce vitellogenesis and spawning in prepubertal females of the red seabream, *P. major* (Kumakura et al. 2003). The effects of GnRHa implants on teleost ovarian maturation are preceded by changes in various levels of the hypothalamic–pituitary–gonadal axis, and growth hormone (Gh). Moreover, GnRH may also act on the synthesis and release of Gh, which can be considered as a "co-gonadotropin" in teleosts, participating on the gonadal axis, contributing to gametogenesis and steroidogenesis (Mosconi et al. 2002; Chang and Wong 2009; Pérez et al. 2016).

The ability to accurately predict time a spawning event would facilitate and allow *Piaractus mesopotamicus* farmers to organize the next stages of the fry production process, which are the hatchery and nursery stages. Often, nursery ponds are fertilized and prepared to receive the fry, but breeders frequently fail to respond to hormonal inductions. Therefore, in this study, aiming to make the reproduction of this species more secure and predictable, we evaluated the use of salmon gonadotropin-releasing hormone analogue [D-Arg^{6}Pro^{9}NEt]-sGnRHa sustained release cholesterol pellets implants (150 μg – Ovaplant®, Western Chemical Inc., USA) in increasing the number of suitable *P. mesopotamicus* females for induced spawning in a predictable period.

Materials and methods

Maintenance, hormonal induction, and sampling

Microchipped (AnimallTAG® – Korth RFID Ltda, São Carlos, SP) pacu females were reared in 200 m^3 outdoor earthen ponds, stocked at a density of 0.25 fish/m^3, at the Aquaculture Center, Sao Paulo State University (Jaboticabal, SP, BR); the females were obtained by induced spawning at the same institution. The earthen ponds received constant flowing water at the rate of ≅ 20 L/min. The physical and chemical analyses of water were carried out fortnightly before 09:00 h. A YSI model 55 oximeter and a YSI model 63 multiparameter sounder (Yellow Springs Instruments, Yellow Springs, OH, USA) to determine the dissolved oxygen, pH, conductivity levels, and temperature; the concentration of N-ammonia was determined according to the method of Solórzano (1969). The fish were fed a commercially available feed (Guabi, Campinas, SP, BR; composed of 32.0% crude protein, 6.5% fat, 10.0% ash, and 7.0% crude fiber) twice a day ad libitum. All of the fish were treated and euthanized according to the accepted protocols of the Comissão de Ética no Uso de Animais (CEUA) from the Faculdade de Ciências Agrárias e Veterinárias/Unesp (Jaboticabal, SP, BR) approved on February 09, 2010 (protocol n° 02.116/10).

In the middle of spring at "0" days after implantation (DAI), to compose the treated group, nine females randomly selected from the earthen ponds were injected with an Ovaplant® implant (Syndel, Qualicum Beach, BC, CA) at a dosage of 150 μg, using a RALGUN® as the applicator (Syndel, Qualicum Beach, BC, CA). Another nine females were randomly selected as the control group (sham treatment), which received exactly the same procedures as the experimental group, but without the use of implants.

Ovaplant® is a synthetic peptide analogue of salmon gonadotropin-releasing hormone, presented in a cholesterol-based matrix as an intramuscular pellet implant, available in 75, 150, or 250 μg sGnRH per pellet. According to tests performed before in fish species, 40–60% of the sGnRH is released within 24 h, and the remainder is released over the next 7–21 days. Standard hormone dosage rates will be 10–75 μg sGnRHa/kg body weight (U.S Fish & Wildlife Service 2016).

At 0 (n = 9 per group), 21 (n = 4 per group), 35 (n = 7 per group), and 115 DAI (n = 3 per group), the fish were anesthetized with benzocaine (0.1 g/L), the body mass (BM) was weighed (kg), and the fish was photographed. The sample size varied at collection times since we determined that fishing nets would be used no more than two times in the same earthen pond and sampling time; thus, the sample sizes were defined by what was obtained with this effort. This measure was taken to make sure the catch efforts were equal between samplings,

avoiding different levels of stress that could impact the results. From each digital picture that was taken, the following metric sizes (cm) were determined: standard length (SL)—length of the anterior margin of the head to the posterior margin of the body (insertion of the caudal fin); and abdomen width (AW)—maximum distance of the belly transverse to the body axis. The measurements were taken using the free software IMAGE J (US National Institute of Health, http://rsb.info.nih.gov/ij/); each metric size was taken in triplicate and the means of these rejoinders were used for the analysis, and from this measurement the AW/SL index was also calculated.

