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Hormonal induction and semen characteristics of tambaqui *Colossoma macropomum*

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Summary

In the hatchery-bred tambaqui *Colossoma macropomum*, spontaneous semen release does not occur, and hand-stripping produces reduced semen volume. The goal of this work is to evaluate the effects of hormonal induction with carp pituitary extract (CPE) on both qualitative (visual aspect, pH, motility, viability and morphological abnormalities) and quantitative (volume, concentration and number of spermatozoa per ejaculate) traits of tambaqui semen. Eleven males were treated with CPE (induced), and 11 were left untreated as a control (non-induced). All analysed parameters except motility and percentage of viable spermatozoa presented significant differences ($p < 0.05$) between the induced and non-induced treatments. CPE induction resulted in a 25-fold increase in semen volume and a 10-fold increase in the number of spermatozoa collected. However, both sperm concentration and the frequency of sperm with morphological abnormalities (commonly detached heads or bent tails) were significantly lower in CPE-induced fish. The hormonal induction of tambaqui males with CPE is efficient and positively influences some qualitative and quantitative properties of semen. Additionally, semen collection via gentle abdominal massage occurs more readily in CPE-induced fish.

Keywords: Carp pituitary extract, *Colossoma macropomum*, Fish, Semen quality, Sperm morphology

Introduction

The tambaqui (*Colossoma macropomum*) is a characid (Froese & Pauly, 2009), and is considered to be the second largest scaled fish in South America (Isaac & Ruffino, 2000). It is a common target of Brazilian fish farming, especially in the north and north-eastern regions of the country, as a popular food fish that is easy to handle and grows rapidly. The tambaqui is a rheophilic species that undergoes reproductive migration (Froese & Pauly, 2009); deep changes in blood hormone levels occur in males during the spawning season, triggered by gonadotrophic hormones (Schulz & Miura, 2002). However, in captivity, some external stimuli are absent, and the breeders usually lack a proper endocrine response, resulting in reduced semen volume and poor sperm quality (Valdebenito, 2008).

Several hormonal induction techniques have been developed for fish breeding over the past few decades (e.g., Woynarovich & Horváth, 1983; Zohar, 1988; Harvey & Carosfeld, 1993; Behr *et al.*, 2000; Zohar & Mylonas, 2001; Valdebenito, 2008). Treatment with a raw pituitary extract obtained from the common carp (CPE) is the most widespread induction method used with rheophilic species in fish culture facilities, and about 40 economically and/or ecologically important fish species in Brazil have been successfully induced to reproduce using this technique. Artificial reproduction procedures rely on injected hormones to induce spawning so that both males and females complete their reproductive cycle under controlled conditions at a particular, planned moment. In males, the CPE induction causes testicular hydration, accelerating the breeding cycle and favouring both spermiation and the release of a larger-than-normal volume of seminal plasma (Zaniboni-Filho & Weingartner, 2007).

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The relationship between gonadotrophic hormones or testicular steroids and semen quality has been intensively evaluated in fish (Schulz & Miura, 2002). In general, the criteria used to evaluate semen quality in fish are based on analyses of sperm motility, concentration and viability (Viveiros & Godinho, 2009) and, therefore, they disregard any possible morphological abnormalities in the sperm cells that may affect fertility. Morphological studies of male infertility have typically focused on mammalian species that undergo artificial insemination, such as dairy and beef cattle (Chenoweth, 2005). The evaluation of sperm morphology in fish semen is important to screen for abnormalities that can affect male fertility.

The goal of this work is to evaluate the effects of CPE hormonal induction on the qualitative and quantitative features of tambaqui semen.

Material and methods

The present work was carried out during late spring (November) in the Fish Culture Laboratory of the São Francisco and Parnaíba Development Company (CODEVASF) located in Neópolis-SE, Brazil, and in the Laboratory of Biotechnology in Animal Reproduction at Embrapa Tabuleiros Costeiros, Aracaju-SE, Brazil.

