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Sperm abnormalities in matrinxã *Brycon amazonicus* after hormonal treatment with carp pituitary extract and Ovaprim™

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Article

Cite this article: de Oliveira RGS *et al.* (2018) Sperm abnormalities in matrinxã *Brycon amazonicus* after hormonal treatment with carp pituitary extract and Ovaprim™. *Zygote* **26**: 279–285. doi: 10.1017/S0967199418000199

Received: 25 April 2017
Revised: 11 June 2018
Accepted: 18 June 2018
First published online: 17 September 2018

Keywords:
Fish; Inducing hormones; Microscope; Morphological changes; Spermatozoa.

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Summary

The aim of this study was to evaluate if hormonal treatment causes changes in the morphology of matrinxã *Brycon amazonicus* spermatozoa. Twelve males were randomized into three treatment groups: 1, Ovaprim™ (0.5 ml/kg); 2, carp pituitary extract (CPE; 2.0 mg/kg); and 3, NaCl solution 0.9% – control group (0.5 ml/kg); with four replicates. Morphological sperm analysis was performed using an optical and scanning electron microscope. The percentage of normal spermatozoa (49.6 ± 4.6% to 60.8 ± 2.8%), with primary (26.1 ± 6.4% to 45.3 ± 4.5%) and secondary abnormalities (4.4 ± 2.9% to 13.9 ± 3.5%) did not differ significantly between treatment groups. There were no significant differences between treatments in relation to the primary abnormalities found in the head (10.5 ± 3.8% to 25.5 ± 6.3%), the midpiece (0.1 ± 0.1% to 0.2 ± 0.2%) and in the flagellum (18.9 ± 3.8% to 15.5 ± 3.1%), as well as in the secondary abnormalities, located in the head (0.8 ± 0.4% to 7.8 ± 4.6%) and in the flagellum (2.2 ± 1.1% to 6.1 ± 1.5%). When each abnormality was evaluated individually, only the percentage of degenerated head was higher in the CPE-induced group (24.9 ± 5.9%) than the control group (7.2 ± 3.1%). We concluded that the use of pituitary extract of carp or Ovaprim™ under the conditions of this experiment does not influence the percentage of sperm abnormalities in *B. amazonicus*.

Introduction

The matrinxã *Brycon amazonicus* belongs to the Characidae family and can be found in the Amazon River and in some Brazilian tributaries (Lima, 2003). It is a rheophilic fish, with an annual reproductive cycle and total spawning. It presents with good zootechnical performance and acceptance in the consumer market (Ninhaus-Silveira *et al.,* 2006). As it does not reproduce spontaneously in captivity, hormone induction techniques using pituitary fish extract and synthetic inducers have been used to aid artificial reproduction in these fish, and mainly in studies that have focused on oocyte quality (Ramos *et al.*, 1997; Ramos, 2000; Romagosa *et al.*, 2001; Pardo-Carrasco *et al.*, 2002, 2006; Zaniboni-Filho & Weingartner, 2007; De Alexandre *et al.*, 2010; Nakaghi *et al.*, 2014; Bashiyo-Silva *et al.*, 2016).

The pituitary extracts of mature fish used for ovulation induction and spermiation of rheophilic species since the 1930s have more recently been replaced by synthetic analogues of gonadotrophin releasing hormone – GnRHa (Zohar & Mylonas, 2001), such as Ovaprim™, which is a synthetic product of salmon GnRHa with domperidone, a dopaminergic inhibitor, successfully employed in spermiation of salmonids, cyprinids (Mousavi *et al.*, 2011; Cejko *et al.*, 2013) and some Brazilian native species (Viveiros *et al.*, 2013).

Although female gametes have been afforded greater emphasis in breeding studies, it is also necessary to characterize semen samples to verify the potential for fertilizing breeding fish. This task includes the evaluation of any morphological abnormalities in spermatozoa. However, studies on sperm morphology in Brazilian native fish have so far been limited (Kavamoto *et al.*, 1999; Streit Jr *et al.*, 2008; Murgas *et al.*, 2011; Maria *et al.*, 2012). The increase in sperm diseases has led to reduced sperm motility and vigour (Lahnsteiner *et al.*, 1998). As a result, there has been a decrease in fertilization capacity (Rurangwa *et al.*, 2004). Determination of the acceptable percentage change in these parameters is important to gauge semen quality. This information can aid in the selection of hormone therapies for fish artificial reproduction, due the importance of morphological alterations in spermatozoa after hormone treatment (Kavamoto *et al.*, 1999; Moraes *et al.*, 2004; Streit Jr *et al.*, 2012). Therefore, this study aimed to

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investigate if hormone treatment with CPE or Ovaprim™ affected the frequency of sperm abnormalities in *B. amazonicus*.

