

## Gonadal steroids levels and vitellogenesis in the formation of oocytes in *Prochilodus lineatus* (Valenciennes) (Teleostei: Characiformes)

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The objective of this study was to obtain information about the possible mechanisms related to poor reproductive performance in tropical rheophilic fish. To that effect, cages (Cs) and earthen ponds (EPs) were used as experimental systems to provide unsuitable and suitable conditions, respectively, for curimatá (*Prochilodus lineatus*) breeders. Fish were maintained under experimental conditions for 18 months, and during this period females were randomly sampled every two months for biometric analysis (n=30), blood (n=5/sampling) and ovary (n=5/sampling). After this period EPs females (EPFs) and Cs females (CFs) were submitted to the induced breeding experiments. The results showed that rearing curimatá for such long time in a cage at this stocking density, reduces its growth, plasma E2 levels and vitellogenesis. During vitellogenesis, the mean plasma estradiol levels of CFs were three times lower than those of EPFs ( $P < 0.01$ ). CFs presented poorer results than EPFs for all the examined parameters of reproductive performance. Taken together these data showed that the reduced estradiol levels during vitellogenesis (and the consequently less intense transition from the previtellogenic to vitellogenic phase) and reduced amounts of yolk are mechanisms associated with the formation of low quality oocytes and shortened and delayed breeding season in this species. Moreover, our data showed that the onset of vitellogenesis (six months before the spawning season) must be considered as a key period related to the formation of oocytes of good quality, and adequate management should be provided throughout the year.

Este estudo teve como objetivo gerar informações básicas sobre os possíveis mecanismos relacionados com os resultados desfavoráveis obtidos com o desempenho reprodutivo em peixes reofílicos tropicais. Para isso tanques-rede (Cs) e viveiros escavados (EPs) foram utilizados como sistemas experimentais para propiciar respectivamente condições inadequadas e adequadas para reprodutores de *Prochilodus lineatus*. Os peixes foram mantidos por 18 meses em viveiros escavados (EPs) e tanques-rede (Cs), e durante este período, foram aleatoriamente coletados a cada dois meses para análise biométrica (n=30), coleta de sangue (n=5/tratamento) e amostragem do ovário (n=5/tratamento). Após este período as fêmeas mantidas em EPs (EPFs) e as fêmeas mantidas em Cs (CFs) foram submetidas aos experimentos de reprodução induzida. Os resultados mostraram que manter curimatás por este período em Cs, na densidade de estocagem utilizada, reduz seu crescimento, níveis plasmáticos de E2 e vitelogênese. Durante a vitelogênese, os níveis plasmáticos de estradiol das CFs foram três vezes menores do que os das EPFs ( $P < 0,01$ ). As CFs apresentaram resultados inferiores aos das EPFs para todos os parâmetros analisados de desempenho reprodutivo. Em conjunto, estes dados mostram que os níveis reduzidos de estradiol durante a vitelogênese (bem como o consequente atraso na transição da fase previtelogênica para a vitelogênica) e as quantidades reduzidas de vitelo são mecanismos associados com a formação de ovócitos de baixa qualidade; redução e atraso na época de desova nesta espécie. Além disso, nossos dados mostraram que o início da vitelogênese (seis meses antes da época de desova) é um período-chave para formação de ovócitos de boa qualidade, e o manejo adequado deve ser aplicado durante todo o ano.

**Key words:** Reproductive cycle, Spawning performance, Steroid levels, Tropical rheophilic fish.

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## Introduction

Rheophilic fish are among the fresh water species of essential economic importance in tropical regions (Dabrowski *et al.*, 2003; Leonardo *et al.*, 2004; Batlouni *et al.*, 2006; Reidel *et al.*, 2010; Romagosa, 2010). In captivity, the reproduction of South American fresh water rheophilic fish remains an obstacle to successfully farming these species. Among these species, the curimbatá (*Prochilodus lineatus*) is an iliophagic fish that feeds mainly at the bottom of ponds/rivers (Rios *et al.*, 2011). The ovarian development is synchronous and the reproductive maturation occurs at 11 months of age (~ 25 cm in total length and 250 g in body weight) (Godinho & Ribeiro, 1985).

In spite of the induced breeding techniques that have been applied for decades in many freshwater fishes (Dabrowski *et al.*, 2003; Leonardo *et al.*, 2004; Reidel *et al.*, 2010; Romagosa, 2010), the results are still highly variable. Although there is an absence of scientific studies on this topic in South American fresh water rheophilic fishes, it is not uncommon to encounter reports by farmers and researchers regarding breeders belonging to diverse species that do not respond adequately to hormonal treatment, which causes inconsistent availability of fingerlings and thus impairs the overall commercial production process.

According to Zohar & Mylonas (2001), three basic kinds of problems are associated with reproductive difficulties in female broodstocks: a) fish may fail completely to undergo vitellogenesis when maintained in captivity (frequent in freshwater eels); b) typical for South American rheophilic fish, vitellogenesis may progress normally, but the post-vitellogenic oocytes fail to undergo final oocyte maturation, ovulation and spawning (which can only be achieved through hormone treatments); and c) oocytes undergo normal vitellogenesis, final oocyte maturation and ovulation, but ovulated eggs are not released to the water (typical for salmonids).

In this context, recent evidence obtained in another fresh water tropical rheophilic fish species (*Brycon amazonicus*) has shown that during induced breeding procedures, oocytes attain maturation - as indicated by germinal vesicle breakdown (GVBD) - however, frequently ovulation does not occur and these oocytes remain attached to the ovaries (Hainfellner *et al.*, 2012). Moreover, as shown for other fish species, oocytes may be ovulated, but the quality of the eggs may also be impaired affecting fertility (Zohar & Mylonas, 2001).

In this concern, it is quite well established that inadequate broodstock management can negatively affect breeders' reproductive performance through different mechanisms (Mylonas *et al.*, 2010). The available literature for other fish species, mainly native to temperate regions, such as carp (Wu *et al.*, 2003) and salmonid fish (Pickering *et al.*, 1987; Stratholt *et al.*, 1997; Campbell *et al.*, 1992) indicates that in many cases, the consequence of this may be low oocyte quality (mainly associated with yolk content) and spawning failures (Brooks *et al.*, 1997; Wu, 2009; Bobe & Labbé, 2010; Mylonas *et al.*, 2010).

As stated previously, most studies concerning reproductive dysfunctions have been obtained in fish native to temperate regions; however, this knowledge may not be applied directly to tropical species due to marked differences in reproductive biology. South American fresh water rheophilic fishes present particular aspects in their reproductive biology such as: a short breeding season that occurs exclusively in summer months (during rainy season); the absolute necessity of using hormones to induce oocyte maturation and spawning (spontaneous spawning or ovulation in captivity has never been reported) (Zaniboni-Filho & Weingartner, 2007); and most importantly, the obvious differences regarding climate characteristics between tropical and temperate regions. For species native to temperate regions, photoperiod and temperature are the main environmental factors controlling the process of sexual maturation and reproduction (Mañanós *et al.*, 1997; Bromage *et al.*, 2001; Almeida *et al.*, 2011), while pH, fluviometric level, dissolved oxygen, temperature, rainfall, electrical conductivity are considered to be of major importance to the reproduction of South American tropical species (Lowe-McConnell, 1987; Barthem & Goulding, 1997; Silva *et al.*, 2011).