Five- to 6-year-old males were collected from earthen ponds and specimens that released semen after gentle abdominal pressure were selected for the experiments. The selected specimens were weighed (7.0 ± 1.0 kg), measured (65.0 ± 4.0 cm total length) and transported to two 5-m^3 concrete ponds with water flow (1 l/s) and a temperature of 27.5°C.

To verify the effects of hormonal induction on semen, 11 males were selected for hormonal induction with carp pituitary extract (CPE-induced fish); 11 other males were separately maintained under similar conditions in a second 5-m^3 tank but did not receive CPE (non-induced fish). All animals were handled in accordance with the guidelines for animal experiments described in Van Zutphen *et al.* (2001). The induction was performed via two intramuscular injections of CPE (0.25 and 2.5 mg/kg body weight) with an 8-h interval between doses, according to a protocol used in CODEVASF fish culture laboratory. At 12 h after the second injection, all males were individually captured, and the urogenital papilla were cleaned and dried with paper towels. The semen of each male was collected by abdominal pressure and placed in an individual graduated tube. Due to the low semen volume of the non-induced males, their sperm was collected in 1 ml syringes rather than graduated tubes. After collection, the semen was evaluated under a light microscope (×400 magnification) to verify a lack of spontaneous sperm motility that would indicate contamination by water, blood, feces or urine. None of the 22 semen samples presented contamination; all were of sufficient quality to be evaluated for both qualitative (visual aspect, pH, motility, viability and morphology) and quantitative (volume, concentration and number of spermatozoa per ejaculate) criteria.

The analysed parameters in the tambaqui semen were: (i) Semen volume (ml): directly observed in the graduated test tubes (for CPE-induced males) and 1 ml syringes (for non-induced males). (ii) Semen pH: measured by a digital pH meter (pH Master, Gulton do Brasil Ltda®). The semen pH was measured for each of the 11 CPE-induced males. For the non-induced males pH was measures only for four fish because the sperm volume of the other seven fish was too low. (iii) Sperm motility (%): 1 µl of each semen sample (n = 11 for CPE-induced fish and n = 11 for the non-induced fish) was diluted in 100 µl of 125 mM $NaHCO_3$ (229 mOsm/kg), placed onto a glass slide and evaluated under a light microscope (×400 magnification). The percentage of cell motility was estimated on a relative scale from 0 to 100%. (iv) Sperm concentration (number of spermatozoa per ml of semen): semen samples (n = 11 for CPE-induced fish and n = 11 for the non-induced fish) were diluted in a formol-citrate solution (2.9 g sodium citrate, 4 ml formaldehyde and 100 ml distilled water; pH 7.8) and analysed in a Neubauer haemocytometric chamber. (v) Number of spermatozoa per ejaculate: calculated by multiplying the values for seminal volume and sperm concentration. (vi) Sperm viability (%): assessed by eosin–nigrosin staining (5% eosin Y, 10% nigrosin, Sigma-Aldrich, Steinheim, Germany; pH = 6.9) at a 1:10 semen-to-stain ratio. After less than one minute, semen smears were prepared on glass slides, and 300 sperm cells were counted per sample (n = 11 for CPE-induced fish and n = 11 for the non-induced fish). Cells staining pink or red (indicating permeability to eosin) were considered dead while those remaining unstained were scored as living spermatozoa. (vii) Sperm morphological abnormalities (%): semen samples from each male (n = 11 for CPE-induced fish and n = 11 for the non-induced fish) were fixed in a formol–citrate solution. A 15 µl aliquot of each fixed sample was added to 0.5 µl of 3% Rose Bengal stain on a glass slide. The morphological examination was conducted under an optic microscope (×1000 magnification) with 300 spermatozoa examined per male. The following morphological abnormalities were evaluated: detached head, macrocephalic, microcephalic, degenerated head, proximal and distal cytoplasmatic drops, degenerated midpiece and bent, broken, coiled and short tail.

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Table 1 Semen characteristics of tambaqui *Colossoma macropomum* males (mean ± standard deviation) with and without hormonal induction with carp pituitary extract (CPE).