Due to the small sample size, which is common in studies with arrays that require low stocking densities and a large number of earthen ponds, we only used two sampling periods, at 35 DAI (n = 5 per group/n total = 10) and 115 DAI (n = 3 per group/n total = 6), when the females were euthanized (benzocaine at 30 g/L) for organ harvesting. The 35 DAI was strategically set because, in other species where similar implants were used, it was observed that gonadotropin levels were maintained at high levels for approximately 4 weeks (Crim et al. 1988), similar to the period of 35 DAI established by us. In the case of 115 DAI, this period was determined to be the end of the breeding season, and we needed a second point for comparison between groups. The ovary, liver, viscera, and visceral fat were dissected from each fish and weighed to calculate the following indices: gonadosomatic (GSI), hepatosomatic (HSI), viscerosomatic (VSI), and visceral fat (VFI). All of the indices were calculated according to the following formula: $Index = [(organ\ weight\ (g)/BM(g)) \times 100]$. Additionally, before anesthetic overdose, blood samples (3 mL) were collected from the caudal vessels at day 0 (n = 5 controls), 21 DAI (n = 4 per group), 35 DAI (n = 5 per group), and 115 DAI (n = 3 per group) using EDTA-treated syringes. The serum was separated by centrifuging the samples at 1500×g for 15 min at 4 °C and stored at −80 °C until measurement of the steroid hormone concentrations.

In the last two collections (35 and 115 DAI), the cranial, central, and caudal fragments of the ovarian tissue were fixed with phosphate-buffered (0.05 M) 2.5% glutaraldehyde (pH 7.3) for 24 h at 4 °C and embedded in Histosec® (Merck KGaA, Darmstadt, HE, GE). The ≅ 1 g ovarian fragments (medial region) were fixed in modified Gilson solution (100 mL 60% ethanol, 800 mL distilled water, 15 mL 80% nitric acid, 18 mL glacial acetic acid, and 20 g of mercury chloride) to facilitate the calculation of absolute fecundity rates (AF). Whole pituitaries were preserved in RNAlater™ solution (Ambion, Austin, TX, USA) according to the manufacturer's instructions and stored at −80 °C until total RNA extraction.

Absolute fecundity rates

The AF was determined by the number of oocytes counted in the fragments that had been submerged in Gilson solution. From the number of oocytes counted the total number of oocytes per a gram of ovary mass was estimated.

Macro and microscopic ovarian maturation stage classification

The fixed ovarian fragment samples were dehydrated in ethanol, cleared in xylene, and embedded in Histosec® (Merck KGaA, Darmstadt, HE, GE), a paraffin plus plastic resin medium, melted at 58 °C. The samples were sectioned to a thickness of 3.0 μm and stained with hematoxylin and eosin. To determine the ovarian maturation stage, a similar analysis to that used by Leonardo et al. (2006), which considers macroscopic (color, shape, size, and

appearance) and histological aspects, was adopted. To determine the gonadal development stage, we performed a visual analysis and based on the description by Lima et al. (1991), established individually, the frequencies of the oocytes in the following phases: previtellogenic (PV), alveolar cortical (AC), vitellogenic (V), and atresia (AT). Thus, females were classified as one of the following developmental stages: rest, early maturation, advanced maturation, and regression.