Materials and methods

Four-year-old males from a wild stock belonging to the Federal University of Amazonas (UFAM) Experimental Farm Aquaculture Station, Manaus – Amazonas – Brazil (02°38′56.1″S; 060°03′14.7″W) were used in the study. The fish were kept in earthen ponds (550 m^2) and fed twice daily to their apparent satiation with commercial fish feed that contained 32% crude protein.

Twelve specimens were selected for hormone treatment in December 2014, transported to Aquaculture Station reproduction area and placed in two circular glass fibre tanks with a capacity of 3000 litres each. Water was constantly renewed and aerated, and kept at an average temperature of 27.7 ± 1.0ºC and pH 7.9 ± 0.2.

The specimens were identified with transponders (AnimallTAG) and coloured threads attached to the dorsal fin. They were randomized in two treatment groups (inducing hormones) and a control group, with four replicates each. Males received single dose injections intramuscularly in the base of the dorsal fin, according to treatment protocols: (1) Ovaprim™ – 0.5 ml/kg (Syndel Laboratories Ltd, Canada); (2) carp pituitary extract (CPE) – 2.0 mg/kg (Danúbio Aquaculture Ltd, Brazil); and (3) 0.9% NaCl solution (control) – 0.5 ml/kg. Body weight was 1.159 ± 0.15 kg and dosages were calculated per kg body weight of the breeding fish. The animals had not been subjected previously to hormone therapy.

Semen collection occurred 12 h after injection. Each specimen was taken from the tank, placed in a container containing Eugenol™ 50 mg/l solution (Vidal *et al.*, 2007) to reduce stress during this procedure. Semen samples from each specimen were fixed with buffered saline formaldehyde at a ratio of 10:990 (semen:fixative). Two wet preparations were obtained from each animal with a 10-µl aliquot of the fixed semen, stained with 3% Rose Bengal (Streit Jr *et al.*, 2004), placed on a slide and covered with a cover slip.

Images were obtained using an optical microscope (Axiophot2, Zeiss) with an immersion objective, with a digital camera (AxioCam MRC, Zeiss) attached to the microscope and to a computer, and were scanned using Zen Lite 2012 software using Thematic Laboratory of Optical and Electronic Microscopy (LTMOE).

Sperm cells were counted over a minimum of 200 spermatozoa from each male, classifying them into either normal cells or abnormal cells having only one anomaly. Primary and secondary abnormalities were analyzed according to the fish classification model proposed by Miliorini *et al.* (2011) and the data were recorded as percentage of abnormal sperm cells.

Fixed samples of two males from each hormonal treatment group as well as from the control group were used to obtain the electromicrographs in scanning electron microscopy (SEM). The samples were washed in 0.1 M sodium cacodylate buffer and kept refrigerated at 5°C until dehydration, carried out using increasing concentrations of ethanol (30–95%) for 10 min each and in 100% ethanol, twice, for 10 min each. The coverslips were subjected to the Critical Point Dry Cleaner with liquid CO_2, and later assembled into stubs, metallized and electromicrographed on a scanning electron microscope LEO 435VP, Zeiss, in the LTMOE laboratory.

Statistical analysis

The experiment was conducted using a completely randomized design with three treatments (two hormonal treatments groups and one control group without hormonal treatment) and four replicates (each male was considered as one replicate). Data were expressed as mean ± standard error (mean ± SE). To determine statistical significance ($P < 0.05$), analysis of variance (ANOVA) and Holm–Sidak Test were applied to the morphological variables. The analyses were carried out using the SigmaStat 3.5 program.

Results

The percentage of normal spermatozoa was not significantly different between the treatment groups. No significant differences were detected between treatment groups in relation to the total primary abnormalities (Fig. 1) and secondary abnormalities (Fig. 2 and Table 1).

There were no significant differences between the treatment groups in relation to the primary abnormalities found in the head, the midpiece and the flagellum. Also, there were no significant differences between the treatment groups for secondary abnormalities located in the head and in the flagellum (Table 2)

When the sperm alterations were evaluated individually, only the percentage of degenerated head abnormalities presented a significant difference among the spermatozoa in the group treated with CPE compared with those in the control group (Figs 3 and 4 and Table 3).

