TOXICITY, FREEZING AND COLD STORAGE TESTS OF NATIVE SPECIES SEMEN

Tiago Viana da COSTA[1], Lidia Miyako Yoshii OSHIRO[2], Luciana Antunes de MATTOS[2], Laura Susana LÓPEZ GRECO[3], Emanuela Paula MELO[2], Helaine dos Reis FLOR[2]

ABSTRACT

In the first part of this study, toxicity tests were performed on the sperm of *Macrobrachium acanthurus* using four cryoprotectants for periods of 10 and 20 min at concentrations of 10 and 20%. In the second part, cryopreservation was performed by applying the least toxic cryoprotectant, and two freezing methods were tested over 24 hours: automated (protocols A and B) and conventional (protocols C and D). Protocol A exhibited a cooling rate of 0.5°C min^{-1} from -6°C to -32°C; protocol B was similar to A except for the starting temperature, which was room temperature; whereas protocols C and D exhibited a cooling rate of 2 and 10°C min^{-1}, respectively. The third part of the study was conducted to assess the lifespan of the sperm when stored at 5°C, in which sperm viability was evaluated by a semen smear with eosin-nigrosin. The least toxic cryoprotectants were 10 and 20% glycerol, and 10% methanol, and the equilibrium time was 10 minutes. The optimal cooling rate was 2°C min^{-1} for 10% glycerol, which had a sperm survival rate of 21.8%. Cold storage for up to 3 days is recommended, presenting a sperm survival rate of 35.3%.

Key words: caridea; cryopreservation; cryoprotectants; glycerol; methanol; shrimp

TESTES DE TOXICIDADE, CONGELAMENTO E REFRIGERAÇÃO EM SÊMEN DE ESPÉCIE NATIVA

RESUMO

A primeira estapa deste trabalho testou a toxicidade de quatro crioprotetores em espermatozoides de *Macrobrachium acanthurus* durante 10 e 20 min nas concentrações de 10 e 20%. A segunda etapa foi realizar a criopreservação aplicando o crioprotetor com menor grau de toxicidade, testanto dois mecanismos de congelamento, um automatizado (protocolos A e B) e outro convencional (protocolos C e D), durante 24 horas. O protocolo A apresentou velocidade de resfriamento de 0,5°C min^{-1} até alcançar -32°C, partindo de uma temperatura de -6°C e idem o protocolo B, com a diferença de partir de uma temperatura ambiente; os protocolos C e D apresentaram uma velocidade de resfriamento de 2 e 10°C min^{-1}, respectivamente, sendo as palhetas transferidas ao nitrogênio líquido. A terceira etapa foi verificar o tempo de vida do espermatozoide quando refrigerado a 5°C. A viabilidade espermática foi avaliada por meio do esfregaço de sêmen com eosina-nigrosina. Os crioprotetores que se apresentaram menos tóxicos foram o glicerol 10 e 20% e metanol 10%, num tempo de equilíbrio de 10 minutos. A melhor velocidade de congelamento foi a de 2°C min^{-1} para glicerol 10%, com 21,8% de sobrevivência espermática, sendo a refrigeração até três dias recomendada, com uma sobrevivência de 35,3%.

Palavras-chave: caridea; criopreservação; crioprotetores; glicerol; metanol; pitú

Artigo Científico: Recebido em 16/11/2016; **Aprovado em** 13/06/2017

[1]*Universidade Federal do Amazonas, Instituto de Ciências Sociais, Educação e Zootecnia. Estrada Parintins/Macurany, 1805, Jacareacanga, Parintins/AM/Brasil. 69.152-240. E-mail: tvianadacosta@yahoo.com.br (corresponding author)*

[2]*Universidade Federal Rural do Rio de Janeiro, Estação de Biologia Marinha. Rua Sereder, s/n, Itacuruçá, Mangaratiba/RJ/Brasil. 23.860-020*

[3]*Universidad de Buenos Aires, Departamento de Biodiversidad y Biología Experimental, FCEN e IBBEA, CONICET-UBA, Argentina.*

INTRODUCTION

Freshwater prawns of the genus *Macrobrachium* Bate, 1868 are distributed throughout the Americas including Brazil, where they are found from Pará to Rio Grande do Sul States (MELO, 2003; TORATI *et al.* 2011). *Macrobrachium acanthurus* (Wiegmann 1836) is captured and consumed mainly by traditional populations, as well as being a species identified with potential for aquaculture because of its size (VALENTI *et al.*, 1989; ALBERTONI *et al.*, 2002). However, studies on the reproduction of this species are scarce, with their larval stages described by CHOUDHURY (1969), MOREIRA *et al.* (1986), BRAILOVSKY and GALERA (1997) and QUADROS *et al.* (2004), and some aspects of their reproductive biology by CARVALHO (1980), VALENTI *et al.* (1989), MÜLLER *et al.* (1992), ALBERTONI *et al.* (2002) and TAMBURUS *et al.* (2012).