Based on empirical results, it has been considered that rearing of South American rheophilic breeders at relatively low densities in earthen ponds provides ideal conditions for suitable reproductive performance, while cages are normally not recommended, particularly when high stocking densities are used. Thus, in the present study Cs were used as an experimental system to simulate the effect of inappropriate management practices employed in many rheophilic fish farms, on reproductive function and spawning success of curimbatá.

## Material and Methods

### Animals

In March 2009, 600 curimbatá specimens (males and females at a sex ratio of 1:1, 12 months old, mean  $\pm$  SE total length 31.48  $\pm$  1.03 cm, and weight 394.85  $\pm$  29.85 g were maintained in the Centro de Aquicultura da Universidade do Estado de São Paulo (CAUNESP), Jaboticabal, São Paulo, Brazil (21°15'17" S 48°19'20" W). Fish were distributed in four earthen ponds (EPs) of 50 m<sup>3</sup> and in four cages (Cs) of 6 m<sup>3</sup>. Fish were obtained from fish farms produced through induced breeding.

Previously evidence obtained in our laboratory has shown that *P. lineatus* breeders kept at high stocking densities in cages presented poor reproductive performance. On the other hand, in EPs the stocking density of 1.5 fish m<sup>-3</sup> has been shown to be adequate for both male and females for this species, since they undergo oocyte maturation and spermatogenesis regularly (Godinho & Ribeiro, 1985). In this study, an initial stocking density of 1 fish m<sup>-3</sup> was applied in EPs (50 specimens per pond). Since there was no information about stocking densities for this species in Cs, and the objective was to employ conditions that would reflect real aquaculture situations, 100 specimens were placed inside each cage, resulting in a stocking density of 16 fish m<sup>-3</sup>.

### Culture conditions

Fish were manually fed a pelleted balanced commercial diet (moisture content (max.) 10.0%; crude protein (min.) 28.0%; ethereal extract (min.) 5.0%; fibrous matter (max.) 7.0%; ash (max.) 10.0%; calcium (max.) 1.2%; phosphorus (min.) 0.6%) corresponding to 3.0% of total body weight twice a day (the total biomass value was readjusted following each set of biometric measurements every biometrics). Water parameters were measured weekly using a YSI model 55 oximeter and a YSI model 63 multiparameter sonde (Yellow Spring Instruments, Yellow Springs, OH, USA) to determine dissolved oxygen, pH, conductivity levels and temperature. Transparency was measured at 9:00 h am. using a Secchi disk. The N-Ammonia concentration was determined according to the method of Solorzano (1969); additionally, colorimetric methods were employed, and absorbances were measured using a Hach DR 2000 spectrophotometer (Hach, Loveland, CO, USA).

### Sampling

Fish were maintained in these conditions for a period of 440 days, representing 80 days of acclimation and an experimental period of 360 days afterwards. During the experimental period, samples were collected every 60 days (bimonthly). Thirty specimens (males and females) each were randomly selected from one EP and C, and were transported to the laboratory (following a similar methodology applied by Buchet *et al.*, 2008). Total length (cm) and body weight (g) were recorded for each animal. Then, from these 30 animals, 5 females were randomly selected from each rearing system, anaesthetised with a benzocaine solution (2 g ethylaminobenzoate: 150 ml alcohol: 20 L water) and killed by severing the section of the spinal cord next to the operculum. Blood samples were collected for determination of plasma steroid levels, and gonads for estimation of gonadosomatic index (GSI) and for histological evaluation. All procedures used followed approved guidelines for the ethical treatment of animals and national laws. Experimental protocols were submitted to and approved by the Animal Ethics and Welfare Committee (CEBEA, Comitê de Ética e Bem-Estar Animal) of The Faculdade de Ciências Agrárias e Veterinárias (FCAV), UNESP, Jaboticabal, SP, Brazil.

### Histomorphometric analyses

Ovarian samples (cranial, medial and caudal regions) were collected and fixed in a solution of 2.5% glutaraldehyde for 24 hours. After fixation, the material was embedded in paraplast, cut into 3-5  $\mu$ m thick sections and subjected to hematoxylin and eosin staining. Histological sections from EPFs ( $n = 5$ ) and CFs ( $n = 5$ ) were utilised to determine the frequency of different oocyte types, considering all oocytes present in thirty microscopic fields (ten fields per region, 5x magnification).

Morphometric analysis was performed to evaluate and compare the cell sizes of oocytes of EPFs ( $n = 5$ ) and CFs ( $n = 5$ ) in each sampling. In this analysis, the mean cell diameters of previtellogenic ( $n = 120$ , 20x magnification) and vitellogenic ( $n =$

120, 5x magnification) oocytes (40 per region/cranial, middle and caudal) were measured using an Olympus BX41 microscope system with Olympus DP11 capture (with measurements performed using Image-Pro Plus Version 4.1.0.0 software).

### Steroid analysis

Blood samples were collected using heparinised syringes (Liquemine, Roche, Rio de Janeiro, RJ, Brazil) and needles by puncturing the caudal vein. Blood samples were centrifuged at 1300 g for 10 min, and the plasma was separated into aliquots, which were immediately frozen on dry ice and preserved at  $-80^{\circ}\text{C}$  until processing. The plasma levels of  $17\beta$ -estradiol ( $E_2$ ) and  $17\alpha$ -hydroxyprogesterone ( $17\alpha$ -OHP) were quantified using an enzyme-linked immunosorbent assay (ELISA) ( $E_2$  and  $17\alpha$ -OHP: Interteck, Virginia, USA). Plasma samples were run in duplicate, and validations of both kits ( $E_2$  and  $17\alpha$ -OHP) were determined by calculating the intra- and inter-assay coefficients of variation (% CV) and spike recovery (%). The acceptable limit for the intra- and inter-assay % CV was  $\leq 20.0$  and for spike recovery, 90-110% (Sink *et al.*, 2008). Absorbance measurements were conducted using a microplate reader (Molecular Devices, CA, USA).

