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Inappropriate management conditions, especially for the regressed class, are related to sperm quality in *Prochilodus lineatus*

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ABSTRACT

The aims of this study were to evaluate the characteristics of the reproductive classes and semen quality in curimbatá (*Prochilodus lineatus*) breeders maintained in two different rearing systems. To achieve this goal, cages (Cs) and earthen ponds (EPs) were used as experimental systems to provide unsuitable and suitable conditions, respectively. The fish were maintained under the experimental conditions for 18 months. During this period, males were randomly sampled every 2 months for biometric analysis (n = 30 per sample) and for an evaluation of selected characteristics of the testes (n = 5 per sample). After this period, males maintained in EPs and males maintained in Cs (CMs) were evaluated in induced breeding experiments. We observed that rearing *P. lineatus* in a C at a high stocking density for the long 18-month period of study produced reductions in growth, testis development, gonadosomatic index values, and sperm quality in the fish. We found differences between the groups in all the reproductive classes examined, especially in the regression class, which showed a pronounced accumulation of immature germ cells in the CMs. In this group, we also noted a less intense transition from a continuous to discontinuous germinal epithelium, with an extended and abnormal but less intense spermatogenic period resulting in decreases in semen volume and sperm concentration in the breeding season. Together, such dysfunctions resulted in the production of low-quality sperm in the CMs, as demonstrated by lower-quality DNA (as evaluated by the comet assay), low fertilization success, and low hatching success. In conclusion, to ensure high-quality semen in *P. lineatus*, appropriate management conditions must be provided throughout the reproductive cycle, especially for the regressed class, even in winter, two seasons before the breeding season.

1. Introduction

In continental aquaculture, fish are commonly maintained under artificial conditions, exposed to various adverse circumstances, such as high stocking densities, and fed an artificial diet [1]. In this context, the influence of these characteristics on the quality of the gametes is poorly understood, and there is a need for a better understanding of the relationship [2]. Concerning this issue, although it is known that the quality of both sperm and oocytes can affect the success of fertilization and larval survival, the fish industry has focused much more on the quality of oocytes [1]. Under such conditions, the milt may be inadequate in both quantity and quality because of physiological and environmental factors, and

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successful fertilization is, therefore, not always achieved with the artificial insemination procedures commonly used for aquaculture species [1].

The stresses caused by inappropriate farming conditions, overcrowding, or transport between farms are poorly understood. Often, little semen is obtained, and fertilization success rates and embryo viability are low. These problems have, in certain cases, been attributed to reproductive problems in females (review in [1]). Recent investigations using the comet assay have shown that sperm DNA undergoes depletion and decreases in quality. However, most of these studies have involved experiments to assess cryopreservation procedures (review in [3]), and virtually nothing is known about the relationship between sperm DNA quality and the characteristics of the breeding ponds in which the fish are kept [1,2]. Moreover, it is known that males of most commercially produced species regularly undergo testicular maturation in captivity [4], and successive classes defined as maturation, regression, regressed, and recrudescence are regularly described during the reproductive cycle [5]. However, virtually nothing is known about the effects of husbandry conditions on the development of reproductive classes [1,2].

Rheophilic fish are among the freshwater species of essential economic importance in tropical regions [6–10]. Among these species, the curimbatá (*Prochilodus lineatus*), a total spawner, is an iliophagic fish that feeds mainly at the bottom of ponds and rivers [11]. The average age of sexual maturity in *P. lineatus* males is 11 months (26 cm total length and 240 g body weight) [12]. Despite the induced breeding techniques that have been applied for decades with relative success in this species [6,7,9,10], the results of spawning in rheophilic fishes are still highly variable and unpredictable [13,14]. In this context, it has been reported that husbandry conditions [13] and hormonal induction protocols [7,14] may negatively interfere with the ovulation process, but the effects of husbandry conditions on the male reproductive classes and semen quality are unknown.

We have reported that rearing *P. lineatus* breeders at relatively low densities in earthen ponds (EPs) provide ideal conditions for suitable female reproductive performance, whereas cages (Cs) are not recommended, particularly if high stocking densities are used [13]. In that study [13], we observed adequate values for *P. lineatus* females maintained in EPs (EPFs) but not for females maintained in Cs. All observed parameters were significantly higher for EPFs. Specifically, the relative fecundity (oocytes per gram of fish), fertilization success, hatching success, and larval survival were 140 ± 16 and 230 ± 12, 43 ± 2.8% and 80 ± 1.4%, 63 ± 8.7% and 90 ± 5.0%, and 77 ± 3.6% and 97 ± 0.6%, respectively. Thus, in the present study, Cs were used as an experimental system to simulate the effect on the reproductive classes and quality of curimbatá spermatozoa of the unsuitable conditions used on many farms for rheophilic fish.

2. Materials and methods

2.1. Animals

In March 2009, 600 curimbatá specimens (males and females at a sex ratio of 1:1; 12-month old; total length [mean ± standard error], 31.48 ± 1.03 cm; and weight, 394.85 ± 29.85 g) were maintained at the Centro de Aquicultura da Universidade Estadual Paulista, Jaboticabal, São Paulo, Brazil (21° 15′ 17″ S 48° 19′ 20″ W). For this study, fish were raised in four EPs of 50 m^3 and four Cs of 6 m^3. The fish were obtained from fish farms and had been produced by induced breeding. Previous evidence obtained in our laboratory has shown that *P. lineatus* breeders maintained at high stocking densities in Cs have poor reproductive performance [13]. In contrast, in EPs, a stocking density of 1.5 fish/m^3 has been shown to be adequate for both males and females of this species because they undergo oocyte maturation and spermatogenesis regularly [12]. In the present study, an initial stocking density of 1 fish/m^3 was applied in the EPs (50 specimens per pond). Because there was no available information about stocking densities for this species in Cs, and our objective was to use conditions that would reflect real aquaculture conditions, 100 specimens were placed in each C, resulting in a stocking density of 17 fish/m^3.

2.2. Culture conditions

The fish were manually fed twice a day with extruded balanced commercial diet (moisture content [maximum], 10.0%; crude protein [minimum], 28.0%; ether extract [minimum], 5.0%; fibrous matter [maximum], 7.0%; ash [maximum], 10.0%; calcium [maximum], 1.2%; phosphorus [minimum], 0.6%) corresponding to 3.0% of their total body weight (the total body weight value was readjusted following each set of biometric measurements). Water parameters were measured weekly at 9 AM using a YSI model 55 oximeter and a YSI model 63 multiparameter sounder (Yellow Springs Instruments, Yellow Springs, Ohio, USA) to determine the dissolved oxygen, pH, conductivity levels, and temperature. Transparency was measured at 9 AM using a Secchi disk. The N-ammonia concentration was determined according to the method of Solorzano [15]; additionally, colorimetric methods were used, and absorbances were measured using a Hach DR2000 spectrophotometer (Hach, Loveland, Colorado, USA).

