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Semen cryopreservation in *Piaractus mesopotamicus*: the effect of different diluents and freezing equipment[1]

Criopreservação seminal de *Piaractus mesopotamicus*: efeito de diferentes diluentes e equipamentos de congelação

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ABSTRACT - *Piaractus mesopotamicus* (Holmberg, 1887) is a fish native to the Basin of the River Plate and which has great economic importance. In view of this, there has been interest in developing biotechnologies that aim to optimise reproduction in captivity. The aim of this study was to verify the effect of freezing the semen of the pacu using different diluents and freezing equipment. The semen, after pooling, was diluted in the proportion 1:3 (semen: diluent) in two different freezing media, resulting from combining 10% DMSO with Glucose 5% or with ACP-104; it was then cryopreserved in a Dry Shipper or programmable freezer (PF). The samples were later thawed and evaluated for sperm kinetics, membrane integrity and sperm morphology using the Computer Assisted Sperm Analyser (CASA). The data were submitted to ANOVA, and the mean values compared by F-test ($P < 0.05$). There was no interaction between the Glucose 5% or ACP-104 diluents, and the Dry Shipper or PF equipment. However, Glucose 5% showed better results for sperm kinetics (motility: 40.52 ± 7.1%; VCL: 28.53 ± 4.07 $\mu m\ s^{-1}$; VSL: 9.38 ± 3.54 $\mu m\ s^{-1}$ and VAP: 15.24 ± 4.23 $\mu m\ s^{-1}$) compared to ACP-104 (motility: 31.19 ± 3.17%; VCL: 22.76 ± 1.86 $\mu m\ s^{-1}$; VSL: 5.4 ± 1.16 $\mu m\ s^{-1}$ and VAP: 10.06 ± 1.53 $\mu m\ s^{-1}$). It can therefore be concluded that Glucose 5% gives the best results compared to ACP-104, regardless of the equipment used, Dry Shipper or PF, in freezing the semen of *P. mesopotamicus*.

Key words: Pacu. ACP-104. Glucose. Dry shipper. Programmable freezer.

RESUMO - O *Piaractus mesopotamicus* (Holmberg, 1887) é um peixe nativo da Bacia do rio da Prata com grande importância econômica. Diante disso, tem-se despertado o interesse no desenvolvimento de biotecnologias que visam otimizar a reprodução em cativeiro. Portanto, o objetivo desse trabalho foi verificar o efeito da congelação seminal de pacu, em diferentes diluentes e equipamentos de congelação. O sêmen, após a formação dos *pools*, foi diluído na proporção 1:3 (sêmen: diluidor) em dois diferentes meios de congelação, resultantes da combinação de 10% DMSO associado à Glicose 5% ou ao ACP-104 e criopreservado em *Dry shipper* ou máquina de congelação programada (MCP). Posteriormente, as amostras foram descongeladas e avaliadas quanto à cinética espermática, com o *Computer Assisted Sperm Analyser* (CASA), integridade de membrana e morfologia espermática. Os dados foram submetidos à ANOVA, e para comparação de médias, foi realizado o teste F ($P<0,05$). Não houve interação entre os diluentes Glicose 5% ou ACP-104 e os equipamentos *Dry shipper* ou MCP. Porém, a Glicose 5% apresentou melhores resultados de cinética espermática (motilidade: 40,52 ± 7,1%; VCL: 28,53 ± 4,07 $\mu m\ s^{-1}$; VSL: 9,38 ± 3,54 $\mu m\ s^{-1}$ e VAP: 15,24 ± 4,23 $\mu m\ s^{-1}$) quando comparada ao ACP-104 (motilidade: 31,19 ± 3,17%; VCL: 22,76 ± 1,86 $\mu m\ s^{-1}$; VSL: 5,4 ± 1,16 $\mu m\ s^{-1}$ e VAP: 10,06 ± 1,53 $\mu m\ s^{-1}$). Assim, conclui-se que a Glicose 5% apresenta os melhores resultados, em relação ao ACP-104, independente do equipamento utilizado, *Dry shipper* ou MCP na congelação do sêmen de *P. mesopotamicus.*

Palavras-chave: Pacu. ACP-104. Glicose. *Dry shipper*. Máquina de congelação programada.

