Zygote **24** (October), pp. 662–667. © Cambridge University Press 2015
doi:10.1017/S0967199415000635 First Published Online 18 December 2015

Sperm dilution ratio affects post-thaw motility rate and velocity of *Prochilodus lineatus* (Characiformes) sperm

Ana T.M. Viveiros[1] *and Marcelo C. Leal*[2,3]
Department of Animal Science, Federal University of Lavras, Minas Gerais, Brazil

Date submitted: 20.06.2015. Date revised: 16.09.2015. Date accepted: 17.09.2015

Summary

There is a lack of standardization in sperm cryopreservation of aquatic organisms and, thus, a necessity of more accurate investigations in all steps of this process. In this study, the effects of sperm dilution ratio on post-thaw sperm quality of *Prochilodus lineatus* were evaluated. Sperm was diluted in a standard freezing medium (glucose and methyl glycol) at four different ratios (sperm to final volume = 1:5, 1:10, 1:50 or 1:100), frozen in a nitrogen vapour vessel at –170°C and then stored in liquid nitrogen vessel at –196°C. Post-thaw motility rate and velocities (curvilinear = VCL; average path = VAP; straight line = VSL) were determined using a Computer-Assisted Sperm Analyzer (CASA) at 10 and 40 s post-activation. The highest motility rates were observed when sperm was frozen at a ratio of 1:5 (76%) and 1:10 (75%). The highest VCL (225 μm/s) and VAP (203 μm/s) were observed at a ratio of 1:10, while VSL was similar among samples frozen at 1:5, 1:10 and 1:50 (97–124 μm/s). When those parameters were evaluated again 30 s later, motility decreased significantly in samples frozen at a ratio of 1:5 (57%) and 1:10 (61%), while velocities decreased significantly in all samples regardless of dilution ratio (75–85 μm/s of VCL, 38–53 μm/s of VAP and 25–39 μm/s of VSL). *P. lineatus* sperm should be frozen at a ratio of 1:10, where both the number of loaded sperm per straw and the post-thaw quality are maximized.

Keywords: CASA, Cryopreservation, Fish, Neotropical, Semen

Introduction

The streaked prochilod *Prochilodus lineatus* (Prochilodontidae; Characiformes) is a native fish species to South America, accounting for up to 50–90% of the total fish biomass in the lower stretches and in the flood plain lagoons of the Paraná River basin (Cordiviola de Yuan, 1992). This species has great potential for aquaculture and has been used in restocking programs through artificial propagation (Carolsfeld *et al.*, 2003). During the spawning season (October to February), this species migrate to spawning sites in a phenomenon known as 'piracema'. As artificial reproduction methods are well established and prolificacy is high, *P. lineatus* has been used as a model species for research in a number of studies addressing genetic diversity, health, nutrition and reproduction (Orfão *et al.*, 2010).

The cryopreservation of fish sperm provides a tool by which reproduction is optimized, improving breeding and fish conservation programs (Kopeika & Kopeika, 2008). Sperm cryopreservation facilitates procedures for artificial reproduction, as, with viable sperm stored in liquid nitrogen, it is necessary to induce spawning and collect gametes only from females. Cryopreserved sperm may be kept in germplasm banks for an indefinite period, which allows the establishment of breeding programmes, eliminates the problem of asynchronous reproductive activity between males and females, and enables maintenance

[1]All correspondence to: Ana T.M. Viveiros. Animal Science Department, Federal University of Lavras, P.O. Box 3037, Lavras, MG, 37200-000, Brazil. Tel: +55 35 38291223. Fax: +55 35 38291231. E-mail address: anatmviveiros@outlook.com
[2]Department of Animal Science, Federal University of Lavras, Minas Gerais, Brazil.
[3]Present address: Laboratory of Biotechnology Applied to Fish Reproduction, Post-Graduate Program in Aquaculture, Nilton Lins University, Manaus, AM, 69058–030, Brazil.