Cryopreservation is a process of storing genetic material in which cells are dehydrated, frozen and thawed. This technology is important for the process of domestication, and may contribute to animal breeding by enabling hybridization processes, preserving population genetics, facilitating sperm translocation between farms, reducing costs associated with the maintenance of breeding animals, and reducing the seasonal limitations of natural reproduction (GWO, 2000; MEDEIROS *et al.*, 2002; AKARASANON *et al.*, 2004; BENSON *et al.*, 2012; TSAI and LIN, 2012).

According to TIERSCH *et al.* (2007), approximately 200 species in aquaculture had their semen cryopreserved for research purposes. This number tends to rise along with increased production demand for these animals, and as a food safety strategy to preserve genetic resources which has been reported by PANIÁGUA-CHAVES *et al.* (2011). According these authors, the Food and Agriculture Organization of the United Nations has contemplated the cryopreservation of germplasm (embryos, sperm, and oocytes) since 2007.

Although decapod crustaceans have great economic importance, cryopreservation techniques and associated information are limited, hindering their commercial industrialization (SUBRAMONIAM, 1994; BENSON *et al.*, 2012). According to ANCHORDOGUY *et al.* (1988), SUBRAMONIAN (1994), LEZCANO *et al.* (2004) and SALAZAR *et al.* (2008), cryopreservation of shrimp semen is hindered by a lack of reliable methods to determine cell survival after thawing, because they are non motile cells, as well as having a limited viability period of the cryopreserved material compared to other animal species.

AKARASANON *et al.* (2004) cryopreserved semen of *Macrobrachium rosenbergii* De Man 1879 at -20°C for 20 days using glycerol and ethylene glycol at concentrations of 10 and 20%, and they observed a decrease in sperm survival starting at the 10th day of storage. Cryopreservation in liquid nitrogen (-196°C) with 20% ethylene glycol also led to a decrease in survival over time, and the authors found a sperm survival rate of 75% at 200th day of storage; however, no embryo was produced with the cryopreserved sperm during this period. Decreased survival was also reported by NIMRAT *et al.* (2006) and UBERTI *et al.* (2013) for *Litopenaeus vannamei* (Boone 1931), and by BAMBOZZI *et al.* (2014), and CASTELO BRANCO *et al.* (2014) for *L. schmitti* (Burkenroad 1936).

Regardless of the technique used, all cryopreservation methods require cryoprotectants: chemical compounds that protect cells against the damage caused by freezing and thawing (FAHY *et al.*, 1990). Nevertheless, PARKS and GRAHAM (1992) mentioned that during the freezing and thawing processes, cell membranes are compromised because the lipid bonds required for normal functioning are disrupted. According to NIEMANN (1991) and BEIRÃO *et al.* (2006), cryoprotectants can be classified in two groups: intracellular (organic solutes responsible for protecting cell organelles during cooling), and extracellular (macromolecules and sugars responsible for decreasing ice formation, facilitating cell dehydration, and protecting the cell membrane).

BENSON *et al.* (2012) emphasized that an important factor to be considered is the high concentration of cryoprotectants, because it has been shown to be cytotoxic. Thus, toxicity tests specific for each cell type can assist in the optimization of cryopreservation processes, because membrane permeability varies among species (HOLT, 2000). GWO (2000) stated that the choice of cryoprotectant should be in accordance with the crustacean species, and indicated that the temperature at which liquid nitrogen freezes varies with the type of equipment used, size of the straw or cryotube, and position of the sample in the cryogenic tank.

Considering that studies on the cryopreservation of freshwater shrimp semen are restricted to *M. rosenbergii* (CHOW, 1982; CHOW *et al.*, 1985;

AKARASANON *et al.,* 2004), the aim of this study was to test the toxicity of four cryoprotectants (dimethyl sulfoxide - DMSO, glycerol, methanol, and ethylene glycol) on the spermatophores of *M. acanthurus*, a Brazilian native species, as well as to test two cryopreservation systems (conventional and automated), and a cold storage process through the analysis of sperm viability.

METHODS

Males of *M. acanthurus* were captured in the summer months of 2013 and 2014 at the Sahy River (22°56'S, 44°01'W) and transported alive to the Marine Biology Station of the Federal Rural University of Rio de Janeiro (UFRRJ), where they were individually acclimated for one week in 42-L tanks for the toxicity, cryopreservation, and cooling tests. The temperature was monitored daily with a digital thermometer, whereas the ammonia and nitrite concentrations were monitored every two days with the aid of a multiparameter device (Hanna Instruments Brazil, model HI 83203, São Paulo, Brazil). The daily food supply was composed of fish/squid muscle and reproducers feed (50% crude protein) at a ratio of 1:1 and rate of 10% live weight. The water was replaced every two days.