2.3. Sampling

The fish were maintained in the previously mentioned conditions for a period of 440 days, representing 80 days of acclimation and a subsequent experimental period of 360 days. During the experimental period, samples were collected every 60 days (bimonthly). Thirty specimens (males and females) were randomly selected from each EP and C and were then transported to the laboratory (following a methodology similar to that used in [16]). The total length (in centimeter) and body weight (in gram) were recorded for each animal. Then, from these 30 animals, five males were randomly selected from each rearing system, anesthetized with a benzocaine solution (ratio of 2 g ethylaminobenzoate to 150 mL ethanol to 20 L water) and killed by severing the section of the spinal cord next to the operculum. The testes were collected for determination of the gonadosomatic index (GSI) and histologic evaluation. All procedures followed the approved guidelines for the

ethical treatment of animals and national laws. The experimental protocols were submitted to and approved by the Animal Ethics and Welfare Committee (Comissão de Ética e Bem-Estar Animal) of the Faculdade de Ciências Agrárias e Veterinárias, UNESP, Jaboticabal, SP, Brazil.

2.4. Histologic evaluation of the testes

Samples of the testes (cranial, middle, and caudal regions) were collected and fixed in Bouin's solution for 24 hours. After fixation, the material was embedded in paraplast, cut into 5.0-μm-thick sections, and subjected to hematoxylin and eosin staining. Histologic sections from males maintained in EPs (EPMs) (n = 5 specimens per sampling) and males maintained in Cs (CMs) (n = 5 specimens per sampling) were used to determine the mean diameter of the seminiferous tubules (n = 20 tubules per region—cranial, middle, and caudal—totaling n = 60 tubules per fish, ×40 magnification). The seminiferous tubules were characterized according to the presence of different types of germ cells within the tubules. First, it was determined whether the tubules contained only isolated germ cells. In the other tubules, we evaluated the presence of different types of germ cells, including spermatogonia, spermatocytes, spermatid cysts, and spermatozoa (n = 20 tubules per region—cranial, middle, and caudal—totaling n = 60 tubules per fish, ×40 magnification). We also determined the percentage of seminiferous tubule cross-sections showing a discontinuous germinal epithelium (DGE) (n = 20 per region—cranial, middle, and caudal—totaling n = 60 per fish, ×40 magnification). Both evaluations were performed using an Olympus BX-41 (Olympus, Tokyo, Japan) microscope system with Olympus DP-11 (Olympus, Tokyo, Japan) capture (with measurements performed using Image Pro Plus version 4.1 software, Media Cybernetics, Rockville, USA).

2.5. Induced breeding

In the third week of December 2010, EPMs and CMs (n = 4 randomly chosen per treatment) were hormonally induced with a single dose of carp pituitary extract at a dose of 1.5 mg/kg body weight. Seven hours later, the males were hand stripped to collect semen. The volume of semen was recorded for each individual, and aliquots were separated for seminal characterization. Subsequently, two pools were prepared, one from the semen of the EPMs and one from the CMs, using equal amounts of semen from all individuals that spermiated. These pools were used to fertilize an oocyte pool obtained from EPFs (n = 8). To avoid the effects of factors other than the influence of the males during the artificial breeding process, the females used for both experimental groups all came from EPs. The EPFs were hormonally induced as previously described by Hainfellner et al. [13]. The insemination dose used was approximately 4.1×10^5 sperm per oocyte. Soon after fertilization, a pool of fertilized eggs from each treatment was prepared and distributed into eight incubators (four incubators per treatment), and 10 g of hydrated eggs was then placed in each incubator (3 L). The incubators were maintained at a constant water flow of between 8 and 12 L/min, with a dissolved oxygen content of 6.2 ± 0.5 mg/L.

To determine fertilization success, 100 eggs were randomly sampled and counted 8 to 12 hours after fertilization (after the blastopore closure stage), and those that were dividing normally were scored. Four counts were performed to determine the mean fertilization success. At 17 hours after fertilization, the overall hatching success was determined by counting the number of hatched eggs divided by the number of fertilized eggs evaluated multiplied by 100. Four counts were performed to determine the mean hatching success.

2.6. Semen evaluation

The evaluated semen samples were the same samples used for the induced breeding protocol. In this experiment, however, pools were not formed, and males were evaluated individually. Only samples that were free of contaminants such as feces and urine were used. Freshly stripped milt was collected and stored at 4 °C until measurement of the total volume in 15 mL graduated plastic Falcon tubes. All samples were obtained approximately 7 hours after administration of the hormonal doses. The duration of sperm motility was subjectively evaluated as the elapsed time, measured with a stopwatch, from activation until 50.0% of the spermatozoa maintained forward swimming activity. For this assessment, 1 μL of semen from each breeder was aliquoted and applied to a glass slide, after which 20 μL of deionized water was added for activation. The stopwatch was started when the water was added. A coverslip was placed over the solution, as in the methodology described by Baynes et al. [17]. Observations of sperm motility were conducted at room temperature (23.0 °C–25.0 °C) using three replicates per sample, soon after milt collection, with the aid of a light microscope (Olympus BX-41, Olympus) under ×40 magnification. The percentage of live spermatozoa (sperm survival) was evaluated based on the integrity of the membrane. The differential penetration abilities of eosin-nigrosin dyes (5.0% eosin and 10.0% nigrosin) in live cells (unstained) and dead cells (pink stained) were evaluated. A total of 200 cells per slide were counted on a microscope at ×100 magnification. The percentage of live cells was calculated as the number of live spermatozoa divided by the number of total cells evaluated multiplied by 100. The sperm concentration was estimated through standard methods (sperm cells per milliliter of milt) using a counting chamber similar to that described by Buyukhatipoglu and Holtz [18]. For this purpose, a sample of sperm was fixed in buffered formaldehyde saline solution (1:1000).

2.6.1. Comet assay

The collected semen samples (n = 4 males per treatment) were protected from light and subsequently subjected to the comet assay (all semen samples were evaluated in triplicate). The samples were then diluted in phosphate-buffered saline (8.0 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4, and deionized water to 1000 mL; pH 7.0) to a concentration of 10.0×10^6 sperm per milliliter. All samples were evaluated before the comet assay to ensure that the spermatozoa had not been activated. The applied methodology was similar to that used by Cabrita et al. [19], with the following modifications: the

solution containing the sperm suspension was used to cover the first layer of agarose, which was then covered with a coverslip and incubated for 15 minutes at 4 °C, after which the coverslip was removed; next, the slides were submersed in cold lysis solution for 1 hour at 4 °C and then incubated for another hour at 4 °C after the addition of 10 mM dithiothreitol to the lysis solution; finally, the slides were incubated in lysis solution for an additional 90 minutes at 4 °C after the addition of 4 mM diiodosalicylate.

On completion of these steps, the slides were removed from the solution and maintained in horizontal electrophoresis cubes filled with electrophoresis solution (0.3 M of NaOH, 1 mM of Na_2EDTA; pH > 13) for 20 minutes at 4 °C. Electrophoresis was conducted for 20 minutes at 25 V and 300 mA at 4 °C. After this process, the slides were covered with neutralizing solution (0.4 M Tris, pH 7.5) for 5 minutes (3×) and then dried and fixed *via* immersion in cold absolute ethanol (4 °C) for 5 minutes and placed in boxes protected from light and dust.

