

CRYOPRESERVATION AND SPERM STORAGE OF THE WHITE SHRIMP*

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ABSTRACT

This study was performed to evaluate a protocol for sperm cryopreservation of the marine shrimp *Litopenaeus schmitti*, an important species in Brazilian commercial fisheries. No studies or protocols were found for cryopreservation of its spermatid mass. This paper provides information about the technique of semen storage based on protocols applied to other penaeids species. Two cryoprotectants, glycerol and dimethylsulfoxide (DMSO), were tested for sperm cells toxicity in two concentrations (5 and 10%), and two exposure times (10 and 30 minutes). Influence of liquid nitrogen storage in apparent sperm viability (ASV) was also tested in 1, 15 and 30 days of storage. The cryopreservation was performed in a two-step (2 and 0.5°C min⁻¹) freezing protocol. After freezing until the temperature -32°C, sperm mass was immersed in liquid nitrogen (-196°C). Thus, glycerol and DMSO was not toxic to sperm in both concentrations and equilibration times. For all toxicity tests ASV was up to 79.8% after exposure. Liquid nitrogen storage tests indicated no significant difference between glycerol 5 and 10%. Results were significantly different over time between days 15 and 30 of storage in liquid nitrogen, in which ASV was around 42.8% and 16.3% respectively. Thus, it is suggested glycerol 5% (10 minutes) as cryoprotectant for *L. schmitti* spermatid mass cryopreservation. It is also suggested that the *L. schmitti* spermatid mass can be stored during 15 days in liquid nitrogen, using a two-step (2 and 0.5°C min⁻¹) freezing protocol.

Keywords: Dimethylsulfoxide; glycerol; *Litopenaeus schmitti*; marine shrimp; sperm

CRIOPRESERVAÇÃO E ESTOCAGEM DO SÊMEN DO CAMARÃO BRANCO

RESUMO

Este estudo foi realizado para avaliar um protocolo de criopreservação do sêmen do camarão marinho *Litopenaeus schmitti*, uma espécie importante para a pesca comercial no Brasil. Não foram encontrados estudos ou protocolos de criopreservação de sua massa espermática. Este estudo fornece informações sobre a técnica de armazenamento de sêmen com base em protocolos aplicados a outras espécies de peneídeos. Dois agentes crioprotectores, glicerol e dimetilsulfóxido (DMSO), foram testados quanto à toxicidade para as células espermáticas em duas concentrações (5 e 10%) e dois tempos de exposição (10 e 30 minutos). A influência da manutenção em nitrogênio líquido sobre a viabilidade espermática aparente (VEA) foi também testada em 1, 15 e 30 dias de armazenamento. Para criopreservação, utilizou-se um protocolo de congelamento em duas etapas (2 e 0,5°C min⁻¹). Após o resfriamento até a temperatura de -32°C, a massa espermática foi imersa em nitrogênio líquido (-196°C). Assim, glicerol e DMSO não foram tóxicos para o esperma em ambas as concentrações e tempos de equilíbrio. Para todos os testes de toxicidade a VEA foi de até 79,8% após a exposição. Os testes de armazenamento em nitrogênio líquido não indicaram diferença significativa utilizando glicerol 5 e 10%, mas houve diferença significativa entre os dias 15 (42,8%) e 30 (16,3%), para a VEA. Assim, sugere-se a utilização do glicerol 5 ou 10% (10 minutos) como crioprotetor para a criopreservação de massa espermática do camarão *L. schmitti*. Também é sugerido que a massa espermática do *L. schmitti* pode ser armazenada durante 15 dias em nitrogênio líquido, utilizando-se um protocolo de congelamento em duas etapas (2 e 0,5°C min⁻¹).

Palavras chave: Dimetilsulfóxido; glicerol; *Litopenaeus schmitti*; camarão marinho; esperma

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INTRODUCTION

The white shrimp *Litopenaeus schmitti* presents interesting features for shrimp farming, with satisfactory growth at lower salinities. This species is largely captured in Brazilians coast and has good acceptance in its consumer market. However, it is not known commercial breeding systems of *L. schmitti* in Brazil. Semen cryopreservation and long-term storage are biotechnologies useful to perform several processes such as hybridization, genetic resources preservation and gene selection. It can also support breeding programs in species of economic interest such as *L. schmitti*. Furthermore, semen storage in liquid nitrogen ensures cellular stability allowing steady supply of sperm. In breeding programs can exclude reproduction seasonal limitations and reduce manpower, space and storage cost of breeders (GWO, 2000). Studies on cryopreservation of invertebrate sperm are underexplored. CHOW *et al.* (1985) are the forerunners of cryopreservation studies in shrimps, but studies on penaeids are also scarce, especially in Brazilian native species.