The total length and cephalothorax length of the shrimps were measured with a digital caliper (0.01 mm), and the animals were weighed with an analytic scale (0.1 mg). The spermatophore was extracted by electrostimulation (6.0 volts), and weighed individually in an analytic scale. Each shrimp was stimulated only once, therefore the spermatophores used in this experiment were produced in the wild.

Toxicity test

A total of 90 males of *M. acanthurus* were used for this experiment. Initially, the spermatophores of 70 shrimp were distributed among the treatments, which consisted of dimethyl sulfoxide (Sigma D4540), ethylene glycol (Sigma-Aldrich 324558), glycerol (Sigma G6279), and methanol (Sigma-Aldrich 494437) at concentrations of 10 and 20% with an equilibrium time of 10 minutes, and 12 replicates per treatment. Cryoprotectant solutions were prepared using distilled water as the diluter following CHOW *et al.* (1985). Spermatophores were distributed so that those from the same shrimp were not in the same treatment, or in a treatment with the same cryoprotectant at a different concentration.

Spermatophores were placed in 2.0-mL plastic microtubes containing 0.5 mL cryoprotectant solution. After reaching equilibrium at room temperature (25°C), the spermatophores were removed from the solution, rinsed twice with distilled water, and later transferred to another microtube containing 0.5 mL distilled water. The spermatophores were then macerated until complete rupture of the material, and homogenized with the diluter to form a sperm solution. A semen smear stained with eosin-nigrosin (Sigma E6003 and N4763) was prepared for the sperm survival test (BAMBOZZI *et al.,* 2014).

Data were subjected to a Shapiro-Wilk test to evaluate normality, Levene test to evaluate homoscedasticity, and an analysis of variance (ANOVA) and t-test at the 5% level of significance to assess whether there was a significant difference among the treatments. For the treatments that did not show significant differences, but indicated good sperm survival, the same tests were repeated at an equilibrium time of 20 minutes following the same procedures described above with the remaining 20 shrimp captured in the Sahy River. After the toxicity tests, the optimum results were used for the cryopreservation test.

Cryopreservation and cold storage tests

A total of 80 males of *M. acanthurus* were used in the experiments: 45 for the cryopreservation tests and 35 for the cold storage test.

The cryopreservation tests were performed in two freezing systems: the first system used an automated device (TK *Equipamentos para reprodução*, model TK 3000 compact SE, Uberaba, Brazil), and the second used a conventional cryopreservation device (polystyrene box - 45.5 x 34.0 x 34.0 cm).

The spermatophores were placed in 2.0-mL plastic tubes containing cryoprotectant solution, and maintained at room temperature for 10 minutes for stabilization (equilibrium time) in 0.5-mL French straws (IMV®). After this period the straws were placed in the freezing devices.

The devices used in the automated system had different protocols, and different temperatures for semen and embryos cryopreservation. Two freezing protocols, A and B (Table 1), were selected. Protocol A was used by BAMBOZZI *et al.* (2014) and CASTELO BRANCO *et al.* (2014) for *L. schmitti*. In this protocol, cryopreservation was performed in two stages. After an initial stabilization at -6°C, the

straws containing the spermatophores and different cryoprotectant solutions were added. After two minutes, the seeding (cristalization) was performed, at that point the device was stabilized for 10 minutes. The temperature was then reduced from -6°C to -32°C at a cooling rate of 0.5°C min^{-1}. The samples remained in the device for an additional five minutes before being transferred to liquid nitrogen (-196°C). Protocol B followed the same procedures as protocol A; however, the temperature was cooled down to -6°C starting from room temperature, and the straws were positioned on the device.

Table 1. Protocols used in the automated system for the cryopreservation of spermatophores of *Macrobrachium acanthurus*.

	Protocol A	Protocol B
Start	from the 1st plateau	from room temperature
1st Ramp	-	3°C min^{-1}
Temperature 1st plateau	-6°C	-6°C
Seeding	2 min	2 min
Time for 2nd ramp	10 min	10 min
2nd Ramp	0.5°C min^{-1}	0.5°C min^{-1}
Temperature 2nd plateau	-32°C	-32°C
Time for 2nd plateau	5 min	5 min
Transference to LN_2	-196°C	-196°C

For cryopreservation in the conventional system (non-controlled freezing protocols), an adaptation of the procedure of AKARASANON *et al.* (2004) was used. The samples were transferred to straws that were vertically attached to the support, 10 cm apart the straws from the liquid nitrogen surface, and 2 cm among the straws, totaling 32 frozen samples. These samples were subjected to a pre-freezing process that started at room temperature and eventually reached -50°C; subsequently, with a cooling rate that averaged 2°C min^{-1} (protocol C), and 10°C min^{-1} (protocol D). After temperature stabilization, the straws were submerged in liquid nitrogen and stored in a cryogenic tank. The treatments for the cryopreservation test consisted of four different protocols (A, B, C and D) with six replicates each, and the cryoprotectant solutions included those that showed the best results in the toxicity test.