### Induced breeding

In the second week of Dec/10 (water temperature:  $25^{\circ}\text{C} - 27^{\circ}\text{C}$ ), fish were randomly chosen from three units (the same used for sampling) (3 females were randomly collected from two units and two from one unit). While 100% of EPF presented large and swollen abdomen with hemorrhagic papillae (appropriate stage for strip-spawning induction), 100% of CFs presented hard abdomen and definitively no signs to be appropriate for strip-spawning induction (but fish were treated anyway). The successful hormone-induced ovulation and strip-spawning of this species has been previously demonstrated with the usage of human chorionic gonadotropin (HCG) and carp pituitary extract (Fenerich-Verani *et al.*, 1984; Godinho *et al.*, 1984, Zaniboni Filho & Barbosa, 1996). Hormone-induced ovulation and strip-spawning was performed following a routine protocol for hormonal induction using two doses of carp pituitary extract (0.6 and  $5.4 \text{ mg kg}^{-1}$ , 12-hour interval between doses). The total number of oocytes released from each female (absolute fecundity) was estimated. To analyse this data, after ovulation and just before fertilisation, the total mass of eggs released by each female was recorded. Sub-samples ( $\sim 1 \text{ g}$ ) of the egg mass were used to extrapolate the total egg numbers. The relative fecundity (number of eggs released per gram of fish) was also determined. After weighing, the eggs were fertilised using a pool of semen from 8 males. To avoid the effects of factors other than the influence of the females during the artificial breeding process, the males used for both experimental groups all came from EPs. Approximately 0.5 ml of semen was used to fertilise 50 g of oocytes. Soon after fertilisation, a pool of fertilised eggs from each treatment was prepared and distributed into 8 incubators (2 treatments x 4 replicates). Then, 10 g of hydrated eggs were placed in each



incubator (3 L). Incubators were maintained at a constant water flow between 8-12 L min<sup>-1</sup>, dissolved oxygen (6.2 ± 0.5 mg L<sup>-1</sup>).

To determine the fertilisation success of the eggs, 8-12 hours post-fertilisation (hpf) (after the blastopore closure stage), 100 eggs were randomly sampled and counted, and those that were dividing normally were scored. Four counts were performed to determine the mean fertilisation success. After 17 hpf, overall hatching success were determined by counting the number of hatched eggs/number of fertilized eggs \* 100. Four counts were performed to determine the mean hatching success.

Two days after hatching, just after the formation of the functional swim-bladder and mouth opening, 2,000 larvae randomly selected from each treatment were equally distributed in four tanks with a 30 L capacity (2 treatments x 4 replicates) with constant water renewal, and the larvae were monitored daily for 30 days. The larvae were fed four times a day (0800; 1100; 1400 and 1700 hrs), as follows: 1-4 days of life (d): 50 *Artemia* nauplii/larvae/day (BioArtemia. Ltda., RN, Brazil); 5-8 d: 100 nauplii/larvae/day; 9-10 d: 150 nauplii/larvae/day; 11-12 d: 200 nauplii/larvae/day; 13-18 d: 300 nauplii/larvae/day; and 19-30 d: 400 nauplii/larvae/day + commercial diet powder (40.0% CP) *ad libitum*. Dead larvae and food debris were removed daily, and 30 days after hatching, the remaining larvae were counted to determine survival rates.

### Statistical analysis

Statistical analysis was performed using the computer program SAS 9.1 (Statistical Analysis Software, 2004). The results are presented as mean ± standard error (SE). Data were analysed for normality and homoscedasticity of variance. Student's t-test and one-way analysis of variance (ANOVA) were applied to check for significant differences between treatments. Additionally, Tukey's multiple comparison test was applied for each treatment during the experimental period.

## Results

Water parameters obtained for EPs and Cs are shown in Fig. 1.

During the experimental period the survival rate of fish kept in EPs and Cs were 100% and 95% respectively. At the end of the experimental period the average weight and total length were 532.0 ± 20.9 g and 339.0 ± 59.8 g, and 34.9 ± 15.6 cm, and 30.9 ± 13.8 cm for EPFs and CFs, respectively (Fig.2).

### Ovarian development: GSI values

The GSI values of the earthen pond females increased progressively from the first (Jul/09) to the fourth sampling (Feb/10), when they reached a peak (representing more than 20% of body weight) (Fig. 3). In the fifth sampling (Apr/10), the values decreased sharply to approximately 5% (P<0.01), showing that between the fourth and fifth sampling (late summer/ early autumn), the reproductive season ended for EPFs, which has started the process of ovarian regression (Figs. 3, 7j). In contrast, the GSI values of CFs only increased

during the three first samplings (from Jul/09 to Dec/09) (P<0.01), stabilising at ~10% in Feb/10 and decreasing to approximately 2% in the last sampling (Jun/10).

### Percentage distribution of the different oocyte types

Analysis of the frequency of the different oocyte types showed that during the first sampling (Jul/09), 100% of the analysed oocytes were at the previtellogenic stage (Fig. 4) for both groups. Two months later (with the onset of the vitellogenic phase), the mean percentages of previtellogenic oocytes (PVOs) in the ovaries of EPFs and CFs decreased to ~40 and ~50%, respectively (P<0.01) (Fig. 4). The mean percentage frequency of PVO in both treatments reached the lowest values during the third sampling, then progressively increased until Apr/10 (end of the spawning season). However, the mean frequency of PVO for CFs was always higher than that of EPFs (P<0.05) (Fig. 4). During the last sampling, nearly 100% of the analysed oocytes were at the previtellogenic stage again, thus beginning the subsequent breeding season.

In both treatments, cortical alveoli (CAO) and vitellogenic oocytes (VO) were first detected during the second sampling (Oct/09) (Fig. 4), and CAO were only detected through the third sampling (early spawning season) (Fig. 4). During vitellogenesis (Oct/09) and the spawning season (Dec/09), the frequency of VO was higher for EPFs (P<0.01) than for CFs. The highest VO values for EPFs and CFs were ~50 and ~45% (P<0.05), respectively, both occurring during the third sampling (early spawning season). The mean frequencies of VO decreased at the fourth sampling in both treatments, though more intensely for CFs (P<0.01) (Fig. 4).

Regarding atretic oocytes (CAO), the mean frequency in the ovaries of EPFs and CFs gradually increased throughout the annual reproductive cycle, reaching a peak at the fourth sampling (Feb/10) for both treatments (Fig. 4). The mean CAO frequencies were higher (P<0.05) in the ovaries of EPFs than CFs during the fourth and fifth samplings (end of the breeding season) (Fig. 4). In both treatments, the vast majority of CAO were fully vitellogenic oocytes that were not released during the spawning season (data not shown).

### Diameter of vitellogenic oocytes

During the entire experimental period, the mean VO diameters of EPFs were statistically higher than those of CFs (Fig. 5). During vitellogenesis (Oct/09), the mean VO diameters were 420.38 ± 26.14 µm and 221.34 ± 56.12 µm for EPFs and CFs, respectively (P<0.01) (Fig. 5). The mean VO diameter of EPFs increased gradually from the first sampling to the breeding season (Feb/10), whereas that of CFs increased only between the second and third samplings and decreased during the spawning season (Dec/09- Feb/10) (Fig. 5).

