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# MILT CRYOPRESERVATION FOR RHEOPHILIC FISH THREATENED BY EXTINCTION IN THE RIO GRANDE, BRAZIL

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## Abstract

**BACKGROUND:** Specific protocols for milt cryopreservation have been established for some freshwater fish species. However, cryopreservation reduces sperm quality, giving unsatisfactory results in reproduction. **OBJECTIVE:** The objective of this work was to evaluate the effect of different cryoprotectants on the quality of *Prochilodus lineatus*, *Brycon orbignyanus* and *Piaractus mesopotamicus* milt after cryopreservation. **METHODS:** The milt was diluted in different cryoprotectant solutions containing 10% methanol, dimethyl sulfoxide, glycerol, propylene glycol or ethylene glycol combined with the Beltsville Thawing Solution extender (5%), then placed in the vapour of a liquid nitrogen (LN) storage tank for 24 h, after which they were immersed in LN. After rewarming, the rate (%) and duration (s) of milt motility and abnormal morphology were evaluated. **RESULTS:** All of cryoprotectant solutions tested used maintained the viability of *P. lineatus* and *P. mesopotamicus* milt. However, in *P. lineatus,* glycerol ensured a lower percentage of abnormal morphology. In case of *P. mesopotamicus*, all of the cryoprotectant solutions tested may be used in the cryopreservation process, with the exception of those containing glycerol. **CONCLUSION:** For *B. orbignyanus,* cryoprotectant solutions containing methanol and ethylene glycol are recommended for use in the cryopreservation process, although they reduced the quality of sperm post-rewarming.

**Keywords:** *Brycon orbignyanus, Piaractus mesopotamicus, Prochilodus lineatus*, reproduction.

## INTRODUCTION

The disappearance of native fish species and declining genetic stocks compromise aquatic ecosystems and commercial fish production. Cryopreservation of fish milt may partially minimises such problems (1). Milt of some fish species can be cryopreserved without losing its quality (3). Cryopreserved milt can be used for reproduction in captivity, allowing the increased production of larvae and the creation of sperm banks to ensure reproductive success and genetic diversity (34).

Specific protocols for milt cryopreservation have been established for some freshwater fish species (2), and studies on Brazilian characids have focused on rheophilic species, such as the dourado *Salminus brasiliensis* (4), piracanjuba *Brycon orbignyanus* (16), matrinxã *Brycon cephalus* (29), and curimba *Prochilodus lineatus* (6, 23). However, it has been shown that cryopreservation reduces sperm quality, giving unsatisfactory results in reproduction. Studies with new cryoprotectants should be performed, and other assessments beyond the rate and duration of motility of the milt should be evaluated, such as sperm morphology and sperm abnormalities (6, 34), as indicators of milt quality. However, to achieve a high fertilization rate, it is also necessary that oocytes be in perfect conditions for use (6). These variables are not yet fully known for the species under

study, but they could help to shed some light on aspects involving milt cryopreservation.

Dimethyl sulfoxide (DMSO) and methanol are the most commonly used cryoprotectants in fish milt cryopreservation (4, 8, 16). However, the best results have not always been obtained with these compounds (2), as cryoprotectants are known to have specific effects on different fish species (6). Therefore, other cryoprotectants have been tested in the search for higher post-rewarming milt viability, including ethylene glycol and propylene glycol, which have been used to cryopreserve sperm of species such as *Colossoma macropomum* (17) and *Leporinus macrocephalus* (27). Reports on the use of ethylene glycol, propylene glycol, and glycerol associated with Beltsville Thawing Solution (BTS; Minitub®) extender for cryopreservation of curimba (*P. lineatus*), piracanjuba (*B. orbignyanus*), and pacu (*Piaractus mesopotamicus*) milt are scarce. BTS was first developed for pig semen in a diluting solution, which deserves special attention because it has produced good results for cryopreservation of 'piracema' fish semen (6, 9, 15, 19, 24). BTS ensures sperm inactivation; because it is isotonic, it provides a favourable osmotic medium that protects the intracellular portion of the cell membrane during freezing (23).