The samples were thawed 24 hours after cryopreservation by submerging the straws in water at 30°C for four minutes (Chow *et al.*, 1985). After thawing, the cryoprotectant solutions were removed by rinsing the spermatophore with distilled water. Sperm survival was evaluated using eosin-nigrosin staining.

For the cold storage test, the spermatophores were stored in 2.0-mL plastic microtubes containing 0.5 mL of distilled water without cryoprotectants for 10 days. Time 0 (zero) was considered the control, and the material was freshly analyzed. After an equilibrium time of 10 minutes at room temperature, the samples were placed in a refrigerator and maintained at 5°C. Six replicates were used for each day of the test for a total of 66 spermatophores. Sperm viability was also analyzed with a sperm smear stained with eosin-nigrosin.

The data were subjected to a Shapiro-Wilk normality test, Levene test to evaluate homoscedasticity and ANOVA (α=0.05) to test the significance of the cryopreservation curves as well as the cooling time.

RESULTS

Toxicity test

The mean water temperature during the acclimatization period was 24.00 ± 1.2°C, ranging from 21.6 to 25.8°C. The ammonia and nitrite levels were 1.1 ± 0.1 ppm and 0.7 ± 0.3 ppm, respectively, and the mean pH was 6.9 ± 0.4, ranging from 6.3 to 7.3.

The mean length and range of the cephalothorax were 25.6 ± 4.0 mm, and 18.7 to 32.9 mm, respectively; the mean total length of the shrimp was 83.3 ± 15.5 mm, and the mean weight and range of the shrimp was 12.2 ± 5.5 g and 3.8 to 26.2 g, respectively. Of the 90 shrimps used in the experiment, 85.0% produced spermatophores (75.5% of these produced two spermatophores), which had a mean weight of 1.1 ± 0.6 mg.

As observed in Table 2, the best survival results were obtained with 10% methanol regardless of equilibrium time ($p<0.05$), however this index decreased significantly at a concentration of 20% and equilibrium time of 20 minutes, which was considered toxic and unfeasible.

At an equilibrium time of 10 minutes, 10% and 20% glycerol showed similar results ($p<0.05$) that methanol and were not considered toxic to sperm. However, when the semen was exposed to these concentrations for an equilibrium time longer than 10 minutes, sperm survival decreased significantly. Ethylene glycol and DMSO were toxic to the sperm of *M. acanthurus* in the conditions tested in this

Table 2. Sperm survival according to equilibrium time, concentration, and cryoprotectant solutions used in the toxicity test for *Macrobrachium acanthurus*.

	10 min		20 min	
	[10%]	[20%]	[10%]	[20%]
Methanol	71.9 ± 2.7^{aA}	49.8 ± 4.9^{aB}	74.9 ± 3.5^{aA}	-
Glycerol	65.7 ± 3.4^{aA}	68.2 ± 3.4^{aA}	26.8 ± 8.9^{bB}	23.7 ± 7.1^{B}
Ethylene glycol	29.5 ± 6.6^{bB}	23.7 ± 8.8^{bB}	-	-
DMSO	24.4 ± 8.9^{bB}	30.2 ± 6.6^{bB}	-	-

Means followed by the same letters (lowercase in column and uppercase in row) do not differ ($p>0.05$).

Cryopreservation and cold storage tests

The mean water temperature and range during the acclimatization period were 26.8 ± 1.7°C and 23.6 to 30.2°C respectively. The ammonia and nitrite levels were 2.7 ± 1.5 ppm and 0.7 ± 0.8 ppm, respectively, and the pH was 7.3 ± 0.3.

The mean length and range of the cephalothorax were 24.4 ± 3.2 mm and 17.7 to 32.18 mm, respectively, and the mean total length of the shrimp was 72.9 ± 7.8 mm. The mean weight and range were 9.8 ± 3.4 g and 3.4 to 17.7 g, respectively. The mean spermatophore weight was 0.6 ± 0.5 mg. The mean sperm survival rate of spermatophores cryopreserved for one day (24 hours) is shown in Figure 1. No sperm survived (0%) in the samples frozen with 10% methanol. The sperm survival of samples cryopreserved in glycerol were significantly lower than that of the samples analyzed while fresh (control - 76.5 ± 2.1%), regardless of the concentration used; therefore glycerol was also considered toxic.