Discussion

Several authors have related the morphological alterations of fish spermatozoa to the use of hormone therapies. Kavamoto *et al.* (1999) found that spermatozoa of *Prochilodus lineatus* presented abnormalities after being treatment with human chorionic gonadotropin. Moraes *et al.* (2004) compared the effects of pituitary extracts of carp, chicken and rabbit, and found that *Cyprinus carpio* and *Leporinus macrocephalus* had high secondary sperm abnormalities when treated with rabbit pituitary extract. In *Pseudoplatystoma reticulatum*, there was a decrease in the occurrence of primary abnormalities after treating males with CPE (Streit Jr *et al.*, 2012).

Colégio Brasileiro De Reprodução Animal (The Brazilian College of Animal Reproduction – CBRA) (2013), an organization that regularizes the semen quality criteria of breeding animals, established that abnormal sperm percentages above 30% for cattle, equine and swine, 20% for sheep and goats, and 10% for poultry, compromise artificial insemination. Although it has not been advocated by CBRA, it is estimated that the percentages of acceptable sperm defects are higher for fish because their sperm concentration is higher than the terrestrial species (Streit Jr *et al.*, 2012). According to Miliorini *et al.* (2011), it is probable that the critical percentage of sperm abnormalities of migratory fishes with external fertilization and artificial reproduction is about 50%, as artificial fertilization involves a large proportion of spermatozoa per oocyte in a controlled environment. In this study, although the percentage of abnormal sperm cells was elevated (39–50.4%), other species also had relatively high rates of deformities, varying from 32 to 65% (Streit Jr, 2002; Moraes *et al.*, 2004; Bombardelli *et al.*, 2006; Streit Jr *et al.*, 2008; Garcia *et al.*, 2015).

Sperm abnormalities may limit sperm motility and vigour, interfering with fertilization rates (Cosson *et al.*, 1999) and, according to Freneau (2011), the primary abnormalities have a greater effect on fertilization. However, the high percentage of primary abnormalities observed may be insignificant, considering

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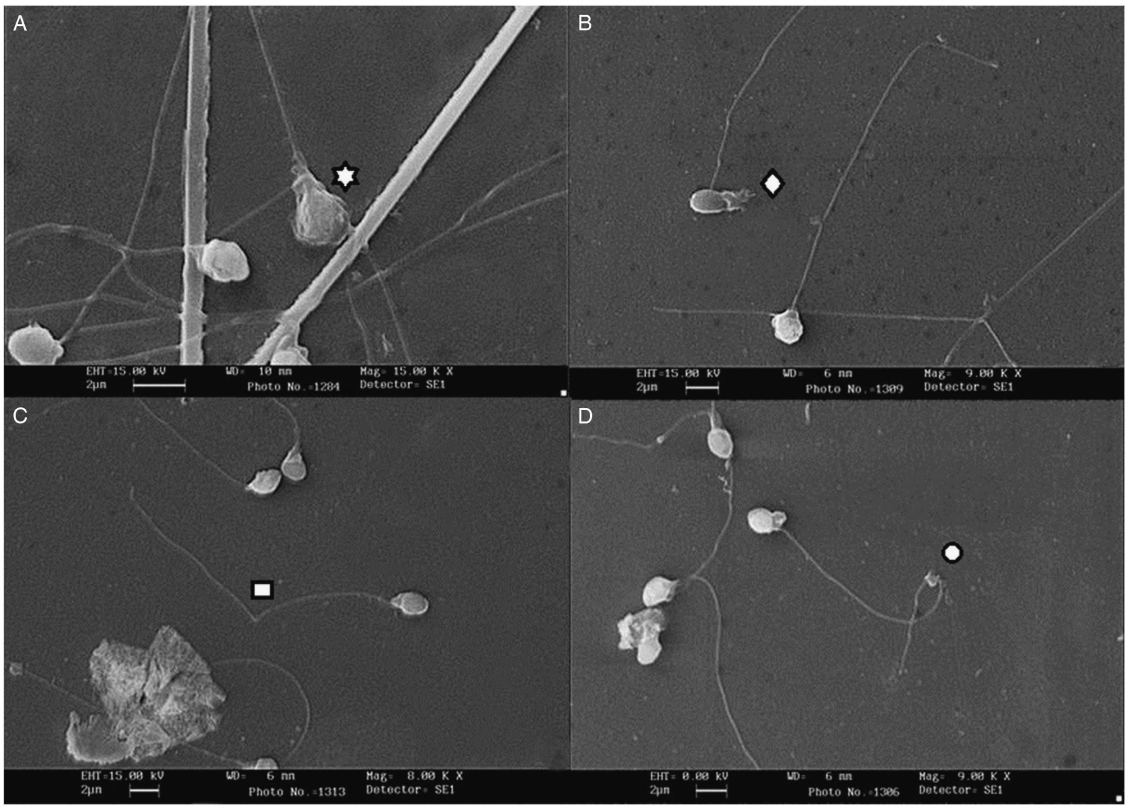