of fewer male broodfish (Viveiros & Godinho, 2009). The cryopreservation technology can be divided in different steps namely: initial sperm quality, freezing medium (extender plus a cryoprotectant agent), dilution ratio, cooling, thawing, and evaluation of post-thaw sperm quality. For most reported studies, the dilution ratio of sperm to final volume ranged from 1:2 to 1:10, including Neotropical fish species (Viveiros & Godinho, 2009). It has been suggested that, depending on the species, a high dilution ratio reduces the motility and viability of cryopreserved sperm (Cabrita *et al.*, 2005). This has been attributed to the dilution of several proteins (Suquet *et al.*, 2000) and anti-oxidative stress compounds (Cabrita *et al.*, 2011) present in the seminal plasma that protect the viability of sperm during the freezing and thawing processes. However, there is a lack of standardization in sperm cryopreservation of aquatic organisms, which is one of the main reasons for inconsistency observed among various studies (Dong *et al.*, 2007; Rosenthal *et al.*, 2010). Therefore, there is a necessity of more accurate investigations in all steps of the cryopreservation process. Although many studies have been performed on *P. lineatus* sperm cryopreservation (Viveiros *et al.*, 2009; Viveiros *et al.*, 2010; Viveiros *et al.*, 2015b, among others), the dilution ratio of sperm to final volume was mostly set at 1:10, without detailed investigation. Thus, the aim of this study was to evaluate the effects of dilution ratio on post-thaw sperm motility and velocity of *Prochilodus lineatus.*

Materials and Methods

Fish handling, sperm collection and sperm features

All fish were handled following the guidelines for animal experimentation described by Van Zutphen *et al.* (2001). *Prochilodus lineatus* males ($n = 10$; mean of 1.1 kg of body weight) were selected from earthen ponds 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), state of Minas Gerais, Brazil, during the spawning season (December to January). Males with detectable running sperm under soft abdominal pressure received two intramuscular doses of catfish pituitary extract (Destiny Land Enterprise Company Ltd, Taiwan; 0.4 and 4.0 mg kg^{-1} BW), with a 12-h interval between injections. After 8 h at ~26–27°C, the urogenital papilla was carefully dried, and approximately 2 ml of sperm were hand stripped directly into glass tubes. Sperm collection was carried out at room temperature (~25°C) and contamination with water, blood, feces or urine was carefully avoided.

Immediately after collection, 5 μl of each sample were placed on a glass slide, motility was triggered in an activating agent (98 mOsm NaCl) and subjectively evaluated using a light microscope (Eclipse E200; Nikon, Tokyo, Japan) at ×400 magnification. All samples possessed a motility rate above 90% and were thus used for the experiment described below. Sperm concentration was determined using a Neubauer-type hemacytometer chamber (Boeco, Hamburg, Germany). Osmolality (Semi-Micro Osmometer K-7400, Knauer, Berlin, Germany) and pH (Digimed DM-22-V1.0, São Paulo, Brazil) of the seminal plasma were measured after the centrifugation of sperm at 2000 *g* for 10 min (MiniStar CM-610, Shanghai, China). All analyses were carried out in duplicate.

Table 1 Proportion of sperm and freezing medium (315 mOsm/kg glucose plus methyl glycol) used in each dilution ratio, for each male

Sperm to final volume	Sperm to freezing medium	Volume in μl (%)		
		Sperm	Glucose	Methyl glycol
1:5	1:4	400 (20%)	1400 (70%)	200 (10%)
1:10	1:9	200 (10%)	1600 (80%)	200 (10%)
1:50	1:49	40 (2%)	1760 (88%)	200 (10%)
1:100	1:99	20 (1%)	1780 (89%)	200 (10%)

Sperm cryopreservation

A glucose solution (Glucose, Glucose Anidra P.A. ACS, $(C_6H_{12})_6$, Vetec Química Fina Ltda™, Duque de Caxias, RJ, Brazil) at 315 mOsm/kg (~5%) and pH adjusted to 7.6, combined with the cryoprotectant agent methyl glycol (CH_3O $(CH_2)_2OH$, Vetec Química Fina Ltda™, Duque de Caxias, RJ, Brazil), always at 10% of the final volume, were used as freezing medium for *P. lineatus* sperm, according to our previous studies (Viveiros *et al.*, 2009, 2015b). Within 10 min after collection, sperm was diluted in this freezing medium at four different ratios (sperm to final volume): 1:5; 1:10; 1:50 and 1:100. Because in some studies dilution ratio is reported as sperm to freezing medium, rather than sperm to final volume, we have given a detailed description of this methodology in Table 1. Diluted sperm was then drawn into 120 unsealed 0.5-ml straws ($n = 3$ replicate straws × 4 dilution ratios × 10 males) and, immediately, without equilibration time, frozen in nitrogen vapour (dry vapour shipper, Cryoport Systems, Brea, CA, USA) at approximately –170°C. Dilution, loading and freezing took about 10 min. Within 24 h, all straws were transferred to a liquid nitrogen vessel (MVE XC 34/18, New Prague, MN 56071, USA) for storage.