Steroid hormones

The plasma levels of 17α-hydroxyprogesterone (17α-OHP), 17β-estradiol (E_2), testosterone (T), and 11-ketotestosterone (11-KT) were quantified by hormone enzyme-linked immunosorbent assays (ELISA) using commercial kits (Cayman Chemical Company, Ann Arbor, MI, EUA for 11- KT; and BioCheck, Inc., Foster City, CA, EUA distributed by Interteck Internacional Imp. e Exp. Ltda. Sao Paulo, SP, BR for all others) according to the manufacturer's instructions. The 17α-OHP, E_2, and T plates were read at 450 nm, and the 11-KT plate was read at 405 nm using a Spectramax 250 ELISA plate reader (Molecular Devices, Inc., Sunnyvale, CA, USA); all of the samples were read in duplicate. The kits were previously validated to pacu by Kuradomi et al. (2016). Females at rest were excluded from the steroid hormones analysis because they could affect the hormones levels since they had not responded to treatment and environmental factors in the control group.

RNA extraction and cDNA synthesis

Total RNA was extracted from the pituitaries using the RiboPure™ Kit (Ambion®, Austin, TX, USA) following the manufacturer's instructions. The total RNA and the presence of DNA were quantified and confirmed by a Qubit™ fluorometer (Invitrogen™) using the Quant-iT™ RNA Assay Kit (Life Technologies™, Eugene, OR, USA) and Quant-iT™ dsDNA High-Sensitivity (HS) Assay Kit (Life Technologies™, Eugene, OR, USA), respectively. The calibrations and quantification were carried out according to the manufacturer's instructions; no sample with DNA contamination was observed. The Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Pico Kit (Agilent Technologies, Palo Alto, CA, USA) were used to evaluate the RNA quality, and RIN values higher than 8.5 were accepted. For reverse transcription quantitative PCR (RT-qPCR), SuperScript™ II Reverse Transcriptase (Invitrogen™, Carlsbad, CA, USA) was applied using random hexamer primers (Invitrogen™, Carlsbad, CA, USA) and 2 μg of total RNA as a template.

Real-time quantitative PCR

Quantitative real-time PCR assays were performed in duplicate using 96-well optical plates on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the default settings. For each 20 μL PCR reaction, 5 μL of cDNA (diluted two-fold) was mixed with 900 nM sense primer and 900 nM antisense primer in 2× SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The primer sequences, except for *gh*, were the same as those used by Kuradomi et al. (2016): 5′-CCTGGTGTTTCAGACCAGCAT-3′/5′-CGTGCTGATAGATGGTGGAGAA-3′; 5′-AAGAATGCGGGAGCTGCA-3′/5′-GGTCTCATACGTCCACTCCCTAAA-3′; 5′-TGGC AAAGATGAGACCCAGAA-3′/5′-GGGTACTCCCAAGACTCGATCAG-3′ and 5′-TCTG

GATGGCACGGAGACA-3′/5′-CAATCTTCCATCCCTTGAACCA-3′ - the sense/antisense to the genes expressions of *lhb*, *fshb*, *gh*, and *elongation factor 1 alpha* (*ef1a*), respectively. Target (*gh, fshb*, and *lhb*) gene expression was normalized to *ef1a* gene expression and calibrated by using the mean of the pituitary *ef1a* expression levels from the control sampling period. PCR efficiency was evaluated according to the equation $Ct = m \times (\log Q) + c$, where *Ct* is the threshold cycle, *Q* is the initial copy number, *c* is the intercept on the *y* axis, and *m* is the slope (Bogerd et al. 2001). A fourfold serial dilution of the pituitary cDNA mixture was performed, and all of the primer sets showed a standard curve with a slope value that was close to 2, indicating exponential PCR amplification, assuming that 2 cycles are required to generate a fourfold increase in the PCR product. To calculate gene expression levels, the ΔΔCt method was used, as previously described (Bogerd et al. 2001) and applied by Kuradomi et al. (2016). The females classified as rest were also excluded from the analyses of gene expression.

Statistical analysis

The statistical analyses were performed using specialized statistical software (STATISTICA–StatSoft, Inc., Tulsa, OK, USA) and Excel (Microsoft, Redmond, USA). Assumptions of normality and homoscedasticity were tested by Shapiro–Wilk's and Levene's tests, respectively. Parametric variables (biometrics data, AW/SL, tangible indices, and fecundity) were assessed using Student's *t* test. For the hormone levels and gene expression data, the non-parametric Mann-Whitney U tests were used. Significance ($\alpha = 0.05$) was determined for all of the datasets, and the results are shown as the mean ± standard error of the mean (SEM).