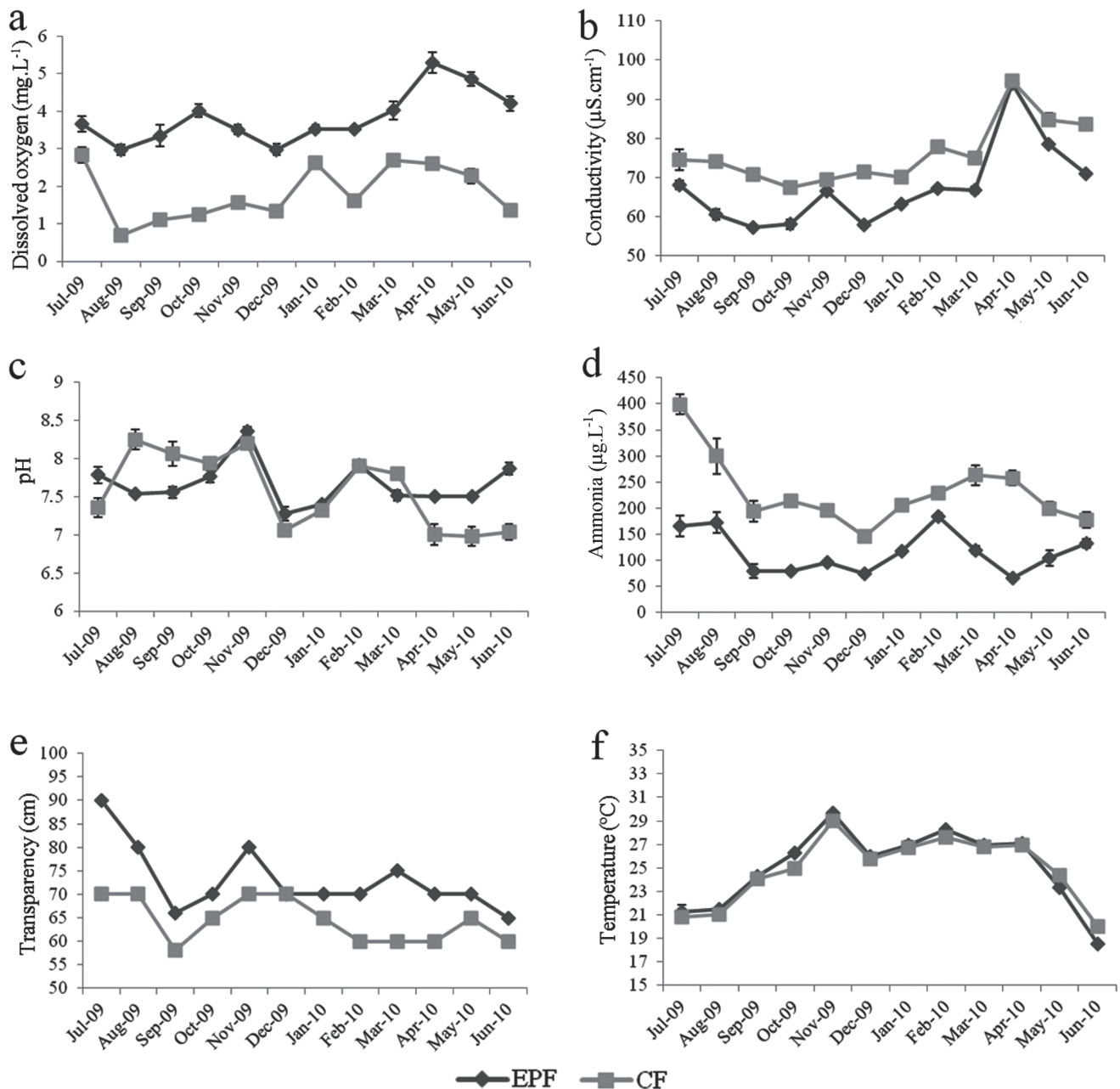
### Diameter of previtellogenic oocytes

In the first reproductive cycle, the maximum diameter achieved by PVO was observed during winter (Jul/09) (Fig. 6) in the previtellogenic phase (Fig. 4) for both treatments. At the onset of vitellogenesis (Oct/09), a reduction of the mean

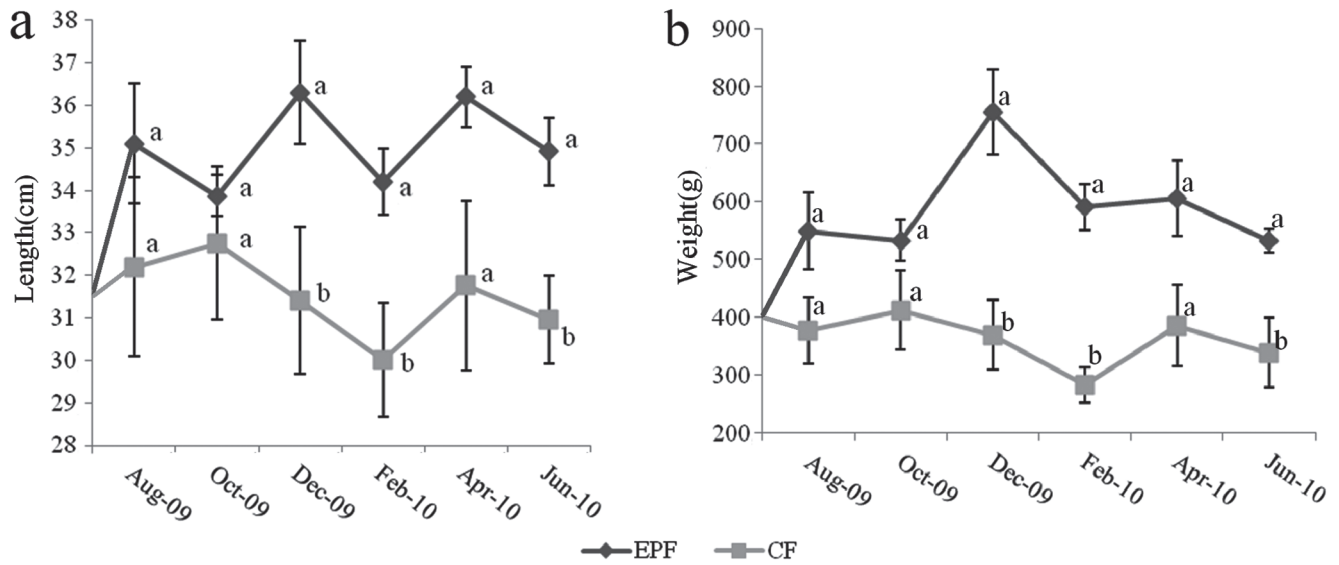
diameter of PVO was detected in both treatments; however, it was more pronounced in EPFs (Fig. 6). One bimester later (Dec/09, breeding season), the mean PVO diameters of EPFs and CFs had decreased and increased, respectively, in comparison to their previous mean values, resulting in higher values ( $P < 0.01$ ) of the former group in comparison with the latter (Fig. 6). At the end of the breeding season of the first reproductive cycle (Feb/10), the PVO diameter increased progressively for three consecutive bimesters through Jun/10, however, the PVO diameters of CFs were higher than those of EPFs ( $P < 0.05$ ) (Fig. 6).