The staining procedure was performed using 50 mL of GelRed solution (Biotium, Hayward, California, USA) diluted in 3 L of Milli-Q water. Images (×40 objective lens) were obtained using a fluorescence microscope (Leica DM 5000B, Leica Microsystems, Wetzlar, Germany) coupled to a digital camera (Leica DFC300 FX, Leica Microsystems, Wetzlar, Germany) and were saved as digital files for later analysis using Leica Application Suite (LAS V2.7.1, Leica Microsystems, Wetzlar, Germany) software for documentation. A total of 100 cells from each individual were analyzed on two slides and were classified visually into five classes according to tail length, designated class 0 through 4, in which class 0 indicated an absence of damage and class 4 indicated maximum damage. The percentage of each class of damage was calculated as the percentage of the occurrence of each class (class 0–4) in the total number of comets counted, using the following formula: damage class (%) = ([n class × 100]/[total number]). Additionally, the damage index (DI) was calculated as the total product of the number of comets in each class and the denominator of the digit class (0, 1, 2, 3, and 4): DI total = (1 × [n class 1] + 2 × [n class 2] + 3 × [n class 3] + 4 × [n class 4]); thus, the total score of 100 comets could range from a minimum of 0 (all undamaged) to a maximum of 400 (all damaged).

2.7. Statistical analysis

Statistical analysis was performed using the computer program Statistica (StatSoft, version 7.0). The results are presented as the mean ± standard error of the mean. Data were analyzed for normality and homoscedasticity of variance. We tested differences between group means using Student's *t* test and a one-way ANOVA followed by Tukey's multiple comparison test. All statistical tests were performed at the 5% level of significance.

3. Results

3.1. Water parameters

The values of the water parameters in the EPs and Cs were as follows: pH (7.7 ± 0.5 and 7.6 ± 0.8, respectively), dissolved oxygen (3.8 ± 1.6 and 2.0 ± 1.2 mg/L, respectively), conductivity (69.3 ± 15.9 and 76.2 ± 9.4 μS/cm, respectively), transparency (68.3 ± 1.8 and 60.0 ± 1.2 cm, respectively), ammonia (129.8 ± 80.8 and 248.5 ± 119.0 μg/L, respectively), and temperature (23.2 ± 3.1 °C and 23.2 ± 2.7 °C, respectively). During the experimental period, the survival rates of the fish maintained in the EPs and Cs were 100% and 95.0%, respectively.

3.2. General characteristics of the breeders

At the onset of the experimental period, the average total weight of the fish was 394.9 ± 29.9 g. The average weight of the EPMs was greater ($P < 0.05$) than that of the CMs throughout the experimental period, except in August 2009. At the end of the experimental period, the average weights of the EPMs and CMs were 448.0 ± 21.1 and 324.0 ± 19.7 g, respectively. When the fish mass in each group was evaluated separately, we observed that the mean values for the EPMs and CMs remained stable during the experimental period except for an increase in the former and a reduction in the latter observed during February 2010 (just after the breeding season) (Fig. 1).

3.3. Testis development: GSI values

Beginning at the second sampling time (October 2009), the GSI values of the EPMs showed a weak tendency to be greater than those of the CMs. This trend was confirmed at the third sampling time and maintained until the end of the experiment ($P < 0.05$) (Fig. 2). We observed the greatest difference between treatments at the beginning of the breeding season (December 2009), when values in the EPMs were more than 2.5 times greater than those in the CMs. The GSI values of the EPMs increased progressively from the first (August 2009) to the third sampling time (December 2010), when they reached a peak (1.3% of body weight) (Fig. 2). At the fifth sampling time (April 2010), the values decreased sharply to 0.3 ($P < 0.05$), showing that between the third and fifth sampling times (late summer to early autumn) the reproductive season ended in the EPMs, which subsequently entered the regression class (Figs. 2, 3E, and 4A, C). In contrast, the GSI values of the CMs only increased between the first and second sampling times

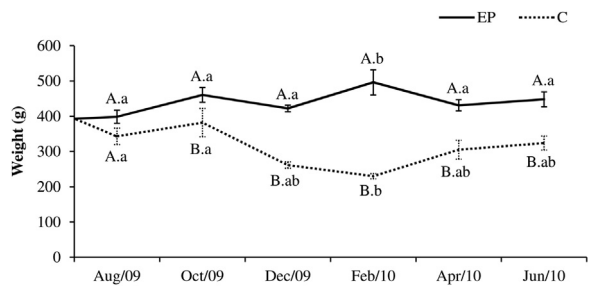

Fig. 1. Mean body weight values (in grams) of *Prochilodus lineatus* maintained in earthen ponds (EPs) and cages (Cs) for 440 days. Capital letters (A and B) indicate significant differences between the groups in the same sample ($P < 0.05$); lowercase letters (a and b) represent significant differences between sampling times in the same group ($P < 0.05$).

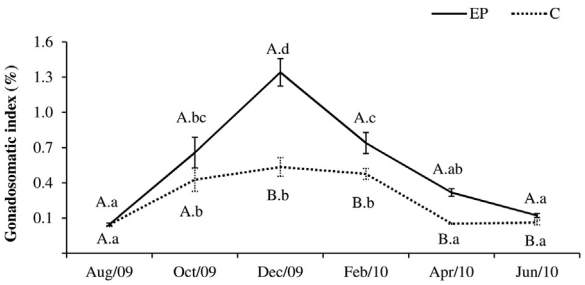

Fig. 2. Gonadosomatic index of *Prochilodus lineatus* males maintained in earthen ponds (EPs) and cages (Cs) for 440 days. Capital letters (A and B) indicate significant differences between the groups in the same sample ($P < 0.05$); lowercase letters (a, b, c, and d) represent significant differences between sampling times in the same group ($P < 0.05$).

(from August 2009 to October 2009) ($P < 0.05$), stabilizing near 0.5 in December 2009 and decreasing to approximately 0.1 at the fifth sampling time (April 2010) (Fig. 2).

3.4. Histomorphometry of the testis

At the first sampling time (August 2009, winter, initial maturation phase), 60 days after the beginning of the experimental period, the general profiles of the seminiferous tubules of the EPMs and CMs were already different, but the differences present at this time were not reflected in their GSI values (which were very similar in the two groups during this period) (Fig. 2). In contrast, although the frequency of tubules containing only spermatogonia (lacking meiotic and spermiogenic phases) in the EPMs was 15.5%, the frequency of this type of tubule in the CMs was much higher, 74.0% ($P < 0.05$) (Fig. 5A). The frequency of other types of germ cells was similar between the groups except that a higher frequency of spermatid cysts was found in EPMs ($P < 0.05$) (Fig. 5B–E). The mean diameters of the seminiferous tubules were similar between treatments during this period ($P > 0.05$) (Fig. 6). In this period, we did not evaluate the frequency of tubules with a DGE