In the automated cryopreservation system, protocol A (10% glycerol - 11.2 ± 9.0% and 20% glycerol - 9.74 ± 5.51%) showed similar results as protocol B (10% glycerol - 4.0 ± 3.1% and 20% glycerol - 11.1 ± 11.1%). The use of 10% glycerol in protocol B produced the lowest rate of sperm survival ($p<0.05$).

For the conventional cryopreservation system, 10% glycerol showed a higher sperm survival ($p<0.05$) in both protocols. Protocol C (21.8 ± 15.2%), which had a cooling rate of 2°C min^{-1} showed better results ($p<0.05$) than protocol D (3.4 ± 3.3%), which had a cooling rate of 10°C min^{-1}. However, 20% glycerol had a significantly reduced sperm survival rate (protocol C - 0.2 ± 0.2%; protocol D - 0.1 ± 0.1%) compared to that of 10% glycerol in both curves.

In the cold storage test, sperm survival was significantly low ($p<0.05$) starting at the third day of storage (27.0%) compared with the survival rate of the control (76.5%) (Figure 2). However, after the first day of cold storage, sperm survival decreased by 42.0%, although this rate was not statistically significant ($p>0.05$).

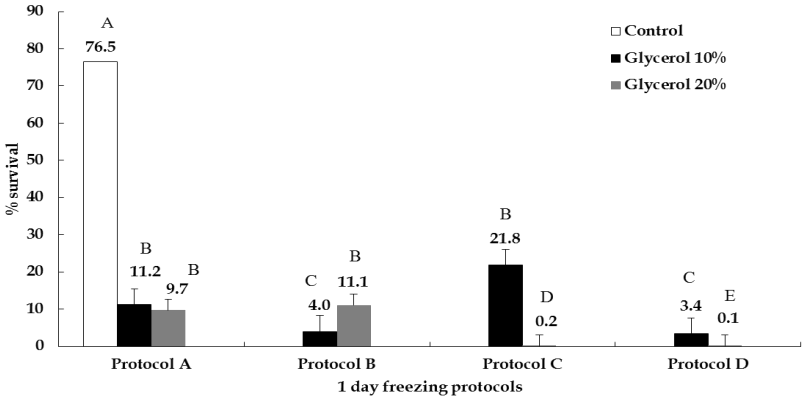
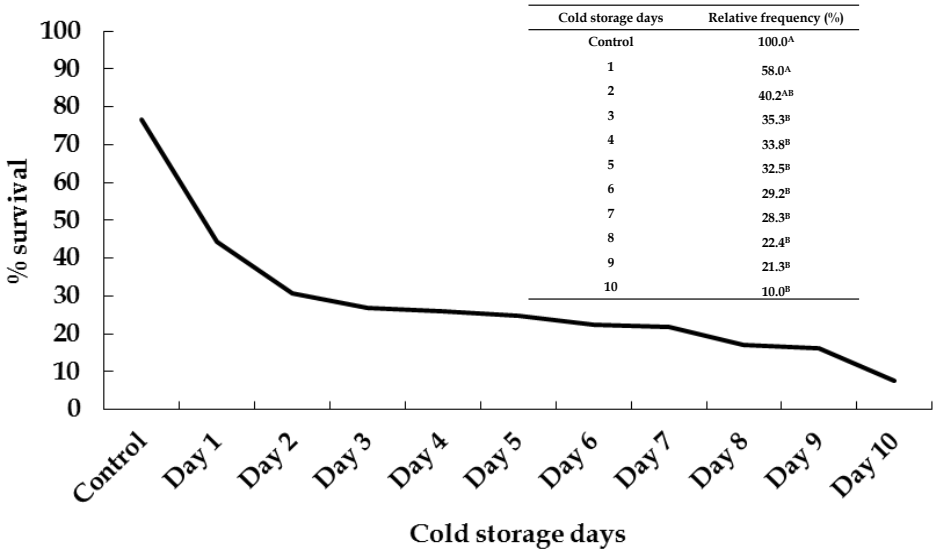


Figure 1. Percentage of sperm survival for spermatophores of *Macrobrachium acanthurus* cryopreserved over 24 hours in 10 and 20% glycerol. The same letters indicate no significant difference ($p>0.05$) when comparing protocols A and B (automated system), and C and D (conventional system).