*P. mesopotamicus, B. orbignyanus* and *P. lineatus* are large, native rheophilic species, with great importance for fishery activity. The populations of these species have been greatly affected by anthropogenic factors, and they are now at risk of extinction (33). The hormonal induction of reproduction is an important issue in research on these species, where the crude extract of carp pituitary is commonly used for induction for reproduction, and alternative hormones have been a topic for discussion. The buserelin acetate hormone (GnRHa) shows a potential for the induction of reproduction of rheophilic species. Thus, the aim of this study was to develop protocols for cryopreservation of sperm of fish species (*P. mesopotamicus, B. orbignyanus* and *P. lineatus*) that are threatened by extinction and are of economic interest in the Rio Grande (Brazil) basin. This was achieved by using different cryoprotectants in association with the BTS extender.

## MATERIALS AND METHODS

Experiments were conducted at the fish farm of the Companhia Energética de Minas Gerais (CEMIG) in the city of Itutinga MG, Brazil, during the breeding season. Curimba breeders were kept in inland hatcheries of approximately 322 $m^2$. The fish were fed *ad libitum* with extruded feed (8 mm in diameter) and 28% raw protein (commercial feed), between March and August. During the reproductive period (between September and February), the animals were fed with extruded feed of equal diameter containing 32% raw protein three times a day.

Breeding individuals were removed from ponds by trawling with a net, where animals were considered able to receive hormonal induction according to the characteristics described by Woynarovich and Horváth (36).

After selection, animals were weighed, identified and transferred to 2,000 L tanks and maintained at a density of seven animals per tank. The weights of the specimens used were 1.1±0.2, 3.2±0.7, and 1.6±0.5 kg for *P. lineatus* (n = 7), *P. mesopotamicus* (n = 6), and *B. orbignyanus* (n = 7), respectively. The tank water received constant aeration and was kept at a temperature of 28±1°C.

***Milt cryopreservation***

For milt collection, animals were wrapped in a towel and its urogenital papilla and surrounding areas were cleaned and dried with paper towels to avoid premature milt activation by water, faeces or urine. Abdomen was pressed toward the craniocaudal portion of the animal's body to stimulate sperm ejection. Milt samples were collected in sterile graduated test tubes, kept protected from light by opaque paper.

To check the rate (%) and duration (s) of sperm motility, 10 µL fresh milt of each animal were placed on a histological slide and then homogenised with 40 µL distilled water (6). Sperm motility was observed under an optical microscope, at a 100x magnification, and the average percentage of motile sperm was calculated in three fields. The duration of sperm motility was estimated by mixing with distilled water until only 10% of the sperm in the field was moving (6). Only samples with a sperm motility rate above 90% were cryopreserved.

Fresh milt was collected for the analysis of concentration and sperm morphology. The sperm concentration was estimated using a Neubauer chamber, with one 10 µL aliquot of fresh milt from each animal being diluted in formalin-citrate [2.9g sodium citrate, 4mL commercial solution of 35% formaldehyde, and

distilled water q.s.p. 100mL (6)], at a milt to formalin-citrate ratio of $1:10^4$, according to the methodology adopted by Silva *et al.* (28).

To analyse sperm morphology, thawed milt was diluted in formalin-citrate at a milt: formalin-citrate ratio of $1:10^4$. For morphological analysis of the milt, 10 μL diluted milt were placed on a histological glass slide and covered with a coverslip. This was examined under an optical composite microscope, with fluorescent episcopic lighting (Nikon, model OPTIPHOT-2). The examination consisted of observation of 100 sperm in various fields across the slide. The bnormalities of head, midpiece, and tail were assessed at 1000x of magnification into primary (degenerate head, abnormal midpiece, broken or curled tail, micro- and macrocephaly) and secondary abnormalities (isolated head, proximal and distal droplet, and simple tail) according to Streit Jr. *et al* (31).

After milt collection and evaluation, a sample of each specimen was diluted in five different cryoprotectant extenders at ratio of 1:5 [milt:cryoprotectant solution – (29)], with the following solutions: Solution A, 5% BTS + 10% methanol; Solution B, 5% BTS + 10% DMSO; Solution C, 5% BTS + 10% glycerol; Solution D, 5% BTS + 10% propylene glycol; and Solution E, 5% BTS + 10% ethylene glycol.

The diluted milt was aspirated into 0.5 mL straws, with two repetitions per treatment, sealed with sterile surgical mass, packed in racks, and placed in LN vapour cylinders (Taylor-Wharton Model CP 300, type *dry shipper*). The cooling rate was 35.6°C $min^{-1}$ between 21°C and -170°C (16). Twenty-four h later, the samples were transferred into LN cylinders at a constant temperature of -196°C and stored for 2 days before rewarming.