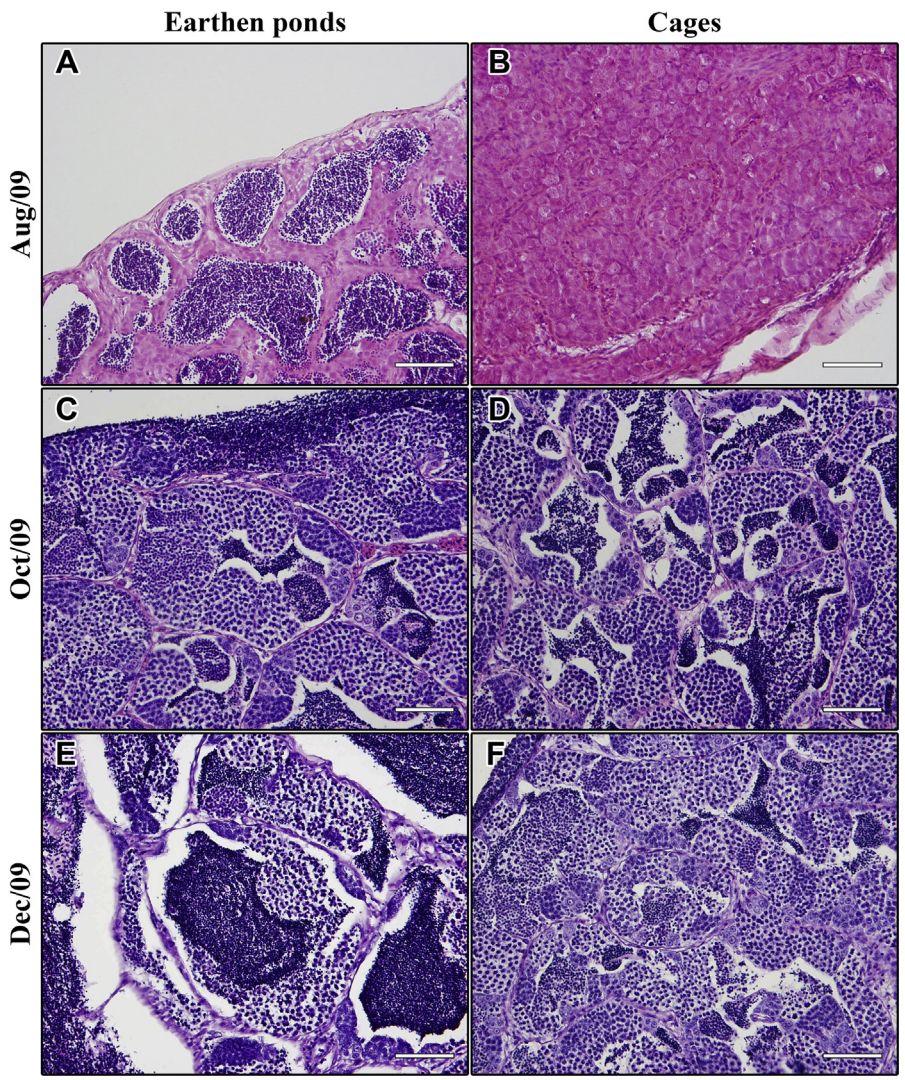

Fig. 3. Cross-sections of the testes of *Prochilodus lineatus* maintained in earthen ponds (A, C, E) and cages (B, D, F) (bar, 50 μm). Hematoxylin-eosin staining. (For interpretation of the references to color in this Figure, the reader is referred to the Web version of this article.)

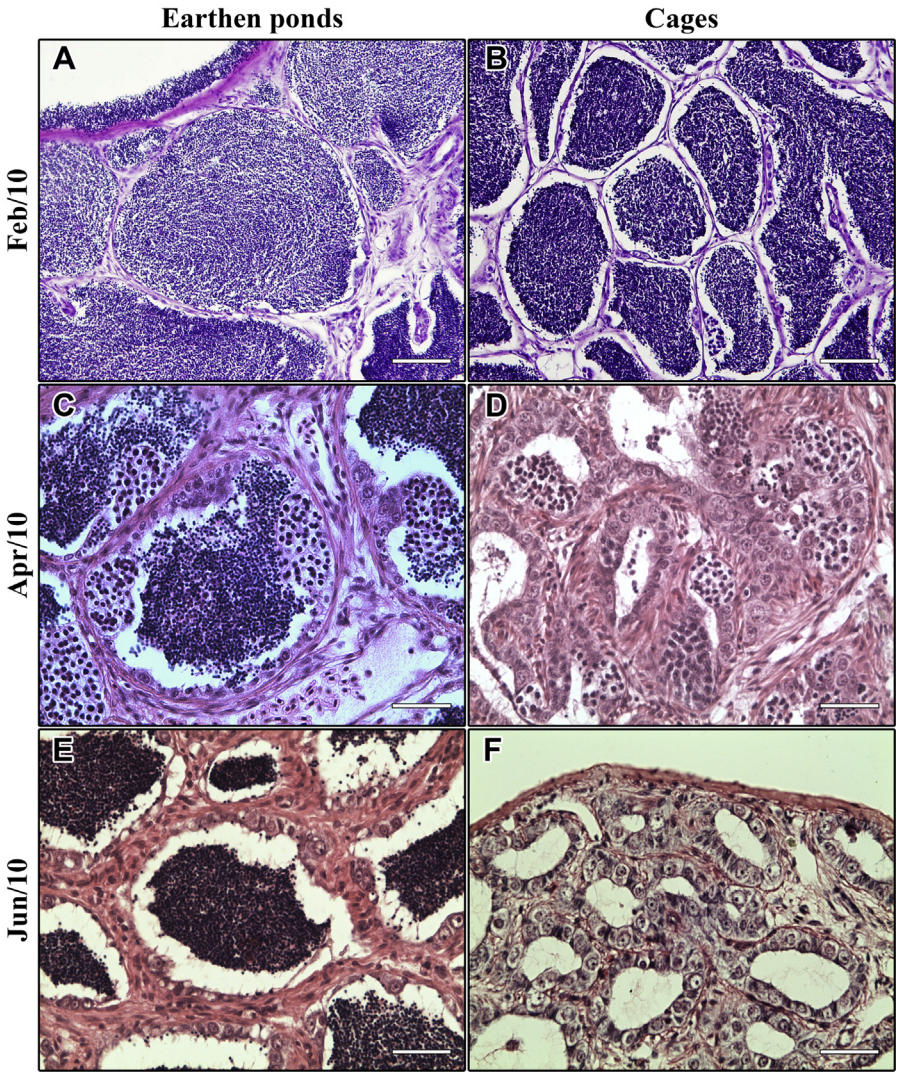

Fig. 4. Cross-sections of the testes of *Prochilodus lineatus* maintained in earthen ponds (A, C, E) and cages (B, D, F) (bar, 50 μm). Hematoxylin-eosin staining. (For interpretation of the references to color in this Figure, the reader is referred to the Web version of this article.)

because most of the tubules lacked a lumen, and an epithelium could not be detected (Fig. 5F).