Cold storage days	Relative frequency (%)
Control	100.0^{A}
1	58.0^{A}
2	40.2^{AB}
3	35.3^{B}
4	33.8^{B}
5	32.5^{B}
6	29.2^{B}
7	28.3^{B}
8	22.4^{B}
9	21.3^{B}
10	10.0^{B}

Figure 2. Mean percentage and relative frequency of sperm survival for spermatophores of *Macrobrachium acanthurus* throughout 10 days of cold storage without cryoprotectant.

DISCUSSION

Toxicity test

Cryoprotectants minimize the deleterious processes that occur in the sperm cell during freezing and thawing. However, few studies have been performed to determine the optimal concentrations for the cryopreservation of freshwater shrimp semen, which is likely because the effect of these substances are species-specific (GWO, 2000), thus limiting research to *M. rosenbergii,* because it is the most important species in the market.

According to KASAI (1996) fast penetrating agents are more favorable, because they require a shorter exposure time to the cryoprotectant before rapid cooling of the sample, which prevents osmotic damage. Intracellular cryoprotectants have colligative properties that reduce the intracellular cryoscopic point, therefore less water remains in the liquid state under low temperatures, thus decreasing the intracellular solute concentration and providing a less deleterious environment to the sperm cell during freezing (WATSON, 1995). SPINDLER *et al.* (2012) mentioned that cell viability is higher in the presence of an intracellular cryoprotectant, and depends on the type and concentration of this substance, and thawing temperature.

HARVEY and ASHWOOD-SMITH (1982), and HARVEY and CAROLSFELD (1993) indicated that methanol most easily penetrates the cell membrane, because of its low molecular weight, however it is also considered the most toxic. In this study, the toxicity of methanol was indicated during the cryopreservation process based on a mortality rate of 100% after 24 hours. This result corroborates the findings of RAPATZ (1973), who studied the effect of methanol on frogs and determined that it was toxic at temperatures close to the freezing point, because its concentration inside the cell increased as the water froze (MERYMAN *et al.,* 1977). Similar results were also reported by BHAVANISHANKAR and SUBRAMONIAM (1997) for the swimming crab *Scylla serrata* (Forskal, 1775). However, 10% methanol showed the most satisfactory results for the cryopreservation of embryos, nauplii, zoea, and sperm of penaeid shrimp (NEWTON and SUBRAMONIAN, 1996; GWO and LIN, 1998; LEZCANO *et al.,* 2004).

PARKS and GRAHAM (1992), and ANGOLA (1994) mentioned that glycerol penetrates and protects the cell membrane by reducing the number of pores and ATP-dependent functions, thus promoting protein aggregation and the formation of lipid blocks. According to JEYALECTUMIE and SUBRAMONIAM (1989), and BHAVANISHANKAR and SUBRAMONIAM (1997), glycerol is adequate for cryopreservation because it is a natural intermediate product in lipid metabolism. Despite its essential effects in the cell cryopreservation process, glycerol can also produce deleterious effects to sperm because its degree of toxicity is dose dependent. Therefore, the dose should be between the minimum required

to promote protection, and the maximum allowed to prevent damage to the sperm (PARKS and GRAHAM, 1992; ANGOLA, 1994; COTORELLO and HENRY, 2002).

In this study, glycerol was not toxic at the concentrations tested except when sperm was exposed for more than 10 minutes. Similar results were reported by CHOW *et al.* (1985), who tested 10% glycerol for *M. rosenbergii* using seven exposure times. This toxic effect might be related to a change in the cell membrane fluidity caused by an intercalation in the lipid bilayer, which may change the cytoplasmic viscosity and affect all metabolic reactions (HAMMERSTEDT and GRAHAM, 1992; McLAUGHLIN *et al.*, 1992). FAHY (2010) mentioned that cells exposed to cryoprotectants for a long time began to degrade and generate toxic metabolites, thus limiting their use. CASTELO BRANCO *et al.* (2014) did not report toxicity at 5 and 10% glycerol with an equilibrium time of 10 minutes for the cryopreservation of *L. schmitti* sperm, obtaining a viability of 79.8%, which is a higher rate than the results of this study.

According to ANCHORDOGUY *et al.* (1987) and DE LEEUW *et al.* (1993), the lipid bilayer interacts more weakly with glycerol than with sucrose and trehalose, which are extracellular cryoprotectants. These authors also mentioned that the interaction with disaccharides is more effective in stabilizing the lipid bilayer than the interaction with monosaccharides, mainly because disaccharides connect to the lipid bilayer by hydrogen bonds, which causes an expansion of the layers, decreases the gelphase transition temperature, minimizes the fusion of the adjacent layers, and stabilizes the process starting at low concentrations. The connections promoted by hydrogen bonds are so strong that they remain intact throughout the entire freezing process.