During freezing and thawing, cell damage is due to the destructive action of concentrate salt solution which cells are exposed when the water is removed as ice (JEYALACTUMIE and SUBRAMONIAM, 1989). There are several cryoprotectants with the function of preventing such damage to the cell wall during cryopreservation. Glycerol and dimethylsulfoxide (DMSO) are used in protocols for semen and embryo cryopreservation of different species. For penaeids sperm cryopreservation, glycerol and DMSO had good results in many studies (LEZCANO *et al.*, 2004; VUTHIPHANDCHAI *et al.*, 2005; 2007; BART *et al.*, 2006; SALAZAR *et al.*, 2008).

The freezing and thawing speed, as well as methods of cryopreservation of spermatophore has a relevant effect on sperm viability. Several protocols have been tested for species of crustaceans, molluscs and fish, and results were quite varied. However it is agreed that the pre-freezing and gradual cooling of the material ensures better survival of sperm cells after thawing (CHOW *et al.*, 1985; JEYALACTUMIE and SUBRAMONIAM, 1989; AKARASANON *et al.*, 2004). SALAZAR *et al.*

(2008) used a programmable equipment for semen and embryo freezing during cryopreservation of the shrimp *L. vannamei* spermatophore mass, a cooling rate of $0.5^{\circ}\text{C min}^{-1}$ until the temperature of -32°C , and then the sperm masses were transferred to liquid nitrogen, reaching the final temperature of -196°C .

No studies or protocols were found for cryopreservation of spermatophore mass of *L. schmitti*. This paper provides information about the technique of semen storage based on protocols applied to other penaeids species. The objective was to evaluate the effect of the cryoprotectants toxicity and the influence of storage time in liquid nitrogen on the survival of *L. schmitti* sperm cells.

MATERIALS AND METHODS

This study was performed in two phases, in the first one was tested the toxicity effect of cryoprotectants over sperm cells apparent viability. The second trial was the cryopreservation test for 1, 15 and 30 days in liquid nitrogen.

Experiment I

In both experimental phases, *L. schmitti* sexually mature wild males were captured from Sepetiba Bay, Rio de Janeiro, Brazil. Shrimps were randomly divided into 500 L polyethylene tanks in a semi-closed water system circulation. They were allocated at a density of 8 shrimp m^{-2} and acclimated for one week in experimental conditions now described: artificial photoperiod of 14 hours of light throughout acclimation and whole experimental period; experimental diet of commercial feed 45% protein, fresh food (sardines, squid and mussels) and *Artemia* biomass offered to satiation 3 times a day. Males ($n = 40$) body weight and total length mean (\pm SD) was $17.9 (\pm 4.1)$ g and $11.7 (\pm 1.0)$ cm, respectively. Shrimps were individually tagged using colorful rings placed on one or both ocular peduncles. Water temperature was kept at $27 \pm 0.33^{\circ}\text{C}$, salinity 33, pH 7.2 ± 0.01 , and dissolved oxygen $\sim 7 \text{ mg L}^{-1}$.

To ensure homogeneity between sperm samples, it was measured the total sperm count of all sperm masses using a hemocytometer. The total sperm cells value was calculated using the

following formula: Cells /mL = Σ Total sperm cells x dilution x 10⁴.

Motility and vigor tests are not used to analyze Penaeids spermatozoa because they are non-motile. Therefore, apparent sperm viability (ASV) for all treatments and control in both experiments was evaluated using a modified eosin-nigrosin (JEYALACTUMIE and SUBRAMONIAM, 1989) staining technique. It was added 25 μ L of 0.5% eosin and 25 μ L of 10% nigrosin in 50 μ L of sperm-cryoprotectant solution. After homogenization, 100 μ L of the resulting colored solution, was used to prepare a smear on a microscope slide, which was air-dried before being examined using an optical microscope with a 40x. The average percentage for ASV was calculated by counting the live and dead cells, at least 100 cells per slide, with two replicates per sample. Dead cells were stained to eosin (pink), whereas live cells were translucent, being translucent in contrast with the dark background of nigrosin. In this work it was not evaluated sperm cells irregularly shaped heads or spikes.