### Ovarian morphology

As previously mentioned, PVOs were the only type of oocyte present in the ovaries of EPFs and CFs during the first sampling (Jul/09) (Figs. 7a- b). At the next sampling (during spring), while there was a predominance of VO in the ovaries of EPFs (Fig. 7d), most of the oocytes in the ovaries of CFs were PVO (Fig. 7c). The CF ovaries only presented a predominance of VOs at the beginning of the summer (Dec/09), (Fig. 7e). From the middle to the end of the summer (Feb/10), most VOs began the atretic process in both treatments (Figs. 7g- h), which extended for more than



**Fig. 1.** Monthly average values and standard errors of dissolved oxygen (mg.L<sup>-1</sup>) (a), conductivity (µS.cm<sup>-1</sup>) (b), pH (c), ammonia (µg.L<sup>-1</sup>) (d), transparency (cm) (e) and temperature (°C) (f).



**Fig. 2.** Bimonthly averages values of total length (a) and weight (b) of EPFs and CFs. Different letters indicate differences ( $P < 0.01$ ) in each treatment during the experimental period. Error bars are SE. EPF: earthen pond females; CF: cage females.

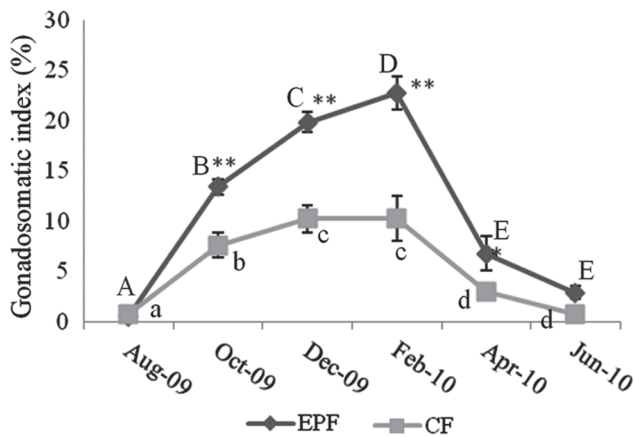
one (Apr/10) or two (Jun/10) bimesters for CFs and EPFs, respectively (Figs. 7k-l).

**Gonadal steroids**

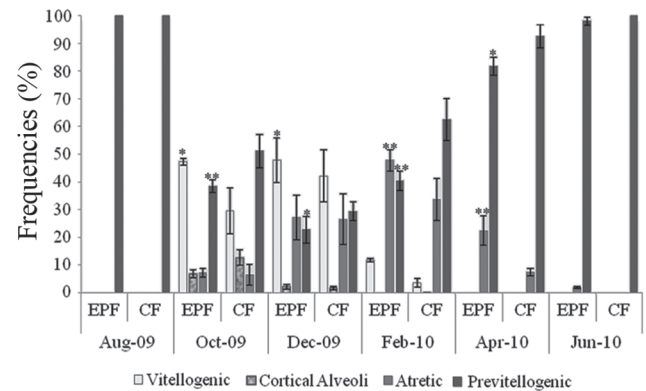
The intra- and inter-assay % CVs, as well as the recovery index were within the acceptable limits initially required (Table 1). The plasma steroid hormone profiles associated with the two treatments showed marked differences regarding the levels during the vitellogenic period and just prior to the spawning period (Figs. 8a- b).  $E_2$  levels increased in both

treatments from the first to the second sampling (Oct/09), but the levels of EPFs at this time were approximately three times higher ( $P < 0.01$ ) (Fig. 8a) than those of CFs. From the second to the third sampling, the plasma levels of  $E_2$  presented a peak (Dec/09) for both groups. After the spawning period, plasma  $E_2$  levels decreased in both treatments, and their profiles remained similar through Jun/10 (Fig. 8a).

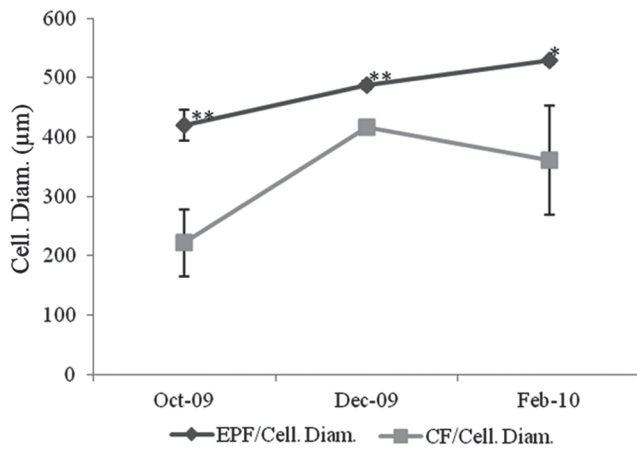
The plasma levels of  $17\alpha$ -OHP decreased slightly in both treatments between the first and second samplings (during the onset of vitellogenesis) (Fig. 8b). During the early breeding season



**Fig. 3.** Mean values of the gonadosomatic index of *P. lineatus* during the experimental period. \* indicates significant differences ( $P < 0.05$ ), or \*\* ( $P < 0.01$ ), between treatments during the same sampling ( $n = 5/\text{sampling}$ ). Different letters indicate differences ( $P < 0.01$ ) in each treatment during the experimental period; capital letters for EPF, lowercase letters for CF. Error bars are SE. EPF: earthen pond females; CF: cage females.



**Fig. 4.** Mean frequencies of different types of oocytes of *P. lineatus* maintained in earthen ponds and cages ( $n = 5/\text{sampling}$ ; 60 fields/ovary). \* indicates significant differences ( $P < 0.05$ ), or \*\* ( $P < 0.01$ ), between the frequencies of each oocyte type between treatments during the same sampling. EPF: earthen pond females; CF: cage females.



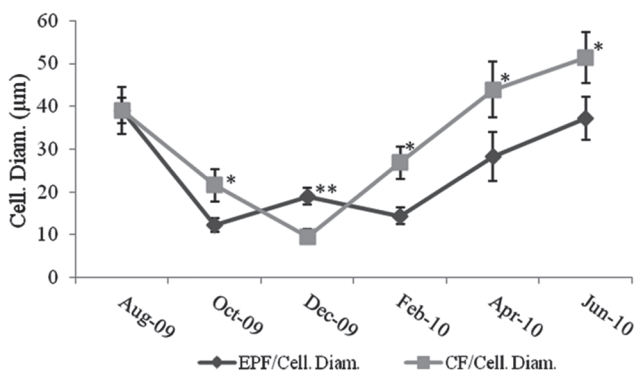
**Fig. 5.** Mean cellular diameters of vitellogenic oocytes of *P. lineatus* during the reproductive cycle. \* indicates significant differences ( $P < 0.05$ ), or \*\* ( $P < 0.01$ ), between treatments during the same sampling ( $n = 5$ /sampling; 120 oocytes/ovary). Error bars are SE. EPF: earthen pond females; CF: cage females.

(Dec/09), the mean values of EPFs peaked at  $\sim 3$  times higher than those of CFs (Fig. 8b), and at the end of the spawning period (Feb/10), this hormone was reduced to similar levels ( $\sim 0.5$  ng ml<sup>-1</sup>) in both treatments, remaining stable through Jun/10 (Fig. 8b).