Samples were rewarmed individually in a water-bath. The method used for rewarming differed for each species, based on preliminary studies: *P. lineatus* was defrosted at 40°C for 8 s (26), *B. orbignyanus* at 60°C for 5 s, and *P. mesopotamicus* at 60°C for 8 s (25). Afterwards, sperm morphology and the rate (%) and duration (s) of sperm motility were evaluated, with the same method for fresh milt evaluation.

***Statistical analyses***

The data were subjected to analysis of variance (ANOVA), using the computational package Sisvar, and a completely randomised design. The assumptions of normality and homoscedasticity were tested using the Shapiro-Wilk and Levene tests, respectively. When a significant difference was observed, the Tukey test was used at a 5% probability. The difference between abnormal sperm in cryopreserved milt and fresh milt was assessed by Dunnett's test at a 5% probability.

## RESULTS

***P. lineatus***

The concentration of *P. lineatus* sperm was $93.6 \pm 14.6 \times 10^9$ sperm/mL. This was higher than that found by other authors for this species, as Felizardo *et al.* (6) and Viveiros *et al.* (34) reported values of $23.4 \pm 18.4 \times 10^9$ and $16.8 \times 10^9$ sperm/mL, respectively. Such variation was expected because sperm concentration can be affected by environmental factors, reproductive phase, milt volume, and the type of hormone used to induce ejaculation (28).

Sperm motility was greater ($P<0.05$) in the treatment containing DMSO (74.6 ± 5.6%) when compared with the treatment containing propylene glycol (52.1 ± 10.2%), but did not differ ($P>0.05$) from the other treatments (Table 1). The rate of motility observed could be considered high, given that a decrease in motility in relation to fresh milt was expected after freezing, as the cryopreservation process may damage the sperm (28). A decrease in the rate of motility after rewarming was reported by Felizardo *et al.* (6) for the same species; they observed a rate of 84.93% in pre-freezing semen and 40.96% in semen post-freezing.

In this study, there was no difference ($P>0.05$) in sperm motility of *P. lineatus* milt that was cryopreserved in solutions containing methanol, glycerol, and DMSO (Table 1). Likewise, Murgas *et al.* (23) found that DMSO and methanol did not affect sperm motility after LN exposure. However, these authors did not evaluate glycerol as a cryoprotectant, which proved to be a good cryoprotectant for milt of this species in this study.

The inclusion of DMSO in the solution led to a longer duration of sperm motility (127.3 ± 47.7 s) compared with the other solutions used, with the exception of glycerol (Table 2). It is noteworthy that the duration of motility of semen cryopreserved with DMSO and fresh semen showed variable results, which could be observed in the SD values. According Murgas *et al.* (22), the reduced duration of sperm motility originates, in part, from the fall in the stock of energy that occurs during the period of motility;

the duration of sperm motility in freshwater fish

**Table 1.** Sperm motility (mean ± SD), reported as rate (%) of post-thaw milt (n)

| Cryoprotectant extenders | Species | | |
|---|---|---|---|
| | *P. lineatus* (n=7) | *P. mesopotamicus* (n=6) | *B. orbignyanus* (n=7) |
| Methanol | 64.2 ± 1.6$^{ABC}$ | 67.1 ± 12.6$^{A}$ | 21.4 ± 9.1$^{A}$ |
| DMSO | 74.6 ± 5.6$^{A}$ | 61.6 ± 8.8$^{A}$ | 0$^{B}$ |
| Glycerol | 71.7 ± 3.6$^{AB}$ | 22.5 ± 18.8$^{B}$ | 0$^{B}$ |
| Propylene glycol | 52.1 ± 10.2$^{B}$ | 52.0 ± 2.0$^{A}$ | 0$^{B}$ |
| Ethylene glycol | 70.3 ± 2.6$^{AB}$ | 42.5 ± 4.7$^{A}$ | 13.5 ± 3.5$^{A}$ |
| Fresh milt | 100.0 ± 0.0 | 98.3 ± 1.3 | 100.0 ± 0.0 |

[1] Means followed by different letters, in columns, differ among themselves in the Tukey test at 5%.