Figure 1. Scanning electromicrographs of *B. amazonicus* spermatozoa. (*A*) Degenerated head. (*B*) Degenerated midpiece. (*C*) Fractured tail. (*D*) Strongly curled tail.

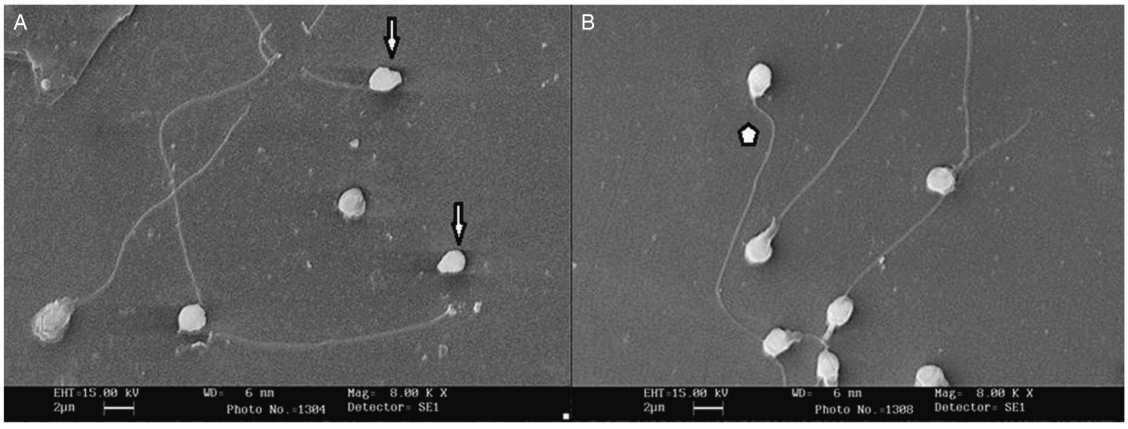

Figure 2. Scanning electromicrographs of *B. amazonicus* spermatozoa. (*A*) Isolated heads (*B*) Folded tail.

that the species presents a high sperm concentration. Fertilization tests are required to confirm this hypothesis.

The percentages of spermatozoa, observed with primary and secondary abnormalities, were similar to those found for other CPE-induced species. In *Rhamdia quelen* it was verified that 5.8% had primary alterations and 26.3% had secondary alterations (Bombardelli *et al.*, 2006). In *Piaractus mesopotamicus*, 12.4% presented primary and 24.6% presented secondary alterations (Streit Jr, 2002). Moraes *et al.* (2004) reported the following percentages of abnormal sperms: *Prochilodus lineatus* 40.2%, *Leporinus macrocephalus* 49% and *Cyprinus carpio* 37.4%. In *Salminus maxillosus*, 56.7% were normal before treatment and 48% were normal after this (Streit Jr *et al.*, 2008). Maria *et al.* (2012) reported 85% of normal spermatozoa after treatment with CPE and 75% normal in the non-hormone-treatment group for *Colossoma macropomum*. Martins *et al.* (2017) in a study on the effects of CPE, Ovopel (GnRHa and metoclopramide) and control group, showed no difference between groups for *C. macropomum*. Garcia *et al.* (2015) reported no significant changes for *Brycon insignis* comparing groups before and after treatment with CPE or GnRH analogues. Possibly, the defects observed are related to factors other than spawning inducing hormones.

There was no observation in the samples of tails with proximal or distal cytoplasmic droplets. The same finding was observed by Moraes *et al.* (2004) in *Prochilodus lineatus*, *Leporinus macrocephalus*, *Cyprinus carpio* and by Streit Jr *et al.* (2008) in *S. maxillosus*.