Therefore, when analysed together, the  $E_2$  and  $17\alpha$ -OHP data showed that there was a higher  $E_2$  concentration in EPFs at the onset of vitellogenesis, as well as higher  $17\alpha$ -OHP levels in EPFs two months before (Dec/09) the peak of the spawning season (Fig. 8b).

### Reproductive performance

All injected females ovulated  $\sim 6$  hours after the second dose was administered. The mean values for the absolute



**Fig. 6.** Cellular mean diameters of previtellogenic oocytes of *P. lineatus* during the reproductive cycle. \* indicates significant differences ( $P < 0.05$ ), or \*\* ( $P < 0.01$ ), between treatments during the same sampling ( $n = 5$ /sampling; 120 oocytes/ovary). Error bars are SE. EPF: earthen pond females; CF: cage females.

fecundity of EPFs were approximately three times higher than those of CFs ( $P < 0.01$ ) (Table 2). Regarding the relative fecundity (the number of oocytes released per gram of fish), the mean values presented by EPFs were approximately 1.7 times higher ( $P < 0.01$ ) than those of CFs. The fertilisation success (5 hpf) were approximately 2 times higher for EPFs compared to those of CFs. The hatching and larval survival success were also higher for EPFs ( $P < 0.01$ ) in relation to those obtained for CFs (Table 2).

### Discussion

This study produced new information about the mechanisms related to the formation of low quality eggs in tropical rheophilic fish. Specifically, the study demonstrated that decreased  $E_2$  levels, less intense vitellogenesis, and reduced amounts of yolk - as indicated by the smaller diameter of the CFs vitellogenic oocytes - are dysfunctions related to the production of low quality eggs.

### Weight and length

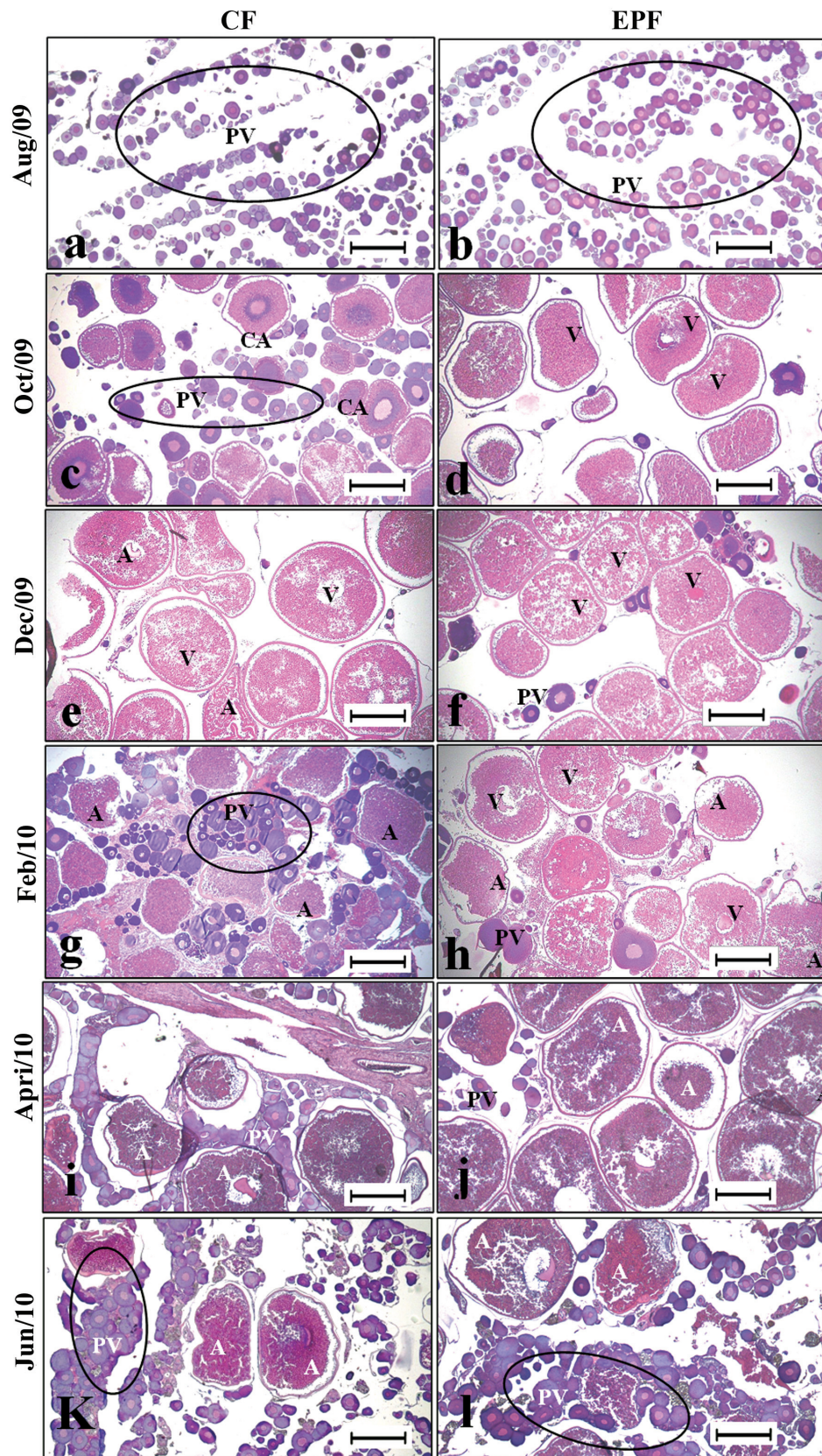
The average length and weight were higher for EPFs than Cs during the whole experimental period. Thus, it was assumed here that the differences observed between groups concerning ovaries characteristics could have been (at least in part) due to nutritional status and reduced growth of CFs. Probably the high / inappropriate stocking density or general rearing conditions provided by the cages could also have affected (reduced) the steroidogenic capacity of the fish, leading to lower plasma  $E_2$  levels (Wu, 2009; Mylonas *et al.*, 2010).

### GSI and spawning season

In this study, the maximum mean GSI value found for CFs ( $\sim 10\%$ ) was only half that found for EPFs ( $\sim 25\%$ ). Moreover, CFs did not display a GSI increase during the spawning season (Nov/09-Feb/10). Thus, considering only the GSI values, we would not assign a spawning season for CFs, and this could only be established with the aid of histological analysis. The majority of studies conducted in this field report reductions in the GSI values of breeders exposed to short or prolonged periods of unfavourable conditions, such as an insufficient tank volume (Buchet *et al.*, 2008), inappropriate photoperiod (Fontaine *et al.*, 2006), poor feed quality (Reidel *et al.*, 2010), fertilisers (Ram & Sathyanesan, 1986) and particularly hypoxia (Wu *et al.*, 2003; Shang *et al.*, 2006; Landry *et al.*, 2007; Thomas *et al.*, 2007; Wu, 2009). However, the effects vary according to the species, the environmental and captivity conditions and the duration of the experimental period (reviewed by Bobe & Labbé, 2010).