At the second sampling time (October 2009, early spring, maturation phase), the frequencies of different types of germ cells became similar between the groups except for the frequency of tubules showing spermatozoa in the lumen, which was higher in the CMs (15.8%) than in the EPMs (0.8%) (Fig. 5A–E). In both groups, most of the evaluated tubules showed a continuous germinal epithelium (CGE), except for a small percentage (1.9%) with a DGE in the EPMs (Fig. 3F). During this period, the mean tubular diameter (Figs. 3C, D and 6) and GSI values increased ($P < 0.05$) compared with the previous sampling time. Moreover, the tubular diameter in the EPMs was 44.0 μm greater than that in the CMs at this time ($P < 0.05$) (Fig. 6).

At the third sampling time (December 2009, summer, breeding season), the frequencies of seminiferous tubules containing only spermatogonia were much reduced (Figs. 3E, F and 5A). Cysts containing meiotic and spermiogenic cells were found to be predominant in both groups, with a slightly increased occurrence of these cells recorded in the CMs ($P < 0.05$) (Fig. 5B–D). The diameter of the seminiferous tubules was 45.7 μm greater in the EPMs than in the CMs ($P < 0.05$) (Fig. 6). The frequency of tubules lined with a DGE in the EPMs (84.0%) was 6.3 times higher than that in the CMs (13.3%) (Figs. 3E, F and 5E). The large amount of sperm stored in the testis of the EPMs (Fig. 3E) contributed to a mean GSI in this group that was 2.5 times greater (Fig. 2) than that in the CMs ($P < 0.05$).

At the fourth sampling time (February 2010, late summer, end of breeding season), both groups entered the regression class (Fig. 4A, B), showing 100% of tubules lined with a DGE (Fig. 5F). In the two groups, the frequency of tubules showing only isolated spermatogonia was ~34.0%, representing a marked increase compared with the previous sampling time (~0%) (Fig. 5A). In contrast, the frequencies of spermatogonia, spermatocytes, and spermatid cysts decreased dramatically (Fig. 5B–D). The mean seminiferous

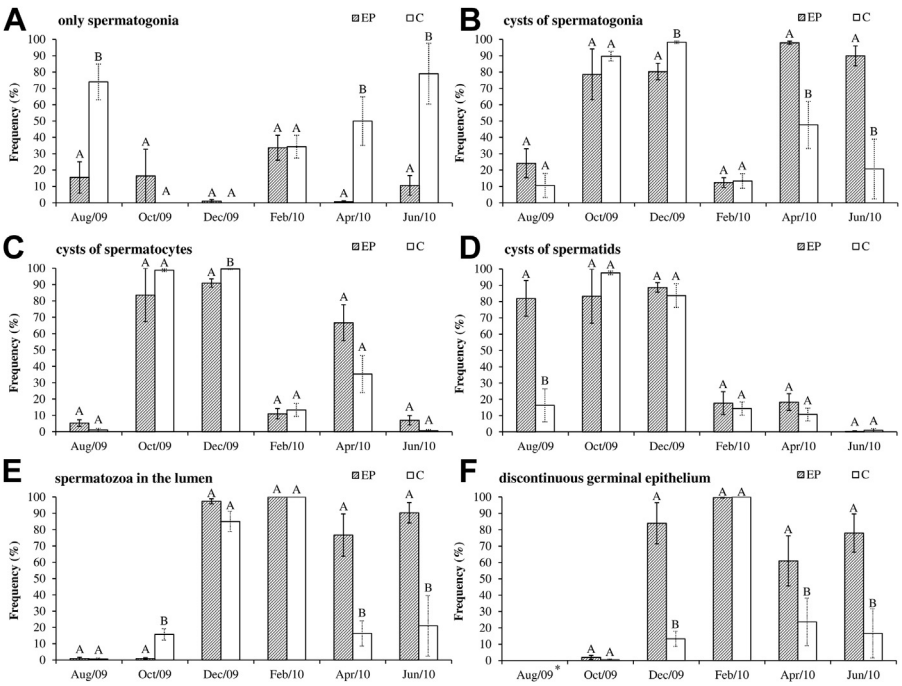

Fig. 5. Frequency of various types of germ cells inside seminiferous tubules in *Prochilodus lineatus* males maintained in earthen ponds (EPs) and cages (Cs) for 440 days. (A) Frequency of tubules lined only with isolated spermatogonia. (B–E) Frequencies of tubules containing spermatogonia, primary or secondary spermatocytes, spermatids, and sperm cells. (F) Frequency of tubules lined with a discontinuous germinal epithelium (DGE). Capital letters (A and B) indicate significant differences between groups ($P < 0.05$). *In this period, we did not evaluate the frequency of tubules with a DGE because most of the tubules lacked a lumen, and an epithelium could not be detected.

tubule diameter and GSI values were greater in the EPMs than in the CMs ($P < 0.05$). However, the GSI values sharply decreased in the former group but remained stable in the latter compared with the previous sampling time (Figs. 2 and 6).

At the fifth sampling time (April 2010, autumn), the regression process was more advanced in the CMs than in the EPMs, causing the mean GSI values to be greater in the latter group ($P < 0.05$). The frequency of tubules lined only by spermatogonia was higher in the CMs (50.0 and 0.7% for the CMs and EPMs, respectively) ($P < 0.05$) (Fig. 5). In both groups, there was a general trend of increasing and decreasing frequencies of immature and mature germ cells, respectively. The frequency of tubules that still contained

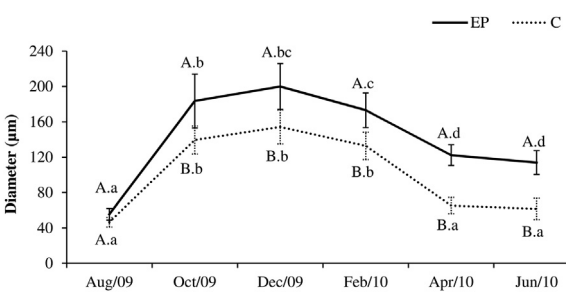

Fig. 6. Diameter of the seminiferous tubules in the testis of *Prochilodus lineatus* maintained in earthen ponds (EPs) and cages (Cs) for 440 days. Capital letters (A and B) indicate significant differences between the groups in the same sample ($P < 0.05$); lowercase letters (a, b, c, and d) represent significant differences between sampling times in the same group ($P < 0.05$).

spermatozoa in the lumen was 4.8 times higher in the EPMs ($P < 0.05$). The frequency of tubules with a DGE and the average seminiferous tubule diameter were higher in the EPMs ($P < 0.05$) (Figs. 4–6).

At the sixth sampling time (June 2010, early winter), the EPMs were still in the regression class, whereas the CMs were completely regressed. The GSI values were greatly reduced in both groups (Fig. 2). The average frequency of tubules lined only by spermatogonia was 7.4 times lower in the EPMs than in the CMs ($P < 0.05$) (Fig. 5F). The frequencies of tubules containing spermatogonia inside cysts and sperm were higher in the EPMs than in the CMs ($P < 0.05$) (Fig. 5B, E). In both groups, almost no meiotic (Fig. 5C) or spermiogenic cells (Fig. 5D) were observed inside cysts. The frequency of tubules with a DGE was higher in the EPMs than in the CMs ($P < 0.05$) (Fig. 5F).