Results

Water parameters

Maximum and minimum variations and mean ± SEM of the temperature (22 to 30 °C; 25.91 ± 2.98), pH (6.59 to 7.46; 6.94 ± 0.13), conductivity (41 to 122 μS/cm; 52.10 ± 10.27) total ammonia (0.00 to 0.50 ppm; 0.25 ± 0.05), and dissolved oxygen (4.26 to 7.46 mg/L; 5.89 ± 0.51) of the earthen ponds water was satisfactory throughout the experimental period, remaining within the recommended levels for South American teleost broodstock (Bock and Padovani 2000; Murgas et al. 2009).

Gene expression by quantitative real-time PCR

The mean values of the relative gene expression of *fshb* at 35 DAI in the treated females was ×1.45 lower than the controls ($P < 0.05$), but similar to all other mean values ($P > 0.05$) (Fig. 1). Additionally, in the same sampling, the mean value of *lhb* expression of the treated females was ×1.64 higher than all other mean values ($P < 0.05$). The mean values of *gh* gene expression were similar in all the periods ($P > 0.05$) (Fig. 1).

Steroid hormones

The mean values of the E_2, T, 17α-OHP, and 11-KT plasma levels were similar ($P > 0.05$) between the groups at same sampling (Fig. 2). However, we observed a trend of gradual

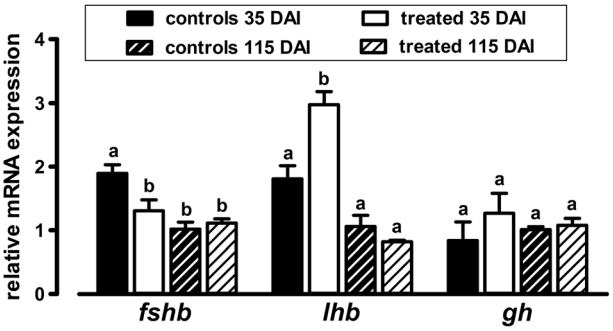

Fig. 1 Relative expression of *fshb*, *lhb*, and *gh* mRNA in the pituitary, comparing treated (sGnRHa – 150 μg) and control pacu (*Piaractus mesopotamicus*) females. Different letters represent significant differences between the groups ($P < 0.05$)

reduction in the E_2 and 11-KT levels in both groups between 21 (onset of spawning season) and 115 DAI (ovarian regression phase) (Fig. 2). Regarding 17α-OHP and T, we observed similar levels between groups during the entire experimental period (Fig. 2).

Morphometry, index, and AF

The means of biometric data are presented in Table 1, which shows that the values were similar ($P > 0.05$) for all variables in both groups (AW, SL, BM, and AW/SL).

The mean values of tangible indices, shown in Table 2, were similar between the groups ($P > 0.05$), except for the HSI which was higher in the treated group at 35 and 115 DAI ($P < 0.05$). The mean values observed for AF data were also similar between groups at 35 and 115 DAI (Table 2).

Ovarian maturation stage

The ovarian maturation, defined both macroscopically and microscopically, is shown in Fig. 3. The percentages of ovaries in different maturation classes in each group are presented in Fig. 4.

Discussion

The use of sGnRHa implants (150 μg Ovaplant®) was associated with a higher frequency of females suitable for hormonal induction at 35 DAI, when 60% (3 of 5) and were in the advanced maturation stage in the treated and 20% (1 of 5) control groups. The higher percentage of females in an advanced stage of maturity at 35 DAI in the treated group was accompanied by higher values of *lhb* expression, higher HSI values, and lower values of *fshb* expression in comparison to the control group. Taken together, these findings indicated that the implants have potential to promote changes on the ovarian maturation process between 0 and 35 DAI in treated females.