A gradual increase in GSI values following the ovarian maturation process has been widely reported for a number of rheophilic species, including *Piaractus mesopotamicus* (Gazola & Borella, 1997), *Pseudoplatystoma fasciatum* (Batlouni *et al.*, 2006; Leonardo *et al.*, 2006; Romagosa, 2010), *Pseudoplatystoma* sp. (Dabrowsky *et al.*, 2008), *Salminus*





**Fig. 7.** Ovary sections illustrating the general appearance during the year showing oocyte types: previtellogenic (PVO); cortical alveoli (CAO); vitellogenic (VO); atretic (A). EPF: earthen pond females; CF: cage females. Hematoxylin and eosin. Scale bars correspond to 500 μm.



**Table 1.** Enzyme-linked immunosorbent assay (ELISA) validation values (%) for *Prochilodus lineatus*.

Hormone	Spike Recovery	CV Intra-assay	CV Inter-assay
Estradiol	103.3 ± 22.97	13.5 ± 3.26	19.1 ± 3.19
17- $\alpha$ -hydroxyprogesterone	104.2 ± 9.7	6.3 ± 0.78	18.7 ± 4.26

*hilarii* (Honji *et al.*, 2009) and *Prochilodus argenteus* (Arantes *et al.*, 2010). Thus, the absence of a GSI peak for *P. lineatus* CFs during the spawning season reflected impairment of the vitellogenic process, such as delayed oogenesis, consequently resulting in vitellogenic oocytes of reduced size. Interestingly, these data may explain or be related to frequent reports from fish farmers that some broodstocks do not “prepare” for reproduction; *i.e.*, they do not show any signs of maturity (such as a swollen abdomen), even during the reproductive season.

#### Percentage distribution of different types of oocytes

In this study, no differences regarding the proportion of atretic oocytes during the reproductive cycle were found between treatments (data not shown), except for related to VOs after the breeding season. We first suspected that the low GSI values of CFs were related to an intense atretic process occurring in their ovaries during vitellogenesis, consequently reducing the number of VOs. In fact, our analysis of the percentage frequencies of oocytes revealed a delay and impairment of oocyte development in CFs, especially during the vitellogenic process. We found that the early spring (September and October) was a key period in determining differences between treatments. At this time, the onset of vitellogenesis was delayed in the ovaries of CFs, as shown

by a greater proportion of remaining previtellogenic oocytes (~50%) compared to EPFs (~40%) ( $P < 0.01$ ).

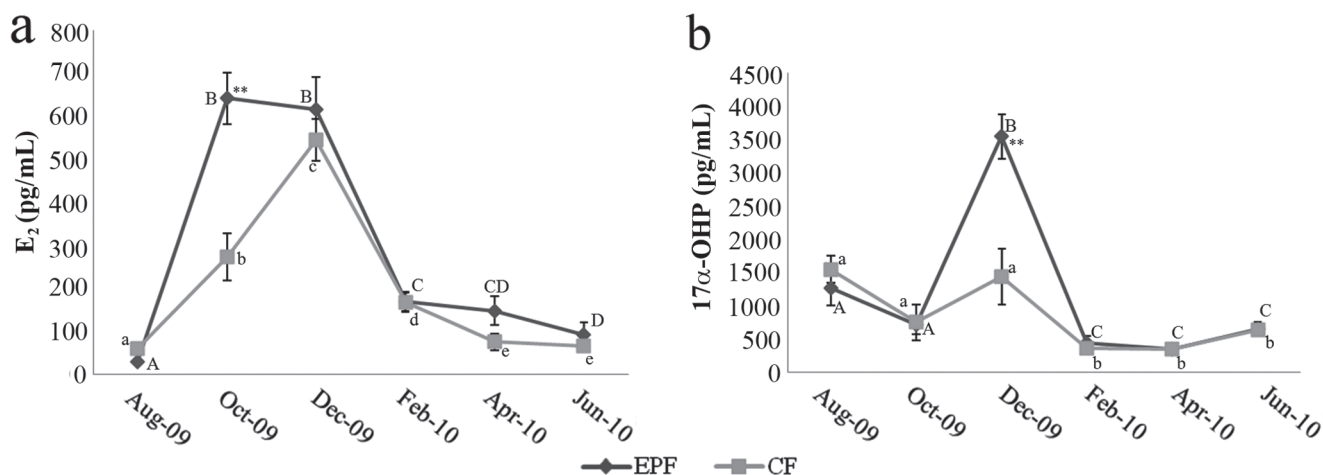
As a consequence of the delayed vitellogenic process in the ovaries of CFs, in early spring (Oct/09), the proportion of vitellogenic oocytes was significantly higher in the ovaries of EPFs (~50%) than in those of CFs (~30%), clearly indicating that vitellogenesis was impaired in ovaries from the latter group. Another striking difference was found in the dynamics of oogenesis between the two treatments; *i.e.*, the proportion of VOs in CFs varied irregularly throughout the year, revealing irregularities in the process of oocyte growth and vitellogenesis in these fish.

#### Oocyte diameter / vitellogenic oocytes

Although CFs were able to produce VOs later than EPFs, these oocytes presented significantly a smaller cytoplasm diameter and, consequently, reduced amounts of yolk. Thus, the reduced mean diameter of VOs in the ovaries of CFs contributed to their lower GSI values and to the lack of a GSI peak during the breeding season.

Simultaneous analysis of the GSI values and mean VO diameters confirmed these findings. Thus, it is clear that the differential profile of the GSI values during the year was not only related to the time of the onset of vitellogenesis, but rather, it was mainly associated with the extension length of the period during which VOs were able to accumulate yolk in each treatment.

Many lines of evidence confirm that adverse conditions may decrease egg diameter and size. The influence of tank volume on egg diameter and spawning performance has also been reported in the sea bass *Dicentrarchus labrax* (Buchet *et al.*, 2008). Similarly, groups of mature male and female rainbow trout subjected to repeated acute stress exhibited a significant delay in ovulation and reduced egg size (Campbell



**Fig. 8.** Mean values of plasma concentrations of E<sub>2</sub> (a) and 17 $\alpha$ -OHP (b) levels during the experimental period in both treatments. \* indicates significant differences ( $P < 0.05$ ), or \*\* ( $P < 0.01$ ), between treatments during the same sampling ( $n = 5$ /sampling). Different letters indicate differences ( $P < 0.01$ ) in each treatment during the experimental period; capital letters for EPF, lowercase letters for CF. Error bars are SE. EPF: earthen pond females; CF: cage females.

**Table 2.** Reproductive parameters of EPF and CF. Mean values and their standard errors; \*\* indicates significant differences ( $P < 0.01$ ), between treatments. Error bars are SE. EPF: earthen pond females; CF: cage females.