3.5. Semen characteristics

The mean volume of semen released by the EPMs was 1.9 times greater than that in the CMs ($P < 0.05$). The motility and the rate of sperm survival were similar between treatments. However, the sperm concentration was 1.7 times greater in the EPMs than in the CMs (Table 1).

3.5.1. Comet assay

There was a significant increase in the frequencies of cells with high DNA fragmentation in the CMs according to the comet length data (scores 3 and 4) (Fig. 7A). A total of 1.7% and 52.0% ($P < 0.05$) of the analyzed cells from the

Table 1
Semen analysis of *Prochilodus lineatus*, maintained for 440 days in earthen ponds (EPs) and cages (Cs).

Prochilodus lineatus	Volume (mL)	Motility (s)	Sperm survival (%)	Concentration (spz/mL)
EP, n = 18	1.5 ± 0.3^{A}	44.2 ± 4.4^{A}	98.9 ± 0.5^{A}	$6.8 \times 10^{10} \pm 1.0 \times 10^{10A}$
C, n = 19	0.8 ± 0.1^{B}	36.3 ± 2.2^{A}	98.9 ± 0.6^{A}	$3.9 \times 10^{10} \pm 0.6 \times 10^{10B}$

Superscript letters (A and B) indicate significant differences within the same column ($P < 0.05$).

EPMs and CMs, respectively, were classified with the highest comet tail length score (score 4). At level 3, the frequencies were 17.0% and 29.0%, respectively ($P < 0.05$) (Fig. 7A). The DI was 2.1 times lower ($P < 0.05$) in the EPMs than in the CMs (Fig. 7B).

3.6. Reproductive performance

Lower fertilization and hatching success were obtained using sperm of the CMs compared with the EPMs (68.4% and 82.8%; and 56.8% and 61.7%, respectively) ($P < 0.05$) (Table 2).

4. Discussion

In this study, we evaluated the reproductive cycle and performance of *P. lineatus* males maintained in two rearing systems that simulated known suitable (EPs) and unsuitable (Cs) conditions for breeders. The CMs exhibited an impaired reproductive cycle and produced low-quality gametes. We observed that the spermatogenic process was less intense in the CMs, accompanied by an accumulation of immature germ cells (especially in the regressed class). The CMs also presented a decreased seminiferous tubule diameter, resulting in the absence of an increase in the GSI values throughout the year in this group.

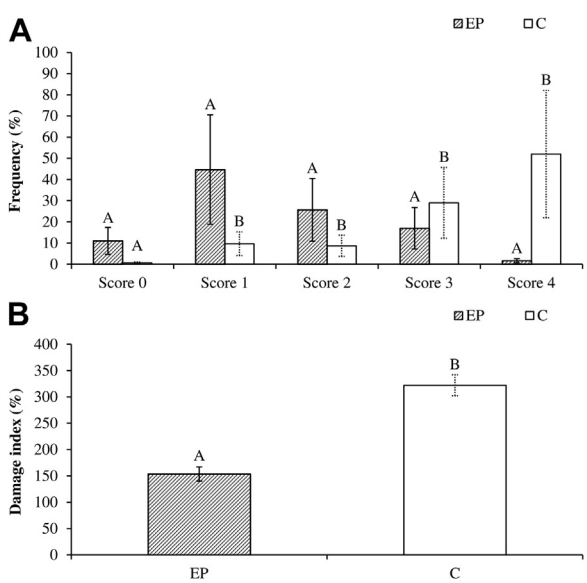

Fig. 7. Analysis of DNA damage in the spermatozoa of *Prochilodus lineatus* maintained in earthen ponds (EPs) and cages (Cs) for 440 days. (A) Frequency of damage classes (%) and (B) rate of damage (%). Capital letters (A and B) indicate significant differences between groups in the same sample ($P < 0.05$).

Consequently, during the breeding season, the CMs presented a reduced semen volume, sperm concentration, spermatozoa DNA quality, and fertilization and hatching success. We observed that the regressed class was a key point of the reproductive cycle because an intense process of spermatogonial proliferation was concentrated in this period, which was affected by inappropriate husbandry conditions. Hence, suitable husbandry conditions should be provided throughout the year for this species to avoid obtaining low-quality semen.

4.1. Reproductive cycle

Considering only the obtained GSI values, we would not assign a spawning season for the CMs, which can be established only through histologic analysis. The CMs did not display a gradual increase in the GSI values during the year or a peak in the spawning season (October 2009-February 2010). In contrast, the mean GSI values of the EPMs gradually increased between October and December, reaching a peak at values that were two and one-half times higher than those of the CMs at the same period. A gradual increase in GSI values after the testis maturation process has been widely reported for a number of tropical rheophilic species that are total spawners, including *Piaractus mesopotamicus* [20], *Pseudoplatystoma fasciatum* [8,21] *Pseudoplatystoma* sp. [22], *Salminus hilarii* [23], and *Prochilodus argenteus* [24] maintained in captivity, and reflects a process of annual germ and Sertoli cell proliferation (reviewed in [25]) that was most likely impaired in the CMs, as will be discussed later.

We should mention that the average length (data not shown) and weight were greater in the EPMs than in the CMs during almost the entire experimental period. Thus, it was assumed that the differences observed between groups concerning testes characteristics could have been (at least in part) because of the nutritional status and reduced growth of the CMs.

In August 2009, during the winter, the EPMs were in the maturation class, but the CMs were still regressed, showing $58.4 \pm 1.4\%$ more and $65.6 \pm 0.8\%$ fewer tubules containing isolated spermatogonia and spermatid cysts, respectively, than the former group. These data indicate that the onset of spermatogonial proliferation and meiosis in the CMs was

Table 2
Mean and standard error of the fertility rates of eggs and hatching larvae of *Prochilodus lineatus* in earthen ponds (EPs) and cages (Cs).

Fertilization rate (%)	Fertilization	Eclosion
EP male × EP female	82.8 ± 03.4^{A}	61.7 ± 3.5^{A}
C male × EP female	68.4 ± 04.5^{B}	56.8 ± 4.1^{B}

Superscript letters (A and B) indicate significant differences within the same column ($P < 0.05$).

less intense. The available information on the relationship between husbandry conditions and spermatogenesis is extremely scarce, but inhibited testicular growth and low sperm production associated with a greater number of immature germ cells are dysfunctions that are also caused by exposure to hypoxia [26–28]. Similarly, tilapia exposed to low temperatures presented an accumulation of intermediate type B spermatogonia [29], and, according to these authors, the cell cycle may have been extended for mitotic activity. Because accumulation of immature germ cells is generally found when fish are subjected to adverse conditions [26–28], and the mitotic proliferation of germ cells takes place at the beginning of the reproductive cycle during the regressed class [29,30], it appears that adequate management conditions should be supplied throughout the reproductive cycle, especially during the onset of the reproductive cycle.