In this context, it is known that the GnRH controls the secretion and release of Lh (Trudeau et al. 1991; Trudeau 1997; Golan et al. 2015), which is responsible for promoting the final maturation processes and ovulation in fish (Lubzens et al. 2010, Ogiwara et al. 2013). Therefore, an intensified *lhb* gene expression at 35 DAI in treated animals (64% more) was

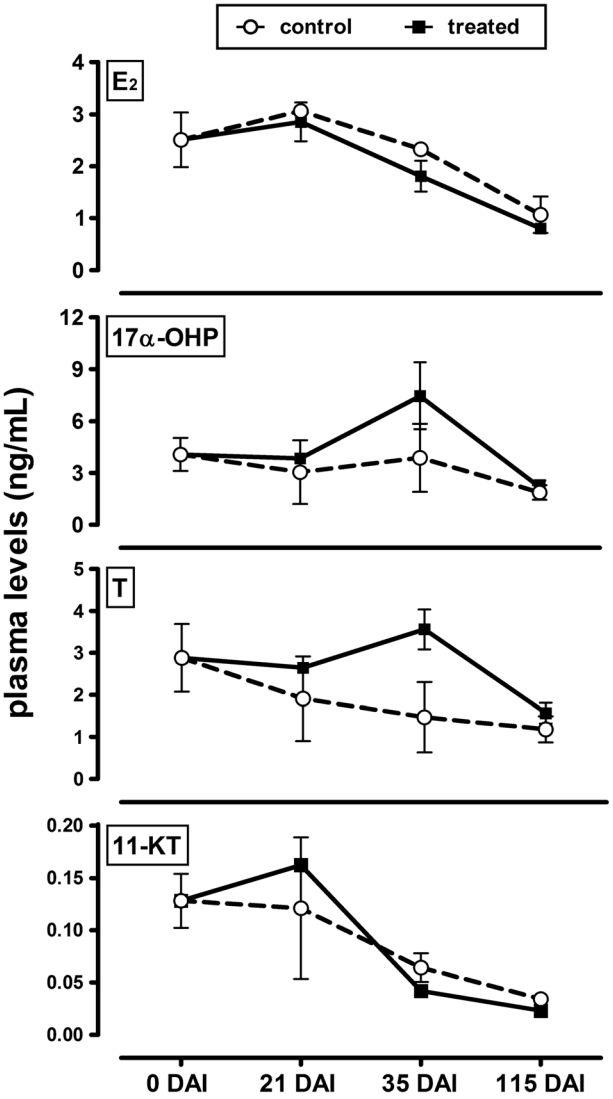

Fig. 2 Plasma 17α-hydroxyprogesterone (17α-OHP), 17β-estradiol (E_2), testosterone (T), and 11-ketotestosterone (11-KT) levels (mean ± SE) in treated (sGnRHa – 150 μg) and control pacu females (*Piaractus mesopotamicus*) throughout the breeding season. *DAI* days after introduction of the implant. *Day* "*0*" beginning of the experiment

associated with a higher frequency of females in the advanced stage of maturation (suitable for hormonal induction). Similar results were observed in sea bass (*Dicentrarchus labrax*) sGnRHa implant treated females, where the synthesis and release of Lh was stimulated (Mateos et al. 2002); and in sockeye salmon (*Oncorhynchus nerka*), in which the use of GnRHa implants induced the Lh transcription level and anticipated the final maturation of mature females (Kitahashi et al. 1998).

Despite being associated with a higher frequency of advanced maturation class females and pronounced changes in *lhb* expression, the use of (sGnRHa – 150 μg) implants applied here

Table 1 Standard length (SL), abdomen width (AW), body mass (BM), and index AW/SL in pacu, *Piaractus mesopotamicus*, females, comparing treated (sGnRHa – 150 μg) and control fish