	Absolute Fecundity (oocyte/ fish)	Relative Fecundity (oocyte/g of fish)	Fertilization	Hatching	Larvae Survival
(CF)	48.309±24.970**	140±16**	43±2.8%**	63±8.68%**	77±3.6%**
(EPF)	158.374±11.486	230±12	80±1.4%	90±5.04%	97±0.6%

*et al.*, 1994). Atlantic croakers chronically exposed to hypoxia presented a marked decrease in their GSI values compared to fish from normoxic areas (Thomas *et al.*, 2007).

### Oocyte diameter / previtellogenic oocytes

Comparative evaluation of the mean diameter of PVOs corroborated the delayed vitellogenesis onset observed in the ovaries of CFs. In EPFs, the marked reduction in the mean diameter of PVOs during early spring indicated that the larger PVOs were transforming into VOs faster than in the ovaries of CFs. Unfortunately, very little is known about the kinetics of oogonia and the early development of oocytes in fish (Lubzens *et al.*, 2010). Regarding rheophilic fish, nothing is known about this process. Nevertheless, this study revealed, in an unprecedented way, that the dynamics of PVO development were indirectly affected by delayed vitellogenesis, which was caused by decreased levels of  $E_2$ , as will be discussed in the following section.

### Gonadal steroids

As expected, an increase in  $E_2$  levels during the vitellogenic period was observed in curimbata, similar to the  $E_2$  profiles described in two other species, such as the *Piaractus mesopotamicus* (Gazola & Borella, 1997) and *Prochilodus argenteus* (Arantes *et al.*, 2010). The three times lower plasma  $E_2$  levels of CFs compared to those of EPFs found in the present study during early spring were probably associated with the delayed and less intense vitellogenesis in this group. Moreover, the low  $E_2$  levels of CFs in early spring were probably the main cause of the significant reduction in the proportion and the mean diameter of VOs in this group. In the present study, it took CFs two months longer to reach their maximum  $E_2$  levels (Dec/09), and similar to EPFs, the greatest diameter and proportion of vitellogenic oocytes occurred concomitantly with the highest mean  $E_2$  levels.

In this regard, our data are in accordance with previous reports showing an association between inappropriate management and reduced  $E_2$  levels (Wu *et al.*, 2003; Fontaine *et al.*, 2006; Wu, 2009). In this context, it would be of interest to develop further studies in south American rheophilic fish to assess the relationships among management,  $E_2$  levels and the quality of the oogenesis process considering isolated variables (*e.g.*, stocking density, rearing system, hypoxia).

The steroid  $17\alpha$ -OHP is the principal precursor of  $17\alpha$ , 20  $\beta$ -dihydroxy-4-pregnen-3-one (DHP), which is in turn the most potent hormone known regarding inducing oocyte maturation and ovulation in fish (Nagahama & Yamashita, 2008). Evaluation

of the levels of DHP and its precursors in plasma breeders has shown that they are related to the success of ovulation and the quality of oocytes and larvae in fish (Dabrowski *et al.*, 2003; Lister & Van Der Kraak, 2008, 2009). In this study, the peak level of  $17\alpha$ -OHP was three times higher in EPFs in comparison to CFs. Studies concerning  $17\alpha$ -OHP or DHP levels in rheophilic species are very scarce, but a few of them indicate that low levels of these hormones may be caused by external stress agents, such as reduced oxygen levels (Lister & Van Der Kraak, 2009) and lower water temperature and oxygen concentration (Arantes *et al.*, 2010). Thus, it is possible that the  $17\alpha$ -OHP levels of *P. lineatus* CFs were related to the inappropriate management conditions provided inside cages. Moreover,  $17\alpha$ -OHP has been measured as a precursor of DHP, as it is difficult to detect DHP levels because no commercial kits are available for this purpose, and no studies have yet been performed to investigate this hormone in *P. lineatus*. However, similarly to the results obtained for *P. brachypomus* (Dabrowski *et al.*, 2003), in this study, inferior reproductive performance was associated with low levels of  $17\alpha$ -OHP in CFs, which will be discussed in the following section.

### Reproductive performance

Data on the effects of broodstock stress on resulting gamete quality are scarce, and different conclusions have been reached depending on the type and intensity of the stressor, the species and the period when the stressor was applied (review in Bobe & Labbé, 2010). However, in this study, for all parameters of reproductive performance considered (absolute and relative fecundity, fertility, hatching and larval survival success), CFs presented lower results than EPFs. In this regard, the inferior values obtained for fecundity of CFs in the present study were expected, due to the lower mean GSI values (~2 times) of this group in comparison to those of EPFs during the reproductive season. Similarly, *Dicentrarchus labrax* females maintained in small tanks (1 m<sup>3</sup>) before and during vitellogenesis presented lower fecundity per spawn compared to fish maintained in larger ponds (8-32 m<sup>3</sup>). (Buchet *et al.*, 2008). Thus, it seems that adverse conditions applied to fish in periods prior to spawning may impair gonad development (changes in the spawning season and size of eggs) and gamete quality in different ways, while adverse conditions applied during hormonal induction (Dabrowski *et al.*, 2003) may be more closely related to the release of lower quality gametes (low fertilisation, hatching and larval survival).

Fertilisation success is probably one of the earliest estimators that can be recorded to accurately estimate egg quality, and it is the most integrative estimator of sperm quality.

Indeed, the ability to fertilise or be fertilised is one of the key components of gamete quality (Bobe & Labbé, 2010). In this context, the fertilisation success observed in this study were almost twice as high for EPFs compared with CFs, and our results are in accordance with previous reports on fish breeders subjected to adverse conditions (Campbell *et al.*, 1992, 1994). However, reports in the literature are very scarce regarding the effects of adverse conditions or inappropriate management in tropical rheophilic fish. The only existing information on this topic is for *Piaractus brachypomus* subjected to hypoxic conditions during hormonal induction experiments (Dabrowski *et al.*, 2003). In our study, the development of larvae until 30 days post fertilisation was also different between treatments (data not shown). Here, we only evaluated the survival rate 30 dpf, when it was statistically higher for EPFs (96.5%) compared to CFs (72.3%). However, we observed a high frequency (though it was not quantified) of larvae with deformities and larvae that were susceptible to diseases only in the CF group.

Concluding, the rearing of curimatá breeders for 440 days in Cs (at the stocking density applied) reduces growth, plasma  $E_2$  levels and vitellogenesis. Moreover, the onset of vitellogenesis early spring (September and October) was a key period in producing eggs of good quality in this species. The reduced  $E_2$  levels, the impaired transition from previtelogenic to vitellogenic oocytes and the reduced amounts of yolk in vitellogenic oocytes were reproductive dysfunctions associated with the formation of low quality oocytes and abnormal breeding season in this species. Our findings indicates that further studies concerning the reproductive management of rheophilic species must give special attention to the beginning of the vitellogenesis period, six months before the spawning season. In this regard, the data obtained here suggest strongly that care must be taken when empirical measures are taken by some fish farmers to provide adequate conditions for rheophilic breeders (such as low stocking densities, good quality water and even better quality diets) only during the months before the spawning season (November and December).

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