Parameters	CPE induced	Non-induced
Visual aspect	White, milky	White, milky
Semen pH[a]	8.0 ± 0.1^{b}	7.7 ± 0.3^{c}
Semen volume (ml)	12.6 ± 4.7^{b}	0.5 ± 0.6^{c}
Sperm concentration ($\times 10^9$/ml)	7.9 ± 2.4^{c}	15.5 ± 4.6^{b}
Number of spermatozoa per ejaculate ($\times 10^9$)	101.1 ± 56.1^{b}	9.7 ± 12.9^{c}
Sperm motility (%)	94.5 ± 6.9^{b}	89.1 ± 5.4^{b}
Sperm viability (%)	97.5 ± 1.6^{b}	96.8 ± 3.5^{b}
Normal spermatozoa (%)	85.0 ± 4.4^{b}	75.0 ± 6.2^{c}

[a]n = 11 for induced males and $n = 4$ for non-induced males. For all other parameters $n = 11$ for both groups.
[b,c]Mean values on the same line followed by different letters are significantly different by Mann–Whitney *U*-test ($p < 0.05$).

Table 2 Percentage of morphologically abnormal sperm (mean ± standard deviation) observed in the semen of tambaqui *Colossoma macropomum* with and without hormonal induction with carp pituitary extract (CPE).

Sperm abnormalities	CPE induced (%)	Non-induced (%)
Detached head	0.18 ± 0.27^{b}	1.60 ± 1.52^{a}
Macrocephalic	0.18 ± 0.22^{a}	0.09 ± 0.20^{a}
Microcephalic	0.01 ± 0.00^{a}	0.12 ± 0.21^{a}
Degenerated head	1.41 ± 0.66^{a}	1.80 ± 1.01^{a}
Proximal cytoplasmatic drops	0.26 ± 0.31^{a}	0.18 ± 0.22^{a}
Distal cytoplasmatic drops	0.01 ± 0.03^{a}	0.03 ± 0.10^{a}
Degenerated midpiece	2.10 ± 1.01^{a}	2.03 ± 1.19^{a}
Bent tail	6.77 ± 3.05^{b}	12.77 ± 3.62^{a}
Broken tail	1.06 ± 0.94^{a}	0.84 ± 0.90^{a}
Coiled tail	1.16 ± 0.59^{a}	2.59 ± 2.30^{a}
Short tail	1.90 ± 1.02^{a}	2.95 ± 4.39^{a}
Total sperm abnormalities	$\mathbf{15.04 \pm 0.86^{b}}$	$\mathbf{25.00 \pm 1.45^{a}}$

[a,b]Mean values in the same line followed by different letters are significantly different by Mann–Whitney *U*-test ($p < 0.05$).

Data analysis

The mean and standard deviation values were calculated and evaluated according to normality criteria and homogeneity of variance (SPSS version 15.0). As pH, volume, sperm concentration and various other semen parameters were not normally distributed, the Mann–Whitney *U*-test was applied to compare the CPE-induced and non-induced groups. The level of significance was set at 5% for all statistical tests.

Results

The values of the seminal parameters analysed for both CPE-induced and non-induced males are shown in Table 1. All evaluated parameters, except motility and viability, presented significant differences ($p < 0.05$) between CPE-induced and non-induced males. CPE-induced individuals had a larger number of spermatozoa and morphologically-normal cells, a higher semen volume and a higher pH value than the non-induced males ($p < 0.05$). On the other hand, the sperm concentration was higher ($p < 0.05$) in non-induced males.

The mean occurrence values of each type of sperm abnormality in CPE-induced and non-induced tambaqui males are shown in Table 2. Of all the abnormalities evaluated, only detached head and bent tail differed significantly ($p < 0.05$) between the CPE-induced and non-induced groups. Bent tail was the most frequently observed abnormality for both groups. The total number of spermatozoa with morphological abnormalities was significantly higher in the non-induced ($p < 0.05$) than in the CPE-induced males.