Variables	Control				Treated			
	0 DAI	21 DAI	35 DAI	115 DAI	0 DAI	21 DAI	35 DAI	115 DAI
SL (cm)	42.83 ± 3.18	42.45 ± 2.45	42.95 ± 1.86	43.61 ± 1.03	41.35 ± 1.79	42.74 ± 2.96	41.16 ± 3.01	40.65 ± 3.76
AW (cm)	5.94 ± 0.76	5.51 ± 0.67	5.53 ± 0.75	5.47 ± 0.71	4.96 ± 0.57	5.02 ± 0.83	4.85 ± 0.67	4.79 ± 0.68
BM (g)	2858 ± 206	2508 ± 424	2593 ± 392	2780 ± 224	2290 ± 537	2468 ± 688	2034 ± 349	2026 ± 533
AW/SL	0.13 ± 0.01	0.12 ± 0.00	0.12 ± 0.01	0.13 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.00	0.12 ± 0.01
n	9	4	7	3	9	4	7	3

DAI days after introduction of the implant. Means ± SE

Table 2 The tangible indices and absolute fecundity – AF (n° of oocytes/g of ovary) (means ± SE) of treated (sGnRHa – 150 μg) and control fish in pacu, *Piaractus mesopotamicus*, females

Variables	Control		Treated	
	35 DAI	115 DAI	35 DAI	115 DAI
HSI	0.78 ± 0.03*	0.68 ± 0.08*	0.94 ± 0.03	0.96 ± 0.08
GSI	3.63 ± 0.60	4.82 ± 1.28	4.47 ± 1.19	4.49 ± 0.21
VSI	6.01 ± 0.48	6.53 ± 2.58	6.77 ± 0.38	4.37 ± 2.37
VFI	2.59 ± 0.38	2.13 ± 1.13	3.02 ± 0.38	1.35 ± 0.55
AF	920.00 ± 80.00	970.00 ± 71.00	880.00 ± 174.00	888.00 ± 260.00
n	5	3	5	3

Asterisks denote significant differences between groups on the same date ($P < 0.05$)

Hepatosomatic HSI, *gonadosomatic* GSI, *viscerosomatic* VSI, *visceral fat index* VFI, and *DAI* days after introduction of the implant

was not able to cause all treated females to reach the advanced maturation class in the same period (35 DAI). However, we also observed that at the concentration applied here, the use of implants did not significantly alter the GSI, the fecundity absolute or the concentrations of gonadal steroids. These results allow us to make some assessments on the dose applied and the results obtained. Considering that it is a pioneering study on tropical total spawning fish and that we showed that the species is probably responsive to the technique and the next steps would be to search for more effective doses followed by pharmacological studies.