According to Hafez & Hafez (2004) and Herman *et al.* (1994), the secondary abnormalities may be related to the preparation of

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Table 1. Percentage of normal spermatozoa, primary and secondary abnormalities (mean ± SE) observed in *B. amazonicus* induced with Ovaprim™, carp pituitary extract (CPE) and control group

		Abnormalities	
Treatment	Normal	Primary	Secondary
Ovaprim™	60.8 ± 2.8	34.7 ± 2.1	4.4 ± 2.9
CPE	49.6 ± 4.6	45.3 ± 4.5	5.1 ± 1.5
Control	59.9 ± 3.1	26.1 ± 6.4	13.9 ± 3.5

Table 2. Percentage of abnormalities in the head, midpiece and flagellum (primary) and in the head and flagellum (secondary) verified in *B. amazonicus* induced with Ovaprim™, carp pituitary extract (CPE) and control group

	Primary abnormalities			Secondary abnormalities	
Treatment	Head	Midpiece	Flagellum	Head	Flagellum
Ovaprim™	18.3 ± 3.9	0.1 ± 0.1	16.4 ± 3.5	0.8 ± 0.4	3.6 ± 2.6
CPE	25.5 ± 6.3	0.2 ± 0.2	18.9 ± 3.8	2.9 ± 1.6	2.2 ± 1.1
Control	10.5 ± 3.8	0.2 ± 0.2	15.5 ± 3.1	7.8 ± 4.6	6.1 ± 1.5

the smears and primary abnormalities are related to failures during spermatogenesis in mammals. These same authors related the origin of sperm abnormalities with nutritional deficiency, age, consanguinity and males diseases, besides the ambient temperature and problems in the spermatic duct. Changes in fish sperm can be caused by xenobiotics, genetic mutation or ageing, or by conservation protocols (Fauvel *et al.*, 2010). The origin of the sperm abnormalities is not fully understood, and it is only possible to assume the same origin (Araújo *et al.*, 2014; Streit Jr *et al.*, 2008; Streit Jr *et al.*, 2006).

Conversely, the collection of semen by extrusion can force the spermatozoa to exit at different stages of development. In this case, some sperm cells may still be immature, and therefore unviable (Asturiano *et al.*, 2006). According to Maria *et al.* (2012), structural damage in spermatozoa can be attributed to increased abdominal pressure exerted at the time of semen extrusion.

The quality of fish gametes depends on the appropriate hormone environment during development, but this may be disturbed by stress (Kime & Nash, 1999). Stress increases the level of cortisol and decreases steroid testosterone and ketotestosterone levels (Rurangwa *et al.*, 2004). These hormones are involved in sperm maturation and testicular hydration (Schulz & Miura, 2002). Stress, in some species, induced changes in plasma osmolarity, which in turn affected sperm quality, reducing sperm motility after activation and also significantly decreased sperm