**Table 2.** Duration (seconds) of sperm motility (mean ± SD) of post-thaw milt

| Cryoprotectant extenders | Species | | |
|---|---|---|---|
| | *P. lineatus* (n=7) | *P. mesopotamicus* (n=6) | *B. orbignyanus* (n=7) |
| Methanol | 50.0 ± 6.8$^{BC}$ | 91.4 ± 20.2$^{A}$ | 34.4 ± 16.6$^{A}$ |
| DMSO | 127.3 ± 47.7$^{A}$ | 71.7 ± 6.3$^{A}$ | 0$^{B}$ |
| Glycerol | 52.2 ± 5.3$^{AB}$ | 33.9 ± 20.4$^{B}$ | 0$^{B}$ |
| Propylene glycol | 29.7 ± 21.2$^{C}$ | 71.0 ± 5.7$^{A}$ | 0$^{B}$ |
| Ethylene glycol | 39.7 ± 14.2$^{BC}$ | 46.0 ± 11.8$^{AB}$ | 15.6 ± 3.3$^{A}$ |
| Fresh milt | 163.6 ± 35.0 | 100.3 ± 16.4 | 84.8 ± 9.6 |

[1] Averages followed by different letters, in columns, differ among themselves in the Tukey test at 5%.

is very short and varies between species and animals due to factors such as nutritional status and health status.

This result conflicts with those obtained by Felizardo *et al.* (6), who observed that methanol led to a longer duration (74 ± 59 s) than DMSO (58 ± 4 s), although the duration of motility in the current study was higher than that in the cited work. This discrepancy may be due to the fact that these authors added egg yolk to the cryoprotectants. Another factor that may have contributed to this result is the high rate of motility in the study (74.6 ± 5.6%) compared with that observed by Felizardo *et al.* (6) (61 ± 24%), as the duration is closely linked to the rate of motility, according to these authors (6).

With regards to sperm morphology in *P. lineatus* milt, the average percentage of total abnormalities observed in fresh milt (28%, Table 3) was higher than that observed by Kavamoto *et al*. (14) and Moraes *et al.* (21), who found an incidence of 7.2% and 9.5% in fresh milt, respectively. According to Herman *et al.* (11), morphological changes in sperm may occur during spermatogenesis as a result of causes that affect breeding, such as disease, inbreeding, food restriction, and environmental stress, in addition to handling procedures during the collection of semen, which may justify the discrepancy with the results of these authors, since that the handling and breeding may have been different. There was no difference ($P$>0.05) in the primary, secondary, and total morphological alterations in the thawed milt treated with the different solutions (Table 3).

The use of the solution containing glycerol and BTS helps preventing osmotic stress in sperm cells and formation of large intracellular ice crystals during the cryopreservation process (35). Another reason for the effectiveness of glycerol may be the fact that it stabilises membranes during freezing. This stabilisation is caused by the binding of hydrogen atoms in the hydroxyl groups of the glycerol with the oxygen atoms in the phosphate groups of the plasma membrane phospholipids (13).

The highest total abnormalities were found in milt cryopreserved with methanol (30.5 ± 3.9%). This value is within the acceptable limits for use in fertilisation according to Miliorini *et al.* (18) who reported that the critical percentage of total milt abnormalities in curimba to be used for artificial fertilisation is below 50%, given that this technique involves a high ratio of sperm:oocytes in a controlled environment.

***P. mesopotamicus***

The concentration of sperm in fresh *P. mesopotamicus* milt was 53.6 ± 3.9 x $10^9$ spermatozoa/mL. Sperm concentration observed was higher than that reported for the same species by Maria *et al.* (15), Silveira *et al.* (30) and Miliorini *et al.* (19), who found values of 13.8 ± 1.2 x $10^9$, 28.0 ± 8.2 x $10^9$, and 18.6 ± 3.3 x $10^9$ spermatozoa/mL, respectively.

Glycerol led to a lower (P<0.05) motility rate (22.5 ± 18.5%) when compared with other solutions (Table 1). The low motility may be correlated with high sperm abnormalities found (Table 4) with the treatment. According to Kavamoto *et al* (14) morphological abnormality of the middle piece and tail cause progressive changes in motility, increasing sperm number with a circular or oscillatory movement and consequently reduces the rate of fertilization.