https://doi.org/10.1017/S0967199415000635 Published online by Cambridge University Press

Post-thaw sperm analysis

A few months later, straws were thawed by immersion in a water bath (Water Bath MA 127, Marconi, Piracicaba, SP, Brazil) at 60°C for 8 s and sperm motility rate and velocities (curvilinear = VCL, average path = VAP and straight line = VSL) were determined using the CASA system (Computer-Assisted Sperm Analyzer) following the methodology described by Viveiros *et al.* (2015a). Briefly, sperm motility was triggered in an activating agent (98 mOsm NaCl), at room temperature, directly in a Makler® counting chamber (Sefi-Medical Instruments ltd, Haifa, Israel), placed under a phase contrast microscope (Nikon™ ECLIPSE E200, Tokyo, Japan) at ×100 magnification with a green filter and pH 1 position. The final ratio of sperm to final volume was 1:500. The microscope was connected to a video camera (Basler Vision Technologies™ A602FC, Ahrensburg, Germany) generating 100 images/s; video recording started at 10 and at 40 s post-activation, in order to follow motility decrease after 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). To determine motility rate and velocities, each individual sperm (a mean of 449 spermatozoa per field) was followed throughout the images and sperm trajectory was calculated. Evaluations were made in triplicates. Furthermore, the actual sperm concentration per straw was also calculated.

Statistical analysis

All values were expressed as means ± standard deviation (SD). Statistical analyses were conducted using the R software program version 2.9.0 (R Development Core Team, 2010). Sperm motility and velocity parameters were tested for normal distribution using the univariate procedure; all parameters showed a normal distribution. Data were tested for significant differences using analysis of variance (ANOVA), followed by the Scott–Knott test, when applicable. The level of significance for all statistical tests was set at 0.05.

Results

The 10 males used in this experiment as sperm donors possessed a mean concentration of $31.9 \times 10^9 \pm 12.4 \times 10^9$ spermatozoa/ml; the seminal plasma had an osmolality of 281 ± 9 mOsm/kg and pH of 8.04 ± 0.4 (Table 2).

Post-thaw motility rates were higher when samples were frozen at a ratio of 1:5 (76%) and 1:10 (75%), compared with 1:50 (59%) and 1:100 (33%). When motility rate was evaluated again 30 s later, the values decreased significantly in samples frozen at a ratio of 1:5 (57%) and at 1:10 (61%). At a ratio of 1:50 and 1:100, the decrease observed after 40 s was not significant (Fig. 1*A*).

Table 2 Body weight and some fresh sperm features of *Prochilodus lineatus* ($n = 10$ males; mean ± standard deviation; minimum–maximum values) after pituitary extract treatment

Parameters	Mean ± standard deviation (SD)	Min–max
Body weight (kg)	1.1 ± 0.4	0.5–1.9
Concentration (spermatozoa × 10^9 /ml)	31.9 ± 12.4	10.6–55.2
Seminal plasma osmolality (mOsm/kg)	281 ± 9	264–298
Seminal plasma pH	8.04 ± 0.4	7.46–8.73

The highest post-thaw VCL (225 μm/s) and VAP (203 μm/s) were observed when sperm was frozen at a ratio of 1:10. VSL, on the other hand, was similar among samples frozen at a ratio of 1:5 (109 μm/s), 1:10 (124 μm/s) and 1:50 (97 μm/s). When velocities was evaluated again 30 s later, the values decreased significantly in all samples regardless of dilution ratio, and varied from 75 to 85 μm/s of VCL, from 38 to 53 μm/s of VAP and from 25 to 39 μm/s of VSL (Fig. 1*B–D*).

The average concentration measured in each straw at a dilution ratio of 1:5 was $6.4 \times 10^9 \pm 2.5 \times 10^9$ spermatozoa/ml, at 1:10 it was $3.2 \times 10^9 \pm 1.2 \times 10^9$ spermatozoa/ml, at 1:50 it was $0.6 \times 10^9 \pm 0.3 \times 10^9$ spermatozoa/ml and at 1:100 it was $0.3 \times 10^9 \pm 0.1 \times 10^9$ spermatozoa/ml.

Discussion

Over the past decade, our research group has been studying the different aspects of cryopreservation on *P. lineatus* sperm, such as extender composition (Viveiros *et al.*, 2009, 2010), cryoprotectant agent (Viveiros *et al.*, 2015b), straw volume, thawing temperature and activating agent (Viveiros *et al.*, 2009). However, the dilution ratio was always set to 1:10. In the present study, the dilution ratio was taken into consideration during the freezing of *P. lineatus* sperm, and was tested from 1:5 up to 1:100.