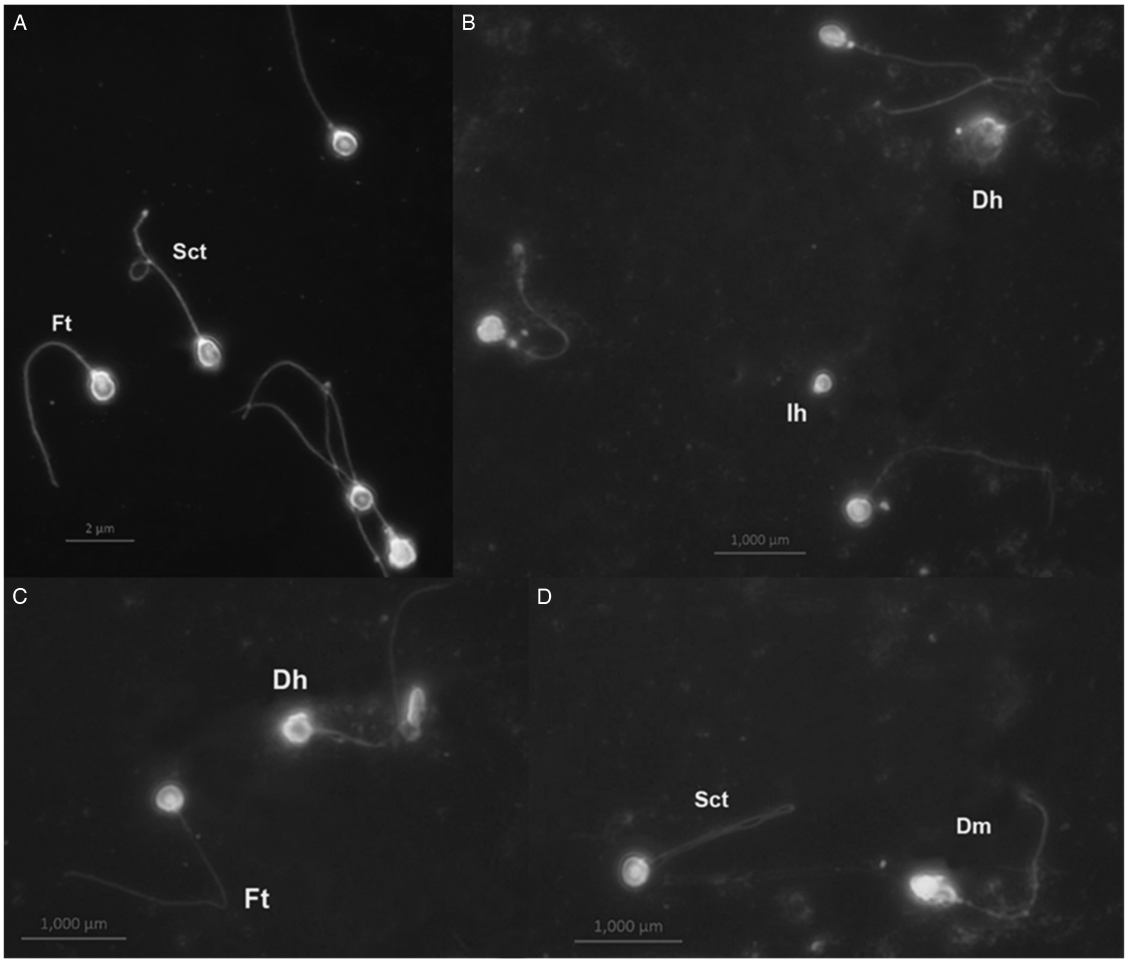

Figure 3. Phase contrast optical microscopy photographs of *B. amazonicus* spermatozoa. (*A*) Sct: Strongly curled tail, Ft: Folded tail. (*B*) Dh: Degenerated head, Ih: Isolated head. (*C*) Dh: Degenerated head, Ft: Fractured tail. (*D*) Sct: Strongly curled tail, Dm: Degenerated midpiece.

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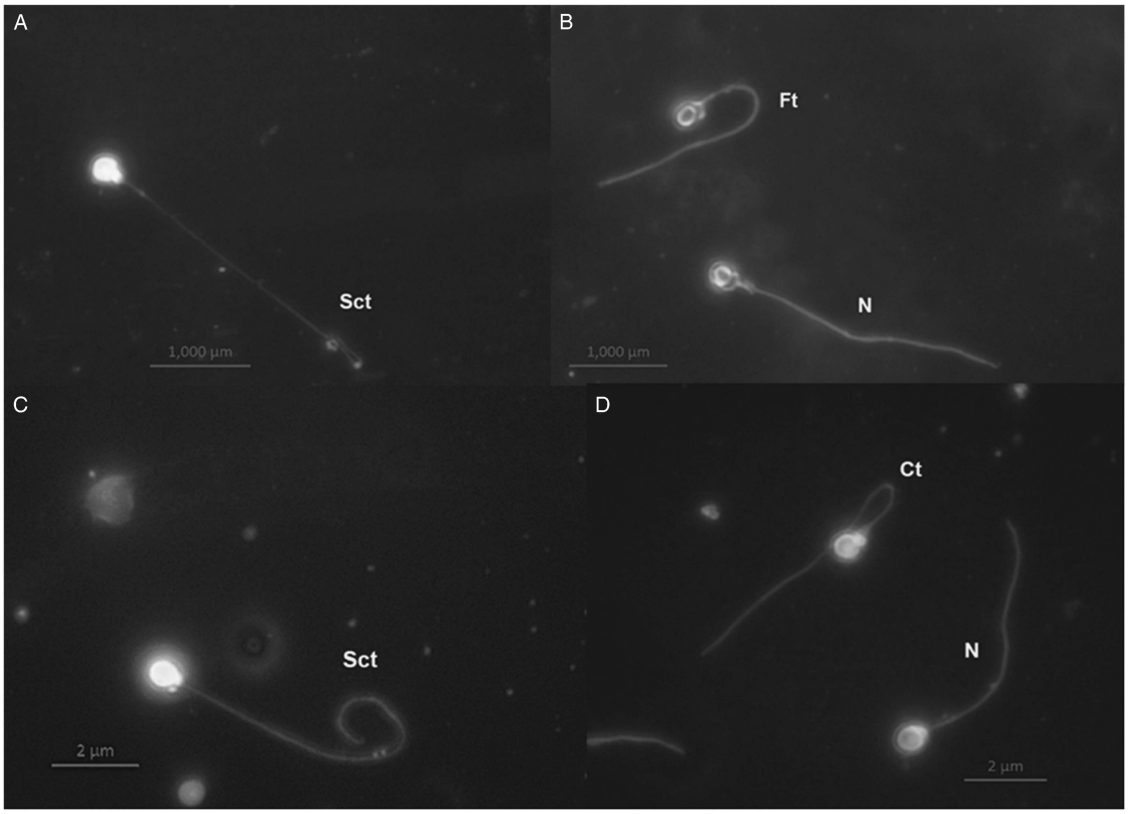