Therefore, an interaction between these cryoprotectants could further stabilize the cell membrane during the entire freezing process, and reduce the required concentrations of intracellular cryoprotectants, thus reducing toxicity and optimizing the cryoprotectant activity (DENNISTON *et al.*, 2000). According to STOREY and STOREY (1996), freezing without the use of cryoprotectants can produce osmotic shock or volume collapse, because of the permanent gel state of the lipid bilayer.

According to AKARASANON *et al.* (2004), ethylene glycol has lower molecular weight and toxicity than glycerol and higher cell permeability. This cryoprotectant is capable of preserving the acrosome, which might indicate a better preservation of other sperm structures, such as the nucleus, essential for perfect embryo development. The authors also reported that such protection can be explained because of ethylene glycol's cell penetration rate, decreasing the high salt concentration in the sperm interior, a harmful feature for the sperm membrane (HOLT *et al.*, 1992; MORAES *et al.*, 1998).

Despite these favorable characteristics, ethylene glycol did not show satisfactory results for *M. acanthurus* compared to glycerol and methanol, which may be explained by the concentration or equilibrium time. AKARASANON *et al.* (2004) used ethylene glycol and glycerol in concentrations of 10 and 20% with an equilibrium time of 60 minutes, and they observed similar toxicity results, which are inconsistent with this study. However, these authors noted a decrease in sperm quality when the spermatophores were cryopreserved with 20% ethylene glycol at -196°C for a long period. UBERTI *et al.* (2013) used ethylene glycol and 10% DMSO with an equilibrium time of 10 minutes for the cryopreservation of *L. vannamei* semen and did not report a significant difference between these two cryoprotectants in terms of sperm survival.

DMSO is a widely used cryoprotectant with high cell permeability. However, its permeability alone does not explain its cryoprotectant effect because DMSO is known to be toxic at high concentrations, despite preserving the integrity of isolated proteins and phospholipids of membranes (ANCHORDOGUY *et al.*, 1991). The toxicity of DMSO at room temperature has been reported by JEYALECTUMIE and SUBRAMONIAM (1989). The negative results reported here for this cryoprotectant were likely produced because of the temperature during the process of strawfilling, room temperature, increasing the potential damage to the sperm during the freezing process, time of exposure to cryoprotectant (10 minutes), or concentration applied (10%). VUTHIPHANDCHAI *et al.* (2007) found a sperm survival rate of less than 20% when using 15% DMSO, and complete mortality when using 20% DMSO in spermatophores of *Penaeus monodon* Fabricius, 1798. These authors also reported that longer equilibrium time increased the sperm mortality, therefore lower concentrations and shorter equilibrium periods might increase the viability of this cryoprotectant.

However, DMSO was successfully used in the cryopreservation of *S. serrata* (JEYALECTUMIE and SUBRAMONIAM, 1989), marine shrimp (ANCHORDOGUY *et al.*, 1988; DIWAN and JOSEPH, 1999; VUTHIPHANDCHAI *et al.*, 2007), and lobster (SASIKALA and MEENA, 2009) at concentrations lower than those of this study or in association with extracellular cryoprotectants, which was consistent with the findings of FAHY (1986). For *Limulus polyphemus* (Lineu, 1758), BEHLMER and BROWN (1984) did not observe sperm survival using DMSO as a cryoprotectant.

Cryopreservation and cold storage tests

The automated semen cryopreservation system has been used for several animal species, including vertebrates (LEITE *et al.*, 2010; ABUD *et al.*, 2014) and invertebrates (BHAVANISHANCAR and SUBRAMONIAM, 1997; VUTHIPHANDCHAI *et al.*, 2007; BAMBOZZI *et al.*, 2014; CASTELO BRANCO *et al.*, 2014).

The cooling rate may have been the dominant factor for the reduced survival in conventional cryopreservation methods, considering that protocol C was significantly better than protocol D. This reduced survival rate may have resulted from damage caused by the formation of intracellular ice crystals as a consequence of inadequate cell dehydration during the fast freezing period, thus increasing the sensitivity of the cell membrane to osmotic changes. According to MAZUR (1984) and ANGOLA (1994), if the cooling rate is fast (higher than 10-20°C min^{-1}), intracellular water does not have time to leave the cell, and it forms ice microcrystals. These microcrystals are unstable and cluster to form large crystals during thawing, thereby leading to cell death.

A comparison of the cooling rates between the conventional and automated systems showed that the automated system (0.5°C min^{-1}) might have cooled too slowly, thus leaving the cryoprotectant solution in contact with the semen for too long before freezing, which was lethal to sperm. However, according to SANTOS (2000), if freezing is slow, damage is primarily caused by excessive dehydration or extensive ice formation outside the cells. In these cases, membrane rupture, solutes concentrations in the cytoplasm reaching toxic levels, and nucleic acid and membrane denaturation might occur. Therefore, cell freezing should take place as quickly as possible to avoid the effects of cryoprotectants but also slowly enough for cells to dehydrate (MAZUR *et al.*, 1972; MEDEIROS *et al.*, 2002).