We obtained a rate of up to 67.1 ± 12.6% using methanol. Streit Jr. *et al.* (31) found a lower motility rate (16.1%) when using DMSO in comparison with the current study (61.6 ± 8.8%). One of the factors that may explain this difference is the overall low motility rate (75 ± 0.7%) of fresh milt reported by these authors, since the cryopreservation process itself leads to a reduction in sperm quality (6). Therefore, the methodology applied for the cryopreservation process, such as sperm collection, dilution ratio, extenders, cryoprotectants, equilibration time, cooling rates, and warming rates can also affect this parameter (20).

**Table 3.** Percentage of sperm abnormal morphology (mean ± SD) of *P. lineatus* post-thaw (n=7).

| Cryoprotectant extenders | Sperm abnormal morphology (%)[1] | | |
|---|---|---|---|
| | Primary | Secondary | Total |
| Methanol | 17.0 ± 0.7$^{A}$ | 13.5 ± 2.9 | 30.5 ± 3.9 |
| DMSO | 13.5 ± 1.6$^{A}$ | 12.2 ± 1.9 | 25.8 ± 0.5 |
| Glycerol | 13.5 ± 1.6$^{A}$ | 11.7 ± 5.4 | 25.2 ± 6.9 |
| Propylene glycol | 19.4 ± 2.5$^{A}$ | 8.2 ± 0.8 | 27.7 ± 1.8 |
| Ethylene glycol | 15.8 ± 0.1$^{A}$ | 11.4 ± 1.3 | 25.8 ± 0.5 |
| Fresh milt | 19 | 9 | 28 |

[1] Averages followed by different letters, in columns, differ among themselves in the Tukey test at 5%.
* Differs from fresh milt in the Dunnett's test at 5%.

**Table 4.** Percentage of sperm abnormal morphology (mean ± SD) of *P. mesopotamicus* post-thaw milt (n=6).

| Cryoprotectant extenders | Sperm abnormal morphology (%)[1] | | |
|---|---|---|---|
| | Primary | Secondary | Total |
| Methanol | 8.6 ± 1.7$^{A}$ | 15.6 ± 2.1$^{A}$ | 24.3 ± 0.3$^{AB}$ |
| DMSO | 6.0 ± 3.6$^{A}$ | 10.5 ± 1.5$^{A}$ | 16.5 ± 5.2$^{A}$ |
| Glycerol | 20.1 ± 6.3$^{B*}$ | 10.6 ± 1.4$^{A}$ | 30.8 ± 4.9$^{B*}$ |
| Propylene glycol | 13.1 ± 1.3$^{AB}$ | 14.3 ± 1.1$^{A}$ | 27.5 ± 2.5$^{AB*}$ |
| Ethylene glycol | 8.0 ± 2.2$^{A}$ | 12.1 ± 0.3$^{A}$ | 20.1 ± 2.6$^{AB}$ |
| Fresh milt | 7.0 | 5.0 | 12.0 |

[1] Averages followed by different letters, in columns, differ among themselves in the Tukey test at 5%.
* Differs from fresh milt in the Dunnett's test at 5%.

The use of the solution containing glycerol resulted in a lower ($P$<0.05) duration of motility in comparison with the use of methanol, DMSO, and propylene glycol (Table 2). Although glycerol has been detected in the seminal plasma of some teleosts, and thus, its use as a milt cryoprotectant is justified for cold weather fish (5), the current study found that glycerol was not an effective cryoprotectant in terms of maintaining the seminal characteristics of *P. mesopotamicus* and *B. orbignyanus*. Carolsfeld *et al.* (4) suggested the use of DMSO for cryopreservation of milt from five species of Characidae, among them *P. mesopotamicus*, which is consistent with the results in this study.

Primary abnormal morphology in thawed milt was higher ($P$<0.05) when using glycerol compared with methanol, DMSO, and ethylene glycol. There was no difference ($P$>0.05) among the solutions tested in terms of secondary abnormalities. With regards to total abnormal morphology, the use of DMSO led to a lower ($P$<0.05) abnormalities in comparison with the use of glycerol (Table 4).