In October 2009, during the spring, the males of both groups were in the initial maturation class. The high frequency of cysts of spermatogonia, spermatocytes, and spermatids, together with the predominance of CGE, indicated that the main function of the testes was producing sperm in this class in both groups [30,31]. However, when the composition of the tubules was similar in the two groups, the average diameter of seminiferous tubules was reduced in the CMs, indicating a less intense Sertoli and germ cell proliferation process in this group. It has been established that a seasonal increase in tubule diameter occurs because of an increased proliferation of Sertoli cells and seminal fluid production [32,33]. In this context, each Sertoli cell is able to support a fixed number of germ cells, and the number of Sertoli cells per testis ultimately dictates testis size and the magnitude of sperm production [25,31]. Thus, the greater the number of Sertoli cells, the higher the final concentration of spermatozoa [25]. Hence, as the sperm concentration, the seminiferous tubule diameter, and the GSI values were reduced in the CMs, it is most likely that the mitotic activity of CM Sertoli cells was also reduced, and the absolute number of Sertoli cells in the CMs was, most likely, less than that in the EPMs. In this context, it should be interesting to address the possible interference of husbandry conditions with the organized and coordinated processes of Sertoli and germ cell proliferation that ensure proper germ cell development [29].

In December 2009, although the EPMs were in an advanced maturation class, the testes of the CMs remained in the initial maturation class, showing a predominance of tubules lined by a CGE. December is the breeding period for this species in captivity [13]. The CM reproductive cycle was, therefore, delayed as the main function of the testes at this time is expected to be sperm storage (DGE predominant) and not sperm production (CGE predominant) [30,31]. A CGE, characterized by the presence of germ cells throughout the length of the seminiferous tubules, occurs in the initial phase of maturation [30]. However, as the cycle progresses, portions of the tubules that are free of germ cells gradually appear and become lined by a DGE [8,34–36].

February 2010 was, most likely, the end of the breeding season for the CMs, as large amounts of spermatozoa were observed in the lumen of the tubules for the last time. The EPMs and CMs were in the regression class at this time, based on the observation that 100% of their seminiferous tubules presented a DGE. The frequency of different types of germ cells was very similar between the groups. However, the higher GSI values observed for the EPMs could be explained by the mean diameter of their seminiferous tubules, which was 1.3 times greater than that of the CMs.

In April and June, the higher frequency of tubules containing single spermatogonia in the CMs and the higher frequency of DGE in the EPMs indicated that the former group presented a shortened breeding season and a delayed onset of the next reproductive cycle. The frequencies of tubules containing proliferating spermatogonia in the EPMs increased in April and June and were two and four and one-half times higher, respectively, compared with the CMs. Moreover, in the same period, the frequency of seminiferous tubules presenting spermatozoa was dramatically decreased in the CMs. Taken together, these characteristics indicated that the EPMs initiated a new reproductive cycle earlier, as they already showed spermatogonia proliferating at the seminiferous tubule borders at this time, and these males could be used as semen donors up to June 2010.

4.2. Sperm quality

Sperm quality can be defined as the ability of sperm to successfully fertilize an egg and subsequently allow the development of a normal embryo (reviewed in [1,2,37]). In this study, the fertilization success and hatchability rates of embryos derived from the CMs were lower than those from the EPMs, and we can therefore conclude that the quality of embryos and sperm in the former group was lower than that in the latter. Induced reproduction of *P. lineatus* is easily accomplished [38], and the fertilization success and hatching rates obtained from EPMs and EPFs are relatively high (80.0% and 72.3%, respectively) [13].

Under a previous approach, eggs obtained through fertilizing oocytes produced by females reared in Cs with EPM spermatozoa also showed reduced fertilization and hatching success (43.2% and 26.5%, respectively) [13], presenting even lower values than those obtained in this study through fertilizing EPF oocytes with CM spermatozoa (68.4% and 56.8%, respectively). In this context, one possible explanation for the poorer results obtained in the previous study is the known ability of oocytes to repair DNA damage present in sperm. In fish, recent studies conducted in rainbow trout reported that some spermatozoa containing damaged DNA are able to carry out fertilization and that the oocyte can repair this damage to a certain degree [39]. According to these authors, when the rate of DNA fragmentation is high, the oocyte repair capacity is insufficient, and the rate of abortions increases considerably.

In this study, the reduced fertilization and hatching success obtained with CM semen showed an association with a reduced semen volume and sperm concentration and increased fragmentation of sperm DNA but not with sperm survival and motility. Motility is often used to evaluate sperm quality, but we did not confirm this relationship

in the present study. This result could be related to the subjective evaluation method used *in lieu* of computational analysis [1]. The decreased semen volume and concentration observed are clearly associated with impaired spermatogenesis, as discussed in the previous section.

4.3. Conclusion

In this study, we observed that the reproductive cycle of *P. lineatus* CMs was impaired. We found differences in the spermatogenic process between groups in all classes of testicular development, but these differences were most obvious in the regressed class in the early reproductive cycle. We showed that the quality of the sperm of the CMs was also reduced, as demonstrated by the low fertility and hatching success achieved in this group. Moreover, the low fertility and hatching success were associated with the semen volume, sperm concentration, and DNA quality but not with sperm motility. In conclusion, suitable conditions must be provided to male breeders of this species throughout their reproductive cycle, in contrast to the empirically accepted notion that appropriate conditions must be provided only before spawning. The regressed class deserves special care even in winter, many months before the breeding season, because inappropriate conditions during this period are related to the accumulation of immature germ cells, resulting in the formation of low-quality sperm.