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INTRODUCTION

The Pacu (*Piaractus mesopotamicus*) is a species native to the Basin of the River Plate (PETRERI JUNIOR, 1989), which enjoys great economic importance due to its wide acceptance on the market and good characteristics for fish farming, where it presents rusticity, rapid growth, precocity and good adaptation to production systems (DIAS-KOBERSTEIN; CARNEIRO; URBINATI, 2005; FRESNEDA *et al*., 2004). As a way of helping to maintain the breeding stock, reduce production costs, and exchange genetic material between fish farms (MARIA; AZEVEDO; CARNEIRO, 2009), it has been sought to apply biotechnologies that optimise reproduction, such as the cryopreservation of semen (SALMITO-VANDERLEY *et al*., 2012).

The freezing of fish semen is a technique which was developed to conserve the genetic material in liquid nitrogen (PEGG, 2007). However, this biotechnology causes cryodamage to sperm cells, requiring the use of substances such as diluents and cryoprotectants. The main function of the diluent is to nourish the cells, while the cryoprotectant protects the cells against damage caused by the wide variation in temperature (SALMITO-VANDERLEY *et al*., 2012).

In Characiformes of genus *Piaractus*, the diluents reported in the literature for the process of freezing semen are: Glucose, Coconut Water Powder (ACP-104; ACP® - ACP Biotechnologia), Andro-Hepes, Beltsville Thawing Solution® (BTS), BTZOR, and modified Zorlesco (ZOR). Among the cryoprotectants, the most widely used are dimethyl sulfoxide (DMSO), egg yolk, glycerol, KCl and methanol. From the equipment used in freezing pacu semen, the Dry Shipper (NASCIMENTO *et al*., 2010; PAULINO *et al*., 2012; STREIT JUNIOR *et al*., 2006; STREIT JUNIOR *et al*., 2009) is important; however, the programmable freezer (PF) has also been used with other species of Characiformes (CARNEIRO *et al*., 2012; OLIVEIRA *et al*., 2016; PINHEIRO *et al*., 2016). The PF is used to allow control of the freezing curve and, consequently, a gradual and homogeneous drop in temperature, which may reduce cryogenic damage caused by the cold (OLIVEIRA *et al*., 2016).

Despite the variety of semen conservation protocols developed for Characiformes, studies related to the reproduction of *P. mesopotamicus* are scarce, with no reports of the use of powdered coconut water specific to fish semen (ACP-104), or of using a PF for freezing the semen of this species. The aim of the present study, therefore, was to test the two diluents (ACP-104 and Glucose) combined with the cryoprotectant dimethyl sulfoxide (DMSO) for freezing pacu semen using different equipment, a Dry Shipper and a PF.

MATERIAL AND METHODS

Location of the experiment and management of the breeding stock

The work was carried out at the Centre for Aquaculture Research of the National Department for Works Against Drought (DNOCS), located in the district of Pentecoste (03°47' S, 39°16' W, altitude 60.0 m), as well as at the Laboratory for Fish Reproduction Biotechnology (LBRP), on the Itaperi campus of the State University of Ceará, in Fortaleza, Ceará, Brazil (03°43' S, 38°32' W, altitude 14.0 m). The work was approved by the Ethics Committee on Animal Use of UECE (3414810/2018).

Twenty-four males of *P. mesopotamicus* from the breeding stock of the DNOCS were selected for the experiment, showing secondary characteristics indicative of reproductive maturity, such as hyperaemic urogenital papillae and easy semen release when subjected to slight abdominal pressure.

The animals were kept in brick tanks with constant water recirculation. All the males were hormonally induced to produce sperm by means of a single dose of common carp pituitary (CCP) in the proportion of 2 mg Kg^{-1} animal weight. Intracoelomic application was made at the base of the pectoral fin, with the semen collected approximately 14 hours after hormonal induction.

Collection and pooling

To facilitate containment, and with a view to animal welfare, the animals had their eyes wrapped in a damp cloth and were restrained in a lateral position on a sponge. During collection of the semen, the urogenital papilla was dried with a paper towel in order to remove any water. Samples contaminated with blood, faeces or urine were discarded. Slight anteroposterior abdominal pressure was given to release the semen, which was collected in graduated polyethylene tubes. The collected material was kept in a thermal box with ice at approximately 4 ºC until processing. Samples that showed less than 80% motility after activation were discarded. After analysing motility, semen samples from 24 animals were used to prepare eight pools, each pool consisting of semen samples from three different males.

Evaluation of sperm kinetics

To analyse sperm kinetics, 1 μL of semen (pool) was homogenised with 100 μL of activating solution (50 mM NaCl - 125 mOsm), placed on a Makler chamber and immediately evaluated under an optical microscope using the Sperm Class Analyser software (SCA v3.2, Microptics, Barcelona, Spain).