BAMBOZZI *et al.* (2014) used the same cooling rate (0.5°C min^{-1}) with 10% glycerol for *L. schmitti* and obtained better results than those of this study. ANCHORDUGUY *et al.* (1988) obtained a post-thaw sperm survival rate of 88% for *Sicyonia ingentins* (Burkenroad, 1938) using a cooling rate of 1°C min^{-1}; however, cell lysis occurred at cooling rates higher than 5°C min^{-1}. With a 10°C min^{-1}, these authors observed a sperm survival rate that was similar to the results of this study. Similarly, LEZCANO *et al.* (2004) reported that a cooling rate of 10°C min^{-1} caused the destruction of all cells of *L. vannamei*. Similarly CHOW *et al.* (1985) reported a negative effect of fast cooling on the sperm survival in *M. rosenbergii*.

Although conventional cryopreservation systems have low costs compared with automated systems, ABUD *et al.* (2014) mentioned that cryopreserved semen might be of lower quality, because of temperature variations during the freezing curve and other factors, such as the quality and dimensions of the Polystyrene box, and nitrogen levels. However, because the experiment in this study was performed in a laboratory with no external temperature variations, the curve might not have undergone great variations, which might have positively affected the results.

Another factor of great importance is the semen thawing protocol. The protocol adopted in this study (30°C for 4 min) is similar to the protocol used by AKARASANON *et al.* (2004) for *M. rosenbergii* (30°C for 5 min) and VUTHIPHANDCHAI *et al.* (2007) for *P. monodon* (30°C for 2 min); however, the results were not positive, which is inconsistent with those studies. UBERTI *et al.* (2013) and CASTELO BRANCO *et al.* (2014) used protocols of 20-25°C for 40 seconds and 20°C for 10 seconds, and reported excellent sperm survival rates for *L. vannamei* and *L. schimitti*, respectively. BHAVANISHANKAR and SUBRAMONIAM (1997) thawed semen of *S. serrata* at 55°C for 10-15 seconds and obtained positive sperm survival results. According to GWO (2000), increasing the rate of the thawing process for aquatic invertebrate semen improves the fertility.

Studies comparing the importance of thawing temperature and time on sperm quality revealed that higher temperatures associated with shorter thawing times promoted adequate post-thawing quality (PEÑA and LINDE-FORSBERG, 2000; MADEIRA *et al.*, 2010). According to SODESQUIST *et al.* (1997), fast thawing reduces the formation of intracellular ice crystals and decreases stress in the sperm cell. Conversely, slow thawing exposes the

sperm cell to higher temperature oscillations, leading to an increase in lipid and protein deposition, and directly affecting the physical structure of the cell, thus reducing sperm vitality (PEÑA and LINDE-FORSBERG, 2000).

Regarding cold storage, CHOW (1982) refrigerated the spermatophore of *M. rosenbergii* and stated that temperatures between 2 and 7°C do not appear to require the presence of cryoprotectants, and are successful for storage periods of up to four days. Similar results were obtained by ISHIDA *et al.* (1986) for lobsters of the genus *Homarus* Weber, 1795. These results are similar to those reported here, because sperm survival only decreased significantly after the third day. Whereas the object of this study is native species being found in estuarine areas, which have been undergoing intense estate speculation, the data found here are relevant to the maintenance of theses crustaceans.

CONCLUSIONS

To develop a protocol for the cryopreservation of spermatophores of *M. acanthurus*, the best cryoprotectant must be determined along with its concentration, maximum and minimum exposure time, and freezing and thawing rates. In this study, 10% glycerol, although toxic, was the best cryoprotectant at an equilibrium time of 10 min. However, further studies should be performed to assess its toxicity at lower concentrations, possible combinations of glycerol with extracellular cryoprotectants, and different equilibrium times. The best freezing rate was 2°C min^{-1}. Additional studies should be performed to determine the optimal thawing temperature and time, because these parameters affect final sperm viability.

Regarding the freezing method, although the automated system is considered more reliable, the conventional system can be used as an alternative because of its low cost, as long as the operator is fully trained to perform the procedure. Despite the efforts to cryopreserve spermatophores of freshwater shrimp, the use of cold storage at 5°C for up to 3 days was the most promising method in this study.

ACKNOWLEDGMENTS

I would like to thank everyone who contributed to this study as well as the Amazonas Research Foundation (FAPEAM - Proc. 2801/2011) for the doctoral scholarship granted and Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES - Proc. BEX 4919/14-9) for their support during the split-site PhD program.

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