***B. orbignyanus***

Fresh *B. orbignyanus* milt had a sperm concentration at 48.2 ± 16.8 x$10^9$ spermatozoa /mL. Others reported sperm concentration for this species ranging from 5.4 x $10^9$ (15) to 10.4 x $10^9$ (7). The high concentration observed in this study may be attributed to the different reproductive hormone used in the current study, buserelin, as the other two studies used crude carp pituitary. According to Silva *et al.* (28), the type of hormones used in hormonal induction can affect sperm concentration, since the application of the hormone will act in altering seminal plasma and can, thereby, further dilute the semen, which will present a greater volume, but will be less concentrated (22).

The utilisation of ethylene glycol (13.5 ± 3.53%) and methanol (21.4 ± 9.1%) led to the highest ($P$<0.05) motility rates (Table 1). Maria *et al.* (16) found seminal motility rates of 21 ± 8% and null (0%), using methanol and DMSO, respectively, whilst trying to determine an efficient protocol for cryopreservation of milt for this species. The results show that although DMSO is the most suitable cryoprotectant for preserving the sperm of several other rheophilic fish species (4), it is not able to maintain sperm viability in this species. The solution containing ethylene glycol may be considered an alternative to DMSO, but other concentrations need further testing, with the aim of increasing viability. Menezes *et al.* (17) observed that the use of propylene glycol for cryopreserving the sperm of *C. macropomum* led to a higher rate of motility in comparison with the use of DMSO.

A longer ($P$<0.05) motility duration was observed when using methanol (34.4 ± 16.6 s) and propylene glycol (15.6 ± 3.3 s) compared with the other solutions, which actually led to immobile sperm (Table 2). Cryopreservation can make fish milt of any desired genetic background available to an aquaculture facility.

This can provide a hatchery with the practical advantages of both reducing the dependence on synchronous maturation between sexes and facilitating selective breeding programmes (4).

Primary and total abnormalities in thawed milt did not differ ($P$>0.05) among the treatments (Table 5). This was not the case for secondary abnormalities, which were less common in the treatment that used DMSO compared with those using methanol, propylene glycol and ethylene glycol.

Taddei *et al.* (32) speculated that injuries in spermatozoa arise from the exposure to cryoprotectant solution and/or low temperature of process, especially at the pre-freezing stage. During cryopreservation, spermatozoa are subjected to stress resulting from the interaction between water and the cryoprotectant solution, which leads to the formation of ice crystals (12) that can cause structural damage in or even the death of the sperm cells.

According to Hafez and Hafez (10), in mammalian sperm, secondary abnormalities such as a broken and loose tail or a loose head may be related to smear preparation, while primary abnormalities are related to failures during spermatogenesis. However, in fish, the origin of such abnormalities is unknown, although the same origin could be assumed.

In conclusion, all cryoprotectants used maintained the viability of *P. lineatus* and *P. mesopotamicus* milt. However, with *P. lineatus* milt, glycerol is recommended to ensure a lower percentage of total abnormal morphology. For *P. mesopotamicus* milt, all the cryoprotectants tested may be used for cryopreservation, with the exception of glycerol. For *B. orbignyanus* milt, the cryoprotectants methanol and ethylene glycol are recommended for use in the cryopreservation process, although they reduce the quality of sperm post-rewarming.

**Table 5.** Percentage of morphological sperm changes (mean ± SD) of post-thaw *B. orbignyanus* (n=7).

| Cryoprotectant extenders | Sperm abnormal morphology (%)[1] | | |
|---|---|---|---|
| | Primary | Secondary | Total |
| Methanol | 12.4 ± 1.0$^{A}$ | 9.5 ± 1.1$^{BC*}$ | 22.0 ± 0.3$^{A}$ |
| DMSO | 11.7 ± 1.5$^{A}$ | 4.2 ± 2.5$^{A}$ | 15.8 ± 4.0$^{A}$ |
| Glycerol | 15.5 ± 1.1$^{A}$ | 6.7 ± 0.8$^{AB}$ | 22.2 ± 0.5$^{A}$ |
| Propylene glycol | 12.0 ± 1.3$^{A}$ | 10.8 ± 2.0$^{C*}$ | 22.8 ± 0.9$^{A}$ |
| Ethylene glycol | 17.8 ± 2.7$^{A}$ | 8.2 ± 0.2$^{BC*}$ | 24.7 ± 2.2$^{A*}$ |
| Fresh milt | 10 | 5 | 15 |

[1] Averages followed by different letters, in columns, differ among themselves in the Tukey test at 5%.
* Differs from fresh milt in the Dunnett's test at 5%.

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