The following parameters were evaluated: total sperm motility (%), curvilinear velocity (VCL - µm s^{-1}), straight line velocity (VSL - µm s^{-1}), and average path velocity (VAP - µm s^{-1}).

Evaluation of sperm concentration

The method adopted was that used by Vieira *et al*. (2011). The fresh semen was fixed in a buffered saline formalin solution in the proportion 1:4000 (semen: fixative), and the sperm count carried out using a Neubauer chamber, as per the Brazilian College of Animal Reproduction (2013).

Evaluation of sperm membrane integrity

Initially, 10 µL eosin, 10 µL nigrosin and 5 µL semen were mixed together, and 10 µL of the mixture was then smeared onto a histological slide and left to dry at room temperature. One slide was prepared per pool, where 200 sperm were evaluated under an optical microscope (400x), and the percentage of sperm with an intact or ruptured membrane was estimated. The sperm were considered intact when colourless, and damaged when they had a reddish colour, indicating rupture of the plasma membrane.

Evaluation of sperm morphology

An aliquot of thawed semen from each treatment was fixed in a 1:10 buffered saline formalin solution (semen:fixative). Rose Bengal stain was then added to a fraction of the fixed semen sample in the proportion 3:20 (dye: semen. A 10-µL aliquot of this solution was smeared onto a histological slide and allowed to dry at room temperature to observe the normality of the morphological pattern, as well as the presence and type of morphological anomalies in the sperm.

The analysis comprised the observation of 100 sperm per slide under an optical microscope (400x), with two slides being evaluated per sample. The sperm was classified as per Miliorini *et al*. (2011) with adaptations: normal, bent-tail, coiled-tail, broken-tail, corrugated-tail, headless, microcephalic and macrocephalic.

Freezing the semen

The pools of fresh semen were diluted in two different freezing media, ACP-104 + 10% DMSO and Glucose 5% + 10% DMSO, in the proportion 1:3 (semen: diluent), and cryopreserved in a Dry Shipper (Taylor-Wharton, model CP 300) or PF (Dominium K BIOCOM®, Brazil), to form four treatments. Four 0.25 mL straws were then filled from each sample, and sealed at each end with polyvinyl alcohol. Two straws were frozen in the Dry Shipper and two in the PF.

For freezing in the Dry Shipper, the straws were kept under refrigeration (~ 4 ºC) for 10 minutes, the time required for equilibration. The straws were then immediately placed into canisters and left in the Dry Shipper (-176 ºC) for 15 minutes (NUNES *et al*., 2016).

For the PF, the methodology adopted by Oliveira *et al*. (2016) was used. For this process, the straws were submitted to a reduction in temperature at a speed of -3 °C min^{-1}, starting at 10 °C and decreasing to -12 °C, considered the crystallisation temperature (step 1). After one minute, the second step began automatically, maintaining the reduction of -3 °C min^{-1}, and continuing the drop in temperature from -12 °C to -60 °C. At the end of the process, the straws remained in the PF for 30 minutes to stabilise, and guarantee that the samples were frozen.

After freezing, all the straws were placed in a liquid nitrogen cylinder at -196 °C. The frozen semen was stored for at least 15 days before defrosting began, which took place in a water bath at 40 °C for 12 s (PAULINO *et al*., 2012). The semen was analysed for sperm kinetics, morphology and membrane integrity, following the same methodology as used for the fresh semen.

Statistical analysis

The data were submitted to the Shapiro-Wilk test to check the residual normality, and the Bartlett test to test the homoscedasticity of the variances between treatments. When the requirements were verified and met, an analysis of variance (ANOVA) was carried out, using the GLM procedure of the SAS Software, to test the effect of the diluent (ACP-104 or Glucose), the effect of the equipment (Dry Shipper or PF), and the interaction between the diluents and equipment. The F-test was applied to compare the mean values at a significance level of 5%, and the results were presented as the mean ± standard deviation.

RESULTS AND DISCUSSION

In this study, the characteristics of fresh pacu semen induced with common carp pituitary are described in Table 1. In general, the results found in the present study agree with those reported in the literature for this species (GALO *et al*., 2018; STREIT JUNIOR *et al*., 2006), except for the sperm concentration, which was lower.