Figure 4. Phase contrast optical microscopy photographs of *B. amazonicus* spermatozoa. (*A*) Sct: Strongly curled tail. (*B*) Ft: Folded tail, N: Normal. (*C*) Sct: Strongly curled tail. (*D*) Ct: Coiled tail; N: Normal.

Table 3. Percentage of sperm abnormalities (mean ± SE) observed in *B. amazonicus* semen after induction with Ovaprim™, carp pituitary extract (CPE) and control group

Sperm abnormalities		Ovaprim™	CPE	Control
Primary	Macrocephaly	3.8 ± 2.9	0.6 ± 0.5	3.3 ± 1.5
	Microcephaly	0.2 ± 0.2	0.0	0.0
	Degenerated head	14.4 ± 2.2^{ab}	24.9 ± 5.9^{a}	7.2 ± 3.1^{b}
	Degenerated midpiece	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2
	Fractured tail	8.4 ± 1.0	12.7 ± 2.8	9.2 ± 2.0
	Strongly curled tail	5.8 ± 2.9	5.9 ± 1.3	4.6 ± 1.6
	Degenerated tail	0.9 ± 0.6	0.0	0.0
	Tail stump	1.3 ± 0.4	1.0 ± 0.4	1.7 ± 0.5
Secondary	Isolated head	0.8 ± 0.4	2.9 ± 1.6	7.8 ± 4.7
	Folded tail	3.6 ± 2.6	2.2 ± 1.1	6.1 ± 1.5

*Different superscript letters on the same line indicate a significant difference ($P < 0.05$).

count compared with non-stressed controls (reviewed in Rurangwa *et al.*, 2004). According to Donaldson *et al.* (2000), artificial reproductive management is a stressful factor and may influence spermiogenesis. Bromage (1995) considers that stress caused by capture and manipulation in the induction tank may influence reproductive rates. Matrinxã is an extremely aggressive fish during capture and in the process of artificial propagation, however detailed studies are lacking to infer that the sperm deformities found could be attributed to stress during handling.

Considering the results of the present study, we can conclude that the hormonal therapy of matrinxã can be performed with both Ovaprim™ and CPE, without influencing the percentage of sperm abnormalities in *B. amazonicus*.

Acknowledgements. The author thanks Dr Elizabeth Gusmão Affonso for acting as an intermediary for Thematic Laboratory of Optical and Electronic Microscopy (LTMOE/INPA).

Financial support. This work was supported by the project grant no. 1271/2012 – Fundação de Amparo à Pesquisa no Amazonas (FAPEAM), and protocol no. 01.09.0472.00 – Financiadora de Estudos e Projetos (FINEP)/Projeto Desenvolvimento da Aquicultura e Recursos Pesqueiros da Amazônia (DARPA).

Ethics statement. The authors assert that all procedures contributing to this work comply with the Ethical Principles of Animal Experimentation, adopted by the Brazilian College of Animal Experimentation (COBEA), and were approved by the Ethics Committee on Animal Use (CEUA) – Federal University of Amazonas (protocol no. 019/2014).

Conflicts of interest. There are no conflicts of interest.

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https://doi.org/10.1017/S0967199418000199 Published online by Cambridge University Press