In this study, sperm cryopreserved at lower dilution ratios (and thus at higher sperm concentration) displayed higher post-thaw motility rate and velocities, compared with those at higher dilution ratios and

https://doi.org/10.1017/S0967199415000635 Published online by Cambridge University Press

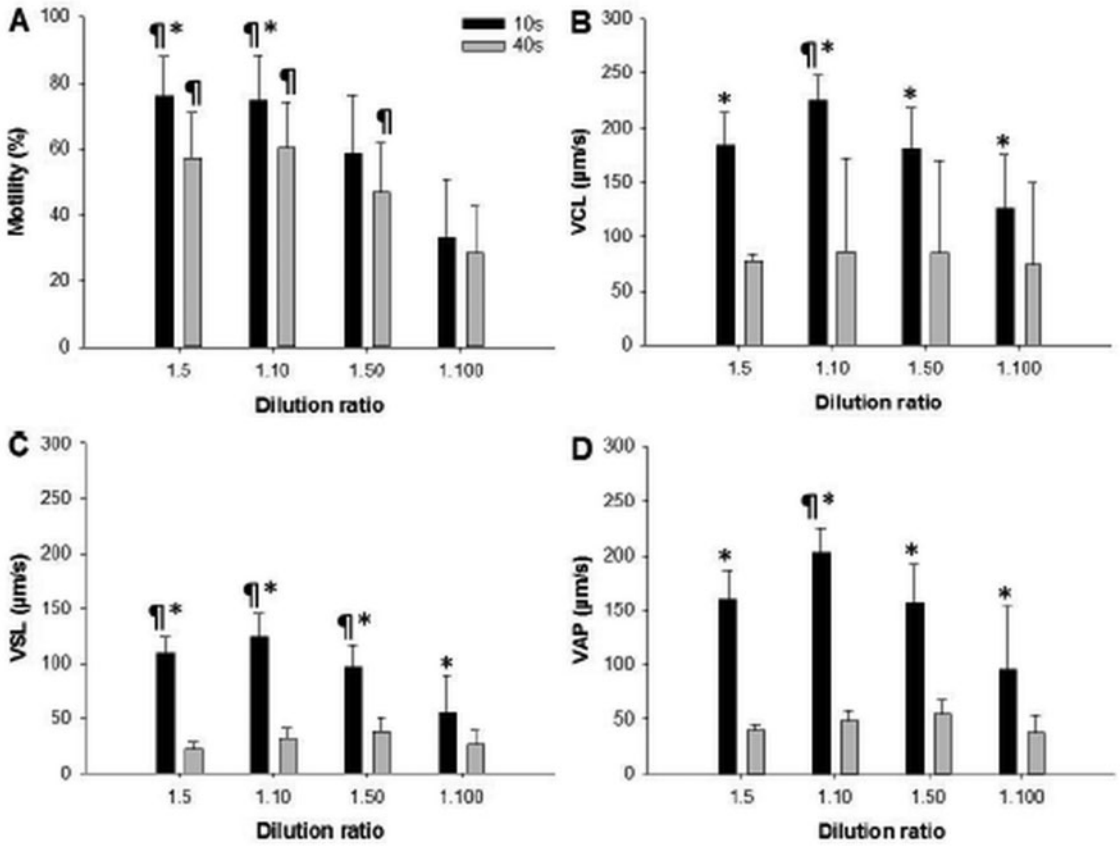

Figure 1 Post-thaw motility rate (*A*), curvilinear (VCL; *B*), straight-line (VSL; *C*) and average path (VAP; *D*) velocities of *P. lineatus* sperm frozen at four dilution ratios (sperm to total volume; 1:5, 1:10, 1:50 or 1:100). Post-thaw motility and velocities were evaluated at 10 s and 40 s post-activation. Each column and error bar represents mean ± standard deviation (SD) (n = 3 replicate straws × 10 males). ¶Differences among dilution ratios ($P < 0.05$; Scott–Knott); *differences between analysis time ($P < 0.05$; ANOVA); values followed by a symbol are significantly higher.

mostly at 1:100. In general, *P. lineatus* sperm possessed higher velocities when frozen after dilution at 1:10, compared with those frozen at 1:5, 1:50 and 1:100. With these results, we can state that too many or too few spermatozoa inside one straw leads to a suboptimal condition for freezing and thawing sperm, and that the ratio of 1:10 seems to fulfill this condition, at least for *P. lineatus.*