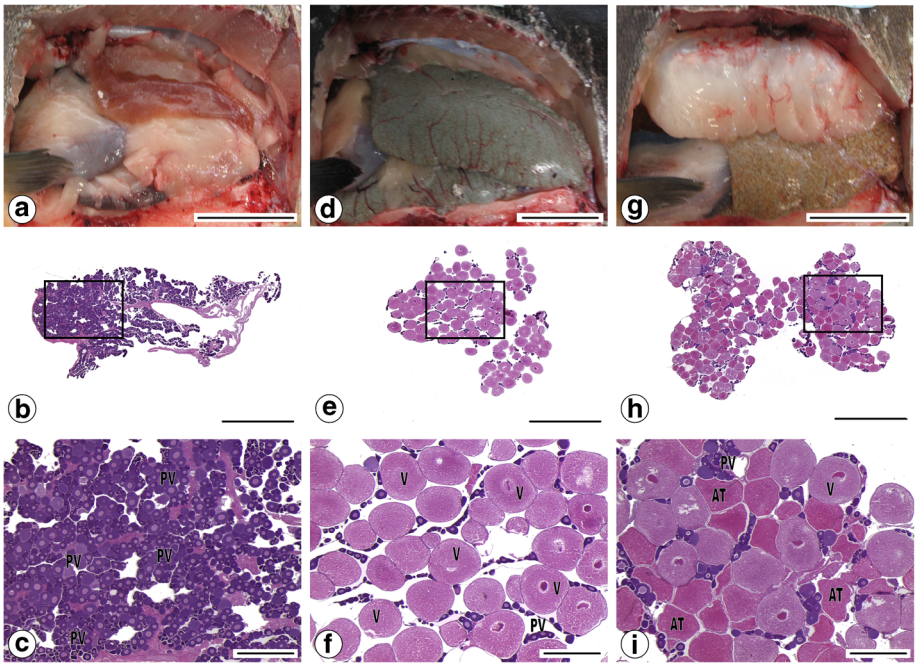

Fig. 3 Illustrative figures of the ovaries (A, D, and G) and photomicrographs of histological sections (B, C, E, F, G, and I) of pacu, *Piaractus mesopotamicus*, in the following stages: rest (A–C), advanced maturation (D–F), and regression (G–I). In the figures, B, E, and H the frame (□) represents the area enlarged in Figs. C, F, I. Pre-vitellogenic (PV) vitellogenic (V), and atretic oocytes (AT). Scale bars = 5 cm (A, D and G), 5 mm (B, E, H), and 1 mm (C, F, I)

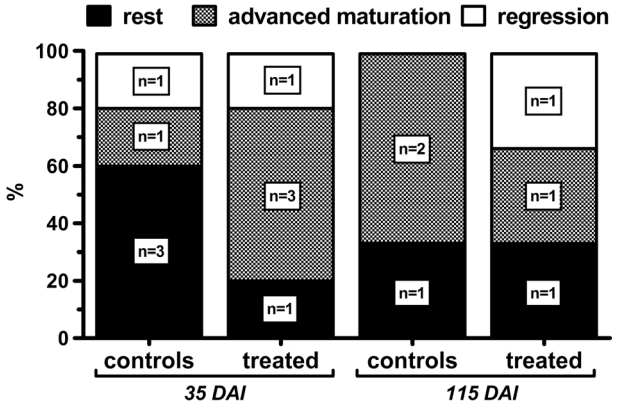

Fig. 4 Frequency of pacu ovaries (*Piaractus mesopotamicus*) classified by histology in the stages of rest, advanced maturation, and regression. *DAI* days after introduction of the implant

In this regard, optimum doses for induced successful spawning using a GnRHa implant have been reported for *Sparus aurata* (Barbaro et al. 1997) and *Lates calcarifer* (Garcia 1989), which spawned successfully using doses from 37.5–80 μg GnRHa/kg of fish BM. In the case of *Lutjanus guttatus*, higher doses ranging from 240 to 280 μg GnRHa/kg of fish BM (Ibarra-Castro and Duncan 2007) were required, as such, the optimum doses seems to be species specific. Moreover, Carolsfeld et al. (1988b) found that the maturation response in *Clupea harengus pallasi* was dose-dependent and the higher tested dose (approximately 800–1000 μg GnRHa/kg of fish BM) was more effective than the lower dose (approximately 170–220 μg GnRHa/kg of fish BM). Therefore, based on the differences among doses previously reported for spawning induction and sexual maturation, it is reasonable to suggest that higher doses than the one applied here for *P. mesopotamicus* could provide better results for inducing sexual maturation. More specifically, in our study, the dose applied was approximately 60–75 μg GnRHa/kg of fish BM, so higher doses could be tested to verify whether a higher proportion of advanced maturation class pacu females at a predictable time would occur, as reported for *Clupea harengus pallasi* by Carolsfeld et al. (1988b).

Concerning gonadal steroid levels, in species treated with GnRHa implants, changes in Lh or *lhb* levels may (Harmin et al. 1995; Kumakura et al. 2003) or may not (Mañanós et al. 2002) be accompanied by changes in levels of the related gonadal steroids, even during the same period, with expressive variations between species and studies. Still in this concern, we must emphasize that, due to the absence of specific kits, we evaluated the values of the precursor 17α-OHP, but not 17α, 20β-dihydroxy-4-pregnen-3-one (DHP). Nevertheless, it is known that, in captivity, DHP peaks are found in the females of most farmed fish close to the ovulation (Nagahama and Yamashita 2008; Mylonas et al. 2010), usually in females that are hormonally induced for spawning. Additionally, eventual peaks of 17α-OHP may have occurred outside of the analysis period (35 and 110 DAI), especially since gonadotropins and steroids may not necessarily have the same patterns of synthesis and release (Mylonas and Zohar 2007).

