Theriogenology 90 (2017) 284–288

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Sperm quality and its freezing ability throughout the spawning season in *Prochilodus lineatus* and *Brycon orbignyanus*

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ARTICLE INFO

Article history:
Received 5 August 2016
Received in revised form
11 December 2016
Accepted 12 December 2016
Available online 18 December 2016

Keywords:
CASA
Cryopreservation
Motility
Neotropical fish
Semen

ABSTRACT

The aim of this study was to determine fresh and frozen sperm quality evaluated over two spawning seasons (2013–2014; 2014–2015) in *Prochilodus lineatus* and *Brycon orbignyanus*. The spawning seasons were divided into two sampling periods: November to December and January to February. Males were hand-stripped after carp pituitary treatment. Fresh sperm motility rate, velocities (curvilinear = VCL; straight-line = VSL; average path = VAP), and the beat cross frequency (BCF) were determined using a Computer-Assisted Sperm Analyzer (CASA). Sperm of each species was frozen using methyl glycol as cryoprotectant and a glucose solution for *P. lineatus* or a NaCl solution for *B. orbignyanus* as extender. Diluted sperm was loaded into 0.25 mL straws, frozen in a nitrogen vapor vessel (dry shipper) and stored in a liquid nitrogen vessel. Six months later, straws were thawed in a water bath at 60 °C for 3 s and sperm quality was determined, as described for fresh sperm. No significant difference was observed for any of the fresh and frozen sperm features between the two spawning seasons or the two sampling periods in *P. lineatus* and in *B. orbignyanus*. Motility rate and velocities, but not BCF, was always higher in fresh sperm when compared with frozen sperm. Comparing both species, higher motility in frozen sperm and higher VCL and VAP in both fresh and frozen sperm were observed for *P. lineatus*, while higher VSL in fresh sperm and higher BCF in both fresh and frozen sperm were observed for *B. orbignyanus*. Sperm quality and its freezing ability of both species were sustained over the spawning season and thus fish farmers can reproduce these species and freeze their sperm in any time throughout the spawning season. *P. lineatus* sperm is more resistant to the cryopreservation process than *B. orbignyanus*.

1. Introduction

The streaked prochilod *Prochilodus lineatus* (VALENCIENNES, 1836) and the piracanjuba *Brycon orbignyanus* (VALENCIENNES, 1849) are fish species from the order Characiformes and are native from South America. These species have a great potential for Brazilian freshwater aquaculture and have been used in restocking programs through artificial propagation for conservation and management [1]. During the rainy season, these species migrate to find clean water and spawn. This migratory behavior is known as *piracema* and occurs when the environment is appropriate to stimulate fish reproduction [2]. Most Brazilian rheophilic fish exhibit annually migration and spawn during the rainy months, in flooding areas and high temperatures [3].

Spermatogenesis in fish is regulated by the reproductive endocrine system (endogenous factors) and environmental (exogenous factors such as thermoperiod or photoperiod) stimulus [4]. The physiological changes that occur regulates gonadal development and spermiation [5]. Thus, it is clear that certain environmental changes define the period and the reproductive success in these species. These changes may result in different fish sperm maturation. In addition, there are also other factors that may decrease sperm quality and even change their freezing ability such as the aging of mature sperm inside the testis and early spermiation in the tank prior to manual collection in captivity. Changes on sperm qualitative and/or quantitative characteristics throughout the spawning season in species with annual spawning cycles have been reported in haddock *Melanogrammus aeglefinus* [6], Atlantic halibut *Hippoglossus hippoglossus* L. [7], common barbel *Barbus barbus* [8], Atlantic cod *Gadus morhua* L. [9] and Caspian roach *Rutilus rutilus caspicus* [5].

Knowledge on the reproductive performance in *Prochilodus lineatus* and *Brycon orbignyanus* over the spawning season is scarce

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http://dx.doi.org/10.1016/j.theriogenology.2016.12.011
0093-691X/

and fish farmers often use excess of sperm collected randomly over the spawning season. For the rational use of male broodfish, it is imperative to know the sperm production capacity during the spawning season [10], so that fish farmers can collect the sperm when the quality is maximum. The aim of this study was to compare motility rate, velocities, beat cross frequency and freezing ability of *P. lineatus* and *B. orbignyanus* sperm collected throughout two spawning seasons.

2. Materials and methods

All fish were handled following the guidelines for animal experimentation described by Van Zutphen et al. [11].