There was no interaction between the diluents and equipment for each parameter under analysis in the present study. Also, no statistical difference was found between the equipment used ($P>0.05$). Using the Dry

Table 1 - Mean ± standard deviation for fresh-semen parameters in *P. mesopotamicus* (n = 8)

Parameter	Mean ± SD.
pH	8.6 ± 0.18
Concentration (x 10^9 spermatozoa mL^{-1})	10.48 ± 5.38
Total motility (%)	98.7 ± 0.006
VCL (µm s^{-1})	108.6 ± 15.50
VSL (µm s^{-1})	63.8 ± 18.09
VAP (µm s^{-1})	87.7 ± 18.96
Intact spermatozoa (%)	100 ± 0.00

Shipper for cryopreservation of the pacu semen, total motility was 34.64 ± 7.01%, while with the PF, total motility was 37.29 ± 7.43%. For semen velocity, the VCL, VSL and VAP with the dry Shipper were 25.93 ± 4.66 µm s^{-1}, 8.18 ± 3.67 µm s^{-1}, and 13.35 ± 4.57 µm s^{-1} respectively. For the PF, these values were 25.55 ± 4.08 µm s^{-1}, 6.77 ± 2.89 µm s^{-1}, 12.16 ± 3.69 µm s^{-1}.

For membrane integrity using the Dry Shipper, 57.60 ± 13.95% spermatozoa with an intact membrane were found (P>0.05), while for the PF, the value was 56.13 ± 12.27%. In relation to sperm morphology, no difference was found between the freezing methods employed, Dry Shipper or PF, as shown in Table 2 (P>0.05).

For total sperm motility after thawing, samples cryopreserved with the 5% Glucose diluent (40.52 ± 7.10%) gave better results (P<0.05) compared to the ACP-104 (31.19 ± 3.17%), as can be seen in Figure 1. Various studies show the success obtained using glucose as diluent in *Brycon opalinus* (VIVEIROS *et al*., 2012), *Prochilodus brevis* (LOPES *et al*., 2014; NUNES *et al*., 2016), and *Oncorhynchus mykiss* (NYNCA *et al*., 2017), among others. Sperm motility is one of the most important factors in analysing semen quality (VIVEIROS; GODINHO, 2009), and is therefore an essential evaluation, whose results deserve to be highlighted.

For sperm velocity, the semen cryopreserved with 5% Glucose (VCL = 28.53 ± 4.07 µm s^{-1}; VSL = 9.38 ± 3.54 µm s^{-1}; VAP = 15.24 ± 4.23 µm s^{-1}) gave better results (P<0.05), compared to the ACP-104 (VCL = 22.76 ± 1.86 µm s^{-1}; VSL = 5.4 ± 1.16 µm s^{-1}; VAP = 10.06 ± 1.53 µm s^{-1}), regardless of the method of freezing (Figure 2). This is due to the ability of Glucose to supply an energy substrate, a cryoprotective agent and an osmotic component, contributing to the osmotic balance due to its high molecular weight, and acting as a substitute for electrolytes (HOLT, 2000). It is worth noting that VCL is important in fish sperm, since it is directly related to good fertilisation rates (VIVEIROS *et al*., 2010); in this study, the best results for VCL were found when Glucose 5% was used.

ACP-104 has been used with good results with the semen of *P. brevis* (NASCIMENTO *et al*., 2017) and *Prochilodus lineatus* (VIVEIROS *et al*., 2010). The present study, however, showed poor results, possibly due to the complexity of ACP-104 not affording the necessary conditions for use in freezing the pacu semen, considering the specificity of the selected species.

No difference was found between treatments (P>0.05) in relation to membrane integrity or sperm morphology. When tail morphopathology was observed,

Table 2 - Mean ± standard deviation for sperm morphology in pacu (*P. mesopotamicus*), cryopreserved using two freezing methods, Dry Shipper and Programmable Freezer (PF)

	Freezing Method	
	Dry Shipper	PF
Normal	40.36 ± 8.35%	45.37 ± 6.72%
Bent tail	11.2 ± 4.22%	8.31 ± 3.47%
Coiled tail	7.3 ± 3.80%	7.75 ± 2.94%
Broken tail	20.96 ± 6.29%	18.31 ± 4.34%
Headless	19.9 ± 6.50%	19.21 ± 3.63%

Figure 1 - Mean ± standard deviation for sperm motility in pacu semen, cryopreserved using two different diluents, ACP and Glucose. Lowercase letters show a difference between diluents by F-test (P<0.05)