Similar results were observed concerning E_2; despite the *fshb* levels being higher in the control then in the treated group at 35 DAI, the E_2 plasma levels were similar between groups. In this concern, one would expect higher levels of E_2 in the control group due to the higher *fshb* levels, but no parallel was found, similar to the *lhb* and 17α-OHP relationship. It is known that, in fish, there is an intensification of vitellogenic processes concurrent to an elevation in E_2 plasma levels (Berlinsky and Specker 1991; Lubzens et al. 2010; Yilmaz et al. 2015). However,

in this study, similar levels of E_2 were in agreement with the results found; at 35 DAI, maturing females (in vitellogenesis) were not found in both groups, and a similar average GSI was found between groups at 35 DAI. Profiles of plasma concentrations of E_2 in the treated and control females of this study were similar to those described for other total spawner fish analyzed during breeding seasons (Arantes et al. 2010; Lubzens et al. 2010; Hainfellner et al. 2012; Yilmaz et al. 2015). However, a clearer understanding of the relationships between the use of implants in *P. mesopotamicus* and the levels of E_2, as well as of 17α-OHP or DHP still depends on further studies using a higher number of implant doses (treatments) with more replicates.

The higher HSI values of treated females at 35 DAI were in accordance with the increased number of advanced maturation females in the treated group since the vitellogenin proteins are synthesized in the liver (Nagahama and Yamashita 2008; Lubzens et al. 2010; Yilmaz et al. 2015). Moreover, and also for this reason, higher HSI values during vitellogenesis than at spawning and/or resting have been reported in several fish species (Santos et al. 2004; Sudarshan and Kulkarni 2013; Hismayasari et al. 2015; Jan and Ahmed 2016).

In this study, we did not observe a relationship between the use of sGnRHa and *gh* expression. Similarly, in African catfish (*Clarias gariepinus*), no association between GnRH and Gh concentrations was found (Lescroart et al. 1996; Bosma et al. 1997). Here, we investigated *gh* expression profiles to have a broad range evaluation of the effects of sGnRH implants since, in other fish species, GnRH has been shown to provoke Gh synthesis and release, which in turn, acts as "co-gonadotropin" over gonadal gametogenesis and steroidogenesis (Chang and Wong 2009; Xu et al. 2011; Sun et al. 2014). Additionally, elevated serum Gh is associated with an inhibition of pituitary *lhb* expression and Lh secretion (Cao et al. 2014). However, we must consider that the action of GnRH on *gh* gene expression, and consequent elevation in Gh plasma concentration, is dependent on the gonad maturation stage (Sumpter et al. 1991; Björnsson et al. 1994; Gomez et al. 1999) or season (Bhandari et al. 2003). Therefore, while we could not demonstrate a clear association between the results found and *gh* gene expression levels in this study, the specific role of this substance in ovarian maturation and its association with the use of implants in this species is a subject for further studies.

The use of slow-release hormone implants has been widely used in freshwater (Mylonas et al. 1992, 1995) and marine fish species (Mylonas et al. 1998; Forniés et al. 2001; Mañanós et al. 2002; Marino et al. 2003; Aramli et al. 2016). Most of the results were obtained with multiple spawner fish that spawn naturally in artificial environment conditions. In these species, the use of implants can increase the number of spawning fish and their associated fertility rates (Mylonas et al. 1998, 2003). In this regard, we found from the present study that GnRH implants can be used for a slightly different purpose in total spawner egg laying fish; that is, promoting a higher number of females suitable for induced spawning at a predictable time.

In summary, we observed that at 35 DAI, the use of 150 μg sGnRHa implants increased *lhb* expression, HSI levels, and the frequency of females in advanced maturation in this study. Although many issues for further testing were raised by this study, the results obtained here indicate that the sGnRHa slow-release implants shows promise for accelerating maturation in *P. mesopotamicus*.

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