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References

[1] Rurangwa E, Kime DE, Ollevier F, Nash JP. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture 2004;234:1–28.
[2] Bobe J, Labbé C. Egg and sperm quality in fish. Gen Comp Endocrinol 2010;165:535–48.
[3] Cabrita E, Sarasquete C, Martínez-Páramo S, Robles V, Beirão J, Pérez-Cerezales S, et al. Cryopreservation of fish sperm: applications and perspectives. J Appl Ichthyol 2010;26:623–35.
[4] Mylonas CC, Fostier A, Zanuy S. Broodstock management and hormonal manipulations of fish reproduction. Gen Comp Endocr 2010; 165:516–34.
[5] Santana JCO, Quagio-Grassiotto I. Extracellular matrix remodeling of the testes through the male reproductive cycle in Teleostei fish. Fish Physiol Biochem 2014;40:1863–75.
[6] Dabrowski K, Rinchard J, Ottobre JS, Alcantara F, Padilha P, Ciereszko A. Effect of oxygen saturation in water on reproductive performances of pacu *Piaractus brachypomus*. J World Aquacult Soc 2003;34:441–9.
[7] Leonardo AFG, Romagosa E, Borella MI, Batlouni SR. Induced spawning of hatchery-raised Brazilian catfish, cachara *Pseudoplatystoma fasciatum* (Linnaeus, 1766). Aquaculture 2004;240:451–61.
[8] Batlouni SR, Romagosa E, Borella MI. The reproductive cycle of male catfish, cachara *Pseudoplatystoma fasciatum* (Teleostei, Pimelodidae) revealed by changes of the germinal epithelium. An approach addressed to aquaculture. Anim Reprod Sci 2006;96:116–32.
[9] Reidel A, Boscolo WR, Feiden A, Romagosa E. The effect of diets with different levels of protein and energy on the process of final maturation of the gametes of *Rhamdia quelen* stocked in cages. Aquaculture 2010;298:354–9.
[10] Romagosa E, Souza BE, Sanches EA, Baggio DM, Bombardelli RA. Sperm motility of *Prochilodus lineatus* in relation to dilution rate and temperature of the activating medium. J Appl Ichthyol 2010;26: 678–81.
[11] Rios FSA, Carvalho CS, Pinheiro GHD, Donatti L, Fernandes MN, Rantin FT. Utilization of endogenous reserves and effects of starvation on the health of *Prochilodus lineatus* (Prochilodontidae). Environ Biol Fish 2011;91:87–94.
[12] Godinho HP, Ribeiro DM. Maturidade sexual de curimbatás, Prochilodus scrofa (Pisces, Teleostei) em viveiros. Arq Bras Med Vet Zoot 1985;34:349–57.
[13] Hainfellner P, De Souza TG, Moreira RG, Nakaghi LSO, Batlouni SR. Low estradiol levels, delayed vitellogenesis and reduced amounts of yolk are dysfunctions associated with the formation of low quality oocytes in *Prochilodus lineatus* (Teleostei: Characiformes). Neotrop Ichthyol 2012;10:601–12.
[14] Criscuolo-Urbinati E, Kuradomi RY, Urbinati EC, Batlouni SR. The administration of exogenous prostaglandin may improve ovulation in pacu (*Piaractus mesopotamicus*). Theriogenology 2012;78:2087–94.
[15] Solorzano L. Determination of ammonia in natural waters by phenol hypochlorite method. Limnol Oceanogr 1969;14:799–801.
[16] Buchet V, Coquard E, Sévère A, Barone H. Influence of tank volume on vitellogenesis and spawning performances in sea bass (*Dicentrarchus labrax* L.). Aquacult Res 2008;9:420–6.
[17] Baynes SM, Scott AP, Dawson AP. Rainbow trout *Salmo gairdneri* Richardson, spermatozoa: effects of cations and pH on motility. J Fish Biol 1981;19:259–67.
[18] Buyukhatipoglu S, Holtz W. Sperm output in rainbow trout (*Salmo gairdneri*): effect of age, timing and frequency of stripping and presence of females. Aquaculture 1984;37:63–71.
[19] Cabrita E, Robles V, Rebordinos L, Sarasquete C, Herráez MP. Evaluation of DNA damage in rainbow trout (Oncorhynchus mykiss) and gilthead sea bream (Sparus aurata) cryopreserved sperm. Cryobiology 2005;50:144–53.
[20] Gazola R, Borella MI. Plasma testosterone and 11-ketotestosterone levels of male pacu *Piaractus mesopotamicus* (Cypriniformes, Characidae). Braz J Med Biol Res 1997;30:1485–7.
[21] Leonardo AFG, Romagosa E, Batlouni SR, Borella MI. Occurrence and significance of ovarian and follicular regression in cachara *Pseudoplatystoma fasciatum* (Linnaeus, 1766): a histology approach. Arq Bras Med Vet Zootec 2006;58:831–40.
[22] Dabrowski K, Arslan M, Rinchard J, Palacios ME. Growth, maturation, induced spawning, and production of the first generation of South American Catfish, *Pseudoplatystoma sp.*, in North America. J World Aquacult Soc 2008;39:174–83.
[23] Honji RM, Narcizo AM, Borella MI, Romagosa E, Moreira RG. Patterns of oocyte development in natural habitat and captive *Salminus hilarii* Valenciennes, 1850 (Teleostei: Characidae). Fish Physiol Biochem 2009;35:109–12.
[24] Arantes FP, Santosa HB, Rizzo E, Sato Y, Bazzoli N. Profiles of sex steroids, fecundity, and spawning of the curimatã-pacu *Prochilodus argenteus* in the São Francisco River, downstream from the Três Marias Dam, Southeastern Brazil. Anim Reprod Sci 2010;118: 330–6.
[25] Schulz RW, França LR, Lareyre JJ, Legac F, Chiarini-Garcia H, Nóbrega RH, et al. Spermatogenesis in fish. Gen Comp Endocr 2010; 165:390–411.
[26] Wu RSS, Zhou BS, Randall DJ, Woo NYS, Lam PKS. Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. Environ Sci Technol 2003;37:1137–41.
[27] Shang E, Wyu R, Wu R. Hypoxia affects sex differentiation and development, leading to a male-dominated population in zebrafish (*Danio rerio*). Environ Sci Technol 2006;40:3118–22.
[28] Thomas P, Rahman MS, Khan IA, Kummer JA. Widespread endocrine disruption and reproductive impairment in an estuarine fish population exposed to seasonal hypoxia. Proc Biol Sci 2007;274:2693–701.
[29] Alvarenga ER, França LR. Effects of different temperatures on testis structure and function, with emphasis on somatic cells, in sexually mature Nile tilapias (*Oreochromis niloticus*). Biol Reprod 2009;80: 537–44.
[30] Grier HJ. The germinal epithelium: its dual role in establishing male reproductive classes and understanding the basis for indeterminate egg production in female fishes. In: Creswell RL, editor. Proceedings

of the fifty-third annual Gulf and Caribbean Fisheries Institute, November 2000. Fort Pierce, Mississippi/Alabama Sea Grant Consortium; 2002. p. 537–552.

[31] Grier HJ, Uribe-Aranzábal MC. The testis and spermatogenesis in teleosts. In: Jamieson BGM, editor. Reproductive biology and phylogeny of fishes (agnathans and bony fishes). New Hampshire: Science Publishers, Endfield; 2009. p. 119–42.

[32] Schulz RW, Menting S, Bogerd J, França LR, Vilela DAR, Godinho HP. Sertoli cell proliferation in the adult testis: evidence from two fish species belonging to different orders. Biol Reprod 2005;73:891–8.

[33] Nóbrega RH, Batlouni SR, França LR. An overview of functional and stereological evaluation of spermatogenesis and germ cell transplantation in fish. Fish Physiol Biochem 2009;35:197–206.

[34] Taylor RG, Grier HJ, Whittington JA. Spawning rhythms of common snook in Florida. J Fish Biol 1998;53:502–20.

[35] Brown-Peterson NJ, Warren JW. The reproductive biology of spotted seatrout, *Cynoscion nebulosus*, along the Mississippi Gulf Coast. GulfMex Sci 2001;1:61–73.

[36] Brown-Peterson NJ, Grier HJ, Overstreet RM. Annual changes in germinal epithelium determine male reproductive classes of the cobia. J Fish Biol 2002;60:178–202.

[37] Bonnet E, Fostier A, Bobe J. Characterization of rainbow Trout egg quality: a case study using four different breeding protocols, with emphasis on the incidence of embryonic malformations. Theriogenology 2007;67:786–94.

[38] Godinho HM, Romagosa E, Cestarolli MA, Narahara MY, Fenerich-Verani N. Reprodução induzida do curimbatá, *Prochilodus scrofa*, Steindachner, 1881 sob condições de cultivo experimental. Rev Bras Reprod Anim 1984;8:113–9.

[39] Pérez-Cerezales S, Martínez-Páramo S, Beirão J, Herráez P. Fertilisation capacity with rainbow trout DNA damaged sperm and embryo developmental success. Reproduction 2010;139:1–10.