2.1. Fish handling and sperm collection

Prochilodus lineatus (107 males, 1.2 ± 0.5 kg of body weight) and *Brycon orbignyanus* (34 males, 1.3 ± 0.5 kg of body weight), with at least three years of age, were selected during spawning season 2013–2014 and 2014–2015 at the Fish Culture Station of the Minas Gerais Power Company (CEMIG) in the city of Itutinga (21°17′36″S, 44°37′02″W), Minas Gerais State, Brazil. Males with detectable traces of sperm released under soft abdominal pressure received intramuscular doses of carp pituitary extract (cPE; Argent Chemical Laboratories, Redmond, Washington, USA). *P. lineatus* males received two doses at 0.4 and 4 mg/kg BW in a 12 h interval and *B. orbignyanus* males received a single dose at 1 mg/kg BW, which is the routine method currently used to induce spermiation in the Fish Culture Station. The urogenital papilla was dried and approximately 2 mL of *P. lineatus* sperm and approximately 10 mL of *B. orbignyanus* sperm were gently hand-stripped directly into test tubes. The difference on the amount of stripped sperm was related to the species characteristics as *P. lineatus* produces a lower sperm volume compared with *B. orbignyanus* [12] and we did not want to force stripping of *P. lineatus* males and end up with blood contamination. Sperm collection was carried out at room temperature (25–27 °C). Contamination of sperm with water, blood, urine or feces was carefully avoided. Sperm was collected during the following dates: (a) season 2013–2014: Nov 21, Dec 05, Dec 12, Dec 19, Jan 07, Jan 14, Jan 23 and Feb 06; and (b) season 2014–2015: Dec 11, Dec 18, Jan 08, Jan 15 and Jan 29. The spawning seasons were divided by Christmas holidays and were referred to as: first sampling period (before Christmas), November and December, with 25 *P. lineatus* and 7 *B. orbignyanus* males for season 2013–2014 and 26 *P. lineatus* and 8 *B. orbignyanus* males for season 2014–2015, and the second sampling period (after Christmas), January and February, with 18 *P. lineatus* and 7 *B. orbignyanus* males for season 2013–2014 and 38 *P. lineatus* and 12 *B. orbignyanus* males for season 2014–2015.

Immediately after collection, each sperm sample was subjectively evaluated for motility rate after activation in 100 mOsm/kg NaCl solution, using a light microscope (Eclipse E200, Nikon, Tokyo, Japan) at magnification: X 200. All samples possessed motility above 90%. Sperm concentration was determined using a Neubauer-type hemacytometer chamber (Boeco, Hamburg, Germany). Tubes containing sperm were placed in a cooler (9–11 °C) containing dry ice foam (Polar Technics CRI Ltd, São Paulo, Brazil) and transported by car in the cooler from CEMIG to the Laboratory of Semen Technology at Federal University of Lavras, in the city of Lavras (~60 km), where sperm analysis took place (see below).

2.2. Fresh sperm analysis

After ~3 h from collection, sperm features were estimated using the Computer-Assisted Sperm Analysis (CASA) system according to the methodology previously validated by our laboratory [13]. Briefly, motility was triggered in a 100 mOsm/kg NaCl solution at approximately 27 °C directly in a Makler™ counting chamber (Sefi-Medical Instruments Ltd, Haifa, Israel) placed under a phase contrast microscope (Eclipse E200, Nikon, Tokyo, Japan), objective magnification: X 10, ocular magnification: X 100, with a green filter and phase one position. The microscope was connected to a video camera (Basler Vision Technologies™ A602FC, Ahrensburg, Germany) generating 100 images/s; video recording started 10 s post-activation. Each image was analyzed using the standard settings for fish by Sperm Class Analyzer™ software (SCA™ 2010, Microptics, S.L. Version 5.1, Barcelona, Spain). Motility rate, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), and the beat cross frequency (BCF) were considered for analysis. For determination of these parameters, an average of 506 *P. lineatus* sperm tracks and 533 *B. orbignyanus* sperm tracks for each straw was followed throughout the recorded video images from which sperm trajectories were evaluated. Sperm was evaluated at a final dilution ratio (v/v) of 1 sperm: 100 activating agent.

2.3. Sperm cryopreservation and post-thaw sperm analysis

Sperm of each species was frozen according to the standard method previously developed in our laboratory throughout the years. *P. lineatus* sperm was frozen in the laboratory ~3 h after collection and transportation according to our previous results [14]. The freezing medium was composed of methyl glycol [CH3O (CH2) 2OH] as cryoprotectant and 325 mOsm/kg glucose solution [15–17]. *B. orbignyanus* sperm, on the other hand, was frozen soon after collection, in the fish farm. The freezing medium was composed of methyl glycol [CH3O (CH2)2OH] as cryoprotectant and 325 mOsm/kg NaCl solution [18]. Both glucose and NaCl solution were adjusted to pH of 7.6. All chemicals were purchased from Vetec Química Fina Ltda (Duque de Caxias, RJ, Brazil). Sperm sample of each male was diluted in the freezing medium to a ratio of 1 sperm: 8 extender: 1 methyl glycol [19]. Sperm was then drawn into unsealed 0.25 mL straws, in duplicate and frozen in nitrogen vapor (dry vapor shipper, Cryoport Systems, Brea, CA, USA) at approximately −170 °C. Final dilution, loading and freezing (equilibration time) took exactly 15 min for *P. lineatus* and 10 min for *B. orbignyanus*. Within 24 h, all straws were transferred to a liquid nitrogen vessel (MVE XC 34/18, New Prague, MN, USA) for storage.

Cryopreserved sperm was first diluted 1:10 in the freezing medium, frozen, thawed and then further diluted 1:100 with the activating agents. Approximately six months later, straws were thawed in a 60 °C water bath (Water-bath MA 127, Marconi, Piracicaba, Brazil) for 3 s and post-thaw sperm motility, velocities and BCF were estimated, as described for fresh sperm. An average of 616 *P. lineatus* sperm tracks and 417 *B. orbignyanus* sperm tracks for each straw was evaluated.

2.4. Statistical analysis

Data were expressed as average ± standard deviation (SD). The statistical analyses were conducted using the R Development Core Team program version 2.11.0 (R Development Core Team, 2014). Data were tested for normal distribution using test Shapiro Wilk and for significant differences using analysis of multiple variance (MR-MANOVA), ANOVA. The level of significance for all statistical tests was set to 5% ($P < 0.05$).

3. Results

The observed mean sperm concentration was of 27.6×10^9 ±