It has been demonstrated that high dilution ratio reduces the motility and viability of frozen sperm (Cabrita *et al.*, 2005) mainly due to the dilution of several proteins (Suquet *et al.*, 2000) and anti-oxidative stress compounds (Cabrita *et al.*, 2011) present in the seminal plasma that protect sperm viability during the freezing and thawing processes. This may explain why we observed the lowest values for motility rate and velocities when *P. lineatus* sperm was frozen at 1:100, at a mean concentration of 0.3×10^9 spermatozoa/ml. From the practical point of view, the use of such a low sperm concentration (and thus a high dilution ratio) demands large volumes of diluted sperm to be loaded in an increased number of straws, as well as to meet sufficient thawed sperm for fertilization (Ciereszko *et al.*, 2013), as it is well known that a higher number of post-thaw sperm is required for fertilization when compared to fresh sperm. This creates a significant problem related to the handling of high number of straws, especially during short-time procedures such as freezing, thawing and fertilization (Lahnsteiner *et al.*, 2002).

Conversely, an increased number of sperm inside each straw when a low dilution ratio is used, may change the conditions of cryopreservation and decrease post-thaw sperm quality (Ciereszko *et al.*, 2013). This phenomenon was observed for Pacific oyster *Crassostrea gigas* (Dong *et al.*, 2007), when agglutination of sperm was found at samples frozen at higher concentrations. Although we have not observed the agglutination phenomenon, when sperm was frozen at a concentrated ratio of 1:5, VCL and VAP were lower compared with those samples frozen at 1:10. Similarly, sperm of the salmoniforme whitefish, *Coregonus lavaretus* frozen at a very concentrated ratio of 3:1 (sperm to freezing medium) yielded similar

https://doi.org/10.1017/S0967199415000635 Published online by Cambridge University Press

motility rate and VSL, and lower VCL and VAP, compared to sperm frozen at a recommended ratio of 1:3 (Ciereszko *et al.*, 2013). Despite the lower results, these authors emphasized the necessity of packing a higher concentration of sperm into the same vessel, by improving the equilibration step and developing cryopreservation technology that is better suited to the cryopreservation of higher concentrations of sperm.

In order to follow motility decrease after activation, sperm quality was evaluated twice with a 30-s interval between analyses. Post-thaw motility rate decreased significantly when evaluated at 40 s compared with at 10 s post-activation, but the values were still above 50% at the concentrated ratios of 1:5 and 1:10, which is a high rate considering frozen sperm. Velocities, however, were all low at 40 s post-activation, with less than 30% of the values observed during the first analysis. Similar decrease of sperm quality was observed when motility rate and motility quality score were subjectively evaluated 30 s after the first analysis, in both *P. lineatus* and *B. orbignyanus* (Gonçalves *et al.*, 2013). Thus, the artificial fertilization using post-thaw sperm should be a short-time procedure, while sperm still possesses high motility rate and fast velocities to find and enter the oocyte micropyle.

P. lineatus sperm should be frozen at a ratio of 1:10 (sperm to final volume), at which both the number of loaded sperm per straw and the quality after freezing and thawing procedures are maximized.

Acknowledgements

The authors thank D. Rossetto and P.A. Piana for statistical support, T.S. França for assistance during sperm analysis and J. Cosson for revising the manuscript.

Financial support

This study received funding from the Brazilian fostering agencies CNPq (A.T.M.V., grant number PQ 302434/2011–9; project 474001/2013–0), and FAPEMIG (A.T.M.V., grant number PPM 00038–13), (M.C.L., grant number CVZ BPD 00167–12; project APQ 01068–12).

Statement of interest

None.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

References

Cabrita, E., Robles, V., Cuñado, S., Wallace, J.C., Sarasquete, C. & Herráez, M.P. (2005). Evaluation of gilthead sea bream, *Sparus aurata*, sperm quality after cryopreservation in 5 ml macrotubes. *Cryobiology* **50**, 273–84.

Cabrita, E., Ma, S., Diogo, P., Martínez-Páramo, S., Sarasquete, C. & Dinis, M.T. (2011). The influence of certain amino acids and vitamins on post-thaw fish sperm motility, viability and DNA fragmentation. *Anim. Reprod. Sci.* **125**, 189–95.