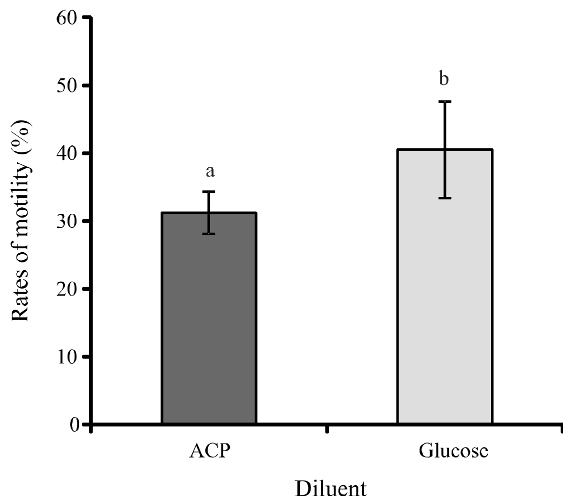
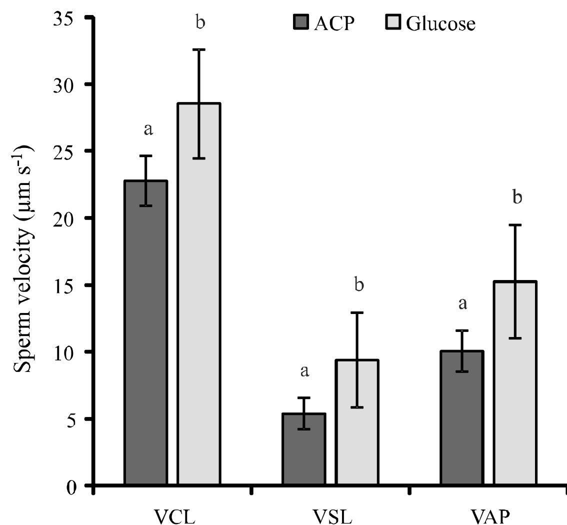


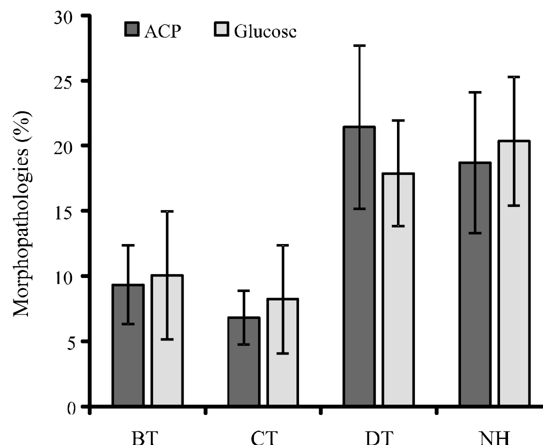
Figure 2 - Sperm velocity (Curvilinear Velocity - VCL; Straight Line Velocity - VSL and Average Path Velocity - VAP) mean ± standard deviation in pacu semen cryopreserved using two different diluents, ACP and Glucose. Lowercase letters show a difference between diluents by F-test (P<0.05)



the most frequent was the broken tail, with the coiled tail occurring least (Figure 3). In work developed by Galo *et al*. (2018), similar behaviour was also seen in the tail morphopathology of pacu semen after thawing.

It should be noted that the percentage of sperm morphopathologies verified under the different treatments, using both ACP-104 and Glucose, whether in the Dry shipper or PF, was lower than that found by Streit Junior *et al*. (2006) in fresh semen, and by Paulino *et al*. (2012) in thawed semen of the same species, thereby giving better results.

Figure 3 - Frequency (%) of morphopathologies seen in the spermatozoa after thawing the semen of *P. mesopotamicus*, using ACP or Glucose as diluent, for the morphopathologies, bent tail (BT), coiled tail (CT), broken tail (DT) and headless (NH)



The present study sought to evaluate the interaction between different diluents and freezing equipment on sperm kinetics, membrane integrity and morphology in the pacu, using the CASA system. In addition, this is the first study with this species to use a PF and show good semen quality after thawing. This study serves as a basis for the development of semen cryopreservation protocols for the pacu, and their application in aquaculture.

CONCLUSION

Of the diluents under test, it was concluded that Glucose 5% is the most suitable for freezing the semen of *P. mesopotamicus*, since it gave better sperm velocity and rates of motility. Both the Dry Shipper and the Programmable Freezer can be used for semen cryopreservation in this species.

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