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Discussion

The present study shows that CPE influences some qualitative and quantitative aspects of tambaqui semen. The mean pH value was higher in the CPE-induced animals (8.0) than in the non-induced animals (7.7). Similarly, in a previous study of *Pleuronectes ferrugineus*, the use of a GnRH-analogue increased seminal pH (Clearwater & Crim, 1998). These increases may be related to the sperm maturation or testicular hydration induced by hormone application. The gonadotrophic hormones present in carp pituitary extract, particularly luteinizing hormone (LH), increase steroid production in the testis (e.g., 11-KT, testosterone and 17α,20β-DP or 20β-S); all of these hormones are involved in sperm maturation and testicular hydration (Schulz & Miura, 2002). Both ionic regulation and the release of seminal plasma are governed by the efferent ducts (Chowdhury & Joy, 2007), with bicarbonate ions directly involved in pH regulation (Alavi & Cosson, 2005). The testicular hydration triggered by hormonal induction putatively affects the secretion of these ions, thereby increasing pH. Nevertheless, other studies are required to confirm the effects of sex hormones on sperm maturation and seminal pH in tambaqui.

Hormonal induction caused a 25-fold increase in seminal volume and a 10-fold increase in the number of spermatozoa collected from tambaqui by abdominal pressure. As spermatozoa production is a long-term process, the increased number of sperm cells collected was possibly caused by the large amount of seminal fluid produced by the seminiferous tubules that washed out the sperm cells present in the lumen, rather than stimulation of spermatogenesis (Viveiros *et al.*, 2002). However, the sperm concentration was reduced by half in animals that received CPE, possibly as a consequence of testicular hydration. Similar results have been reported in other fish species such as piracanjuba *Brycon orbignyanus* (Bedore, 1999), yamú *Brycon amazonicus* (Pardo-Carrasco *et al.*, 2006) and African catfish *Clarias gariepinus* (Viveiros *et al.*, 2002), in which induction via CPE resulted in a two- to 10-fold increase in seminal volume and a two-fold increase in the number of spermatozoa per ejaculate. Hormonal induction was found to reduce sperm concentration by 50% in all three species, as seen in tambaqui.

The main goal of evaluating sperm morphology is to characterize the normal spermatozoa and classify the abnormal forms. Reports addressing the morphological alterations of spermatozoa in Brazilian fish are scarce and include only eight species: curimbatá *Prochilodus lineatus* (Kavamoto *et al.*, 1999), pacu *Piaractus mesopotamicus* (Streit Jr *et al.*, 2006), piavuçu *Leporinus macrocephalus* (Moraes *et al.*, 2004), silver catfish *Rhamdia quelen* (Bombardelli *et al.*, 2006), piracanjuba *Brycon orbignyanus* (Felizardo, 2008), dourado *Salminus maxillosus* (Streit Jr *et al.*, 2008a), piapara *Leporinus elongatus* (Streit Jr *et al.*, 2008b) and jaú *Zungaro jahu* (Drumond, 2008). A remarkable variation in the total number of morphological abnormalities is observed among these species, ranging from 8% in piracanjuba (Felizardo, 2008) to 54.7% in piapara (Streit Jr *et al.*, 2008b). In the present study, the non-induced specimens had a morphological abnormality rate of 25%, while the CPE-induced males had only 15% abnormality. This disparity was largely driven by two abnormality types, detached head and bent tail; both were more common in non-induced specimens, but the causes of this phenomenon are not yet understood. The hydration caused by hormonal induction with CPE increases the semen volume and facilitates both sperm collection through abdominal massage and the manipulation of the material during artificial breeding procedures in a laboratory, probably reducing structural damage. In non-induced males, semen sampling is hindered by the low level of testicular hydration, demanding a higher pressure on the abdominal region. This additional pressure and the difficulties of handling small seminal volumes may result in more structural damage and a higher incidence of sperm abnormalities in non-induced males.

In conclusion, hormonal induction with CPE is efficient for tambaqui males and positively influences some qualitative and quantitative properties of semen, such as volume and the number of spermatozoa per ejaculate. Additionally, CPE-induced fish have a lower frequency of sperm abnormalities, and sperm collection via gentle abdominal massage is facilitated.

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