Carolsfeld, J., Godinho, H.P., Zaniboni Filho, E. & Harvey, B.J. (2003). Cryopreservation of sperm in Brazilian migratory fish conservation. *J. Fish Biol.* **63**, 472–89.

Ciereszko, A., Dietrich, G.J., Nynca, J., Liszewska, E., Karol, H. & Dobosz, S. (2013). The use of concentrated extenders to improve the efficacy of cryopreservation in whitefish spermatozoa. *Aquaculture* **408–9**, 30–3.

Cordiviola de Yuan, E. (1992). Fish populations of lentic environments of the Parana River. *Hydrobiologia* **237**, 159–73.

Dong, Q., Huang, C. & Tiersch, T.R. (2007). Control of sperm concentration is necessary for standardization of sperm cryopreservation in aquatic species: evidence from sperm agglutination in oysters. *Cryobiology* **54**, 87–98.

Gonçalves, A.C.S., Nascimento, A.F., Costa, A.C., Leal, M.C. & Viveiros, A.T.M. (2013). Initiation and suppression of sperm motility is osmolality-dependent in two South American fish species: streaked prochilod (*Prochilodus lineatus*) and piracanjuba (*Brycon orbignyanus*). *Anim. Reprod.* **10**, 62–70.

Kopeika, E. & Kopeika, J. (2008). Variability of sperm quality after cryopreservation in fish. In *Fish spermatology* (eds S.M.H. Alavi, J. Cosson, K. Coward & G. Rafiee), pp. 347–96. Alpha Science Intl Ltd, Oxford.

Lahnsteiner, F., Mansour, N. & Weismann, T. (2002). A new technique for insemination of large egg batches with cryopreserved semen in the rainbow trout. *Aquaculture* **209**, 359–67.

Orfão, L.H., Maria, A.N., Nascimento, A.F., Isaú, Z.A. & Viveiros, A.T.M. (2010). Sperm fertility of the subtropical freshwater streaked prochilod *Prochilodus lineatus* (Characiformes) improved after dilution and cold storage. *Aquac. Res.* **41**, 679–87.

R Development Core Team. (2010). *R: A Language and Environment for Statistical Computing*. Version 2.12.0. R Foundation for Statistical Computing.

Rosenthal, H., Asturiano, J.F., Linhart, O. & Horvath, A. (2010). Foreword on the biology of fish gametes: summary and recommendations of the Second International Workshop, Valencia, Spain, 2009. *J. Appl. Ichthyol.* **26**, 621–2.

Suquet, M., Dreanno, C., Fauvel, C., Cosson, J. & Billard, R. (2000). Cryopreservation of sperm in marine fish. *Aquac. Res.* **31**, 231–43.

Van Zutphen, L.F.M., Baumans, V. & Beynen, A.C. (2001). *Principles of Laboratory Animal Science.* Revised edition. Amsterdam: Elsevier, 428 pp.

Viveiros, A.T.M. & Godinho, H.P. (2009). Sperm quality and cryopreservation of Brazilian freshwater fish species: a review. *Fish. Physiol. Biochem.* **35**, 137–50.

Viveiros, A.T.M., Orfão, L.H., Maria, N.A. & Allaman, I.B. (2009). A simple, inexpensive and successful freezing method for curimba *Prochilodus lineatus* (Characiformes) semen. *Anim. Reprod. Sci.* **112**, 293–300.

Viveiros, A.T.M., Nascimento, A.F., Orfão, L.H. & Isaú, Z.A. (2010). Motility and fertility of the subtropical freshwater fish streaked prochilod (*Prochilodus lineatus*) sperm cryopreserved in powdered coconut water. *Theriogenology* **74**, 551–6.

Viveiros, A.T.M., Gonçalves, A.C.S., Di Chiacchio, I.M., Nascimento, A.F., Romagosa, E. & Leal, M.C. (2015a). Gamete quality of streaked prochilod *Prochilodus lineatus* (Characiformes) after GnRHa and dopamine antagonist treatment. *Zygote* **23**, 212–21.

Viveiros, A.T.M., Nascimento, A.F., Leal, MC., Gonçalves, A.C.S., Orfão, L.H. & Cosson, J. (2015b). Methyl glycol, methanol and DMSO effects on post-thaw motility, velocities, membrane integrity and mitochondrial function of *Brycon orbignyanus* and *Prochilodus lineatus* (Characiformes) sperm. *Fish. Physiol. Biochem.* **41**(1), 193–201.

https://doi.org/10.1017/S0967199415000635 Published online by Cambridge University Press