To evaluate the effect of glycerol and DMSO on ASV a toxicity test of cryoprotectants was performed with 4 replicates in a 2x2x2 factorial in completely randomized trial, of 2 cryoprotectants, 2 concentrations and 2 times of exposure. Fully mature spermatophores (n = 40) were removed by the compression at the base of the coxa of the fifth pereopod below the opening of the gonopore (ARCE *et al.*, 1999; NIMRAT *et al.*, 2006), from where 36 were selected for the trials. Spermatophores presenting the standard features (JAR, 2005) white color and turgidity were then prepared for toxicity tests (n = 32). Spermatophores were dissected with forceps to remove the spermatid mass that was attached to the wing. Only spermatid masses were used as samples in this study. Each sample was placed randomly in an individual microtube and then were added the cryoprotectants. The cryoprotectants tested in this study were, dimethyl sulfoxide (DMSO) and glycerol diluted in calcium-free saline (VUTHIPHANDCHAI *et al.*, 2007) prepared in two concentrations as 5 and 10% (v/v). All sperm-cryoprotectant solutions were kept at room temperature (25°C) at 10 and 30 minutes equilibration periods. As a control,

spermatid masses (n = 4) were diluted only in calcium-free saline and also tested for ASV.

After toxicity tests ASV was not normally distributed, hence data were analysed after transformation to arcseno root of x. Since data remained not normally distributed a nonparametric analysis of variance (ANOVA) was performed, considering 2 cryoprotectants in 2 concentrations by 2 times of stability and 4 replicates. Kruskal-Wallis post-hoc test was used.

Experiment II

Since there was no significant difference when compared to DMSO in the toxicity tests, glycerol was selected to the cryopreservation assays. To evaluate the influence of liquid nitrogen storage time and cryoprotectant concentration on ASV of *L. schmitti*, was conducted in completely randomized design with a split plot design, where the dilution of cryoprotectant were placed in the plot entirely and times of "freezing" in the parcel split. For cryopreservation, the spermatid masses (n = 36) were transferred to glycerol 5% or 10% at room temperature (25°C) for 10 minutes. Spermatid masses were cooled in a two-step freezing protocol in an automatic cryopreservation equipment (Cryogen Neovet®). The cooling process (2°C min⁻¹) started from room temperature (25°C), until stabilization at -6°C. At this point, straws of 0.50 mL containing the sperm-cryoprotectant solution were inserted in the equipment. After two minutes was performed the seeding procedure and the stabilization process continued for 10 minutes. After it started the second cooling process (0.5°C min⁻¹) from -6°C to the temperature of -32°C and another stabilization process goes on for 5 minutes. Then, straws were removed and immediately immersed in liquid nitrogen, reaching the final temperature of -196°C. This freezing protocol was selected because it is similar to the one used by SALAZAR *et al.* (2008) to perform the cryopreservation of *L. vannamei* sperm. Completed the storage times, straws were removed from liquid nitrogen and immediately immersed in water at 20°C for 10 seconds for thawing. Apparent sperm viability tests were performed using semen smears stained with eosin-nigrosin as previously described.

After cryopreservation test apparent sperm viability data was normally distributed, thus were conducted ANOVA and Tukey post-hoc test. Both statistical analysis were performed using the program SAEG 9.1 (Statistical Analysis System and Genetics) (2007). Data are expressed as means (\pm SD).

RESULTS

Total sperm cells count had an average of $13.7 (\pm 0.72) \times 10^6$ and there was no significant difference between samples. In the toxicity tests percentage of ASV of sperm masses exposed to glycerol and DMSO in concentrations of 5 or 10% was not significantly different ($P > 0.05$) from

control with an ASV average of $94.2 (\pm 0.88)$ percent. Apparent sperm viability did not change significantly with exposure times of 10 or 30 min in both concentrations with a mean (\pm SD) percentage of $97.4 (\pm 1.0)$ to DMSO and $79.8 (\pm 4.2)$ to glycerol. Like most sperm cells studied, *L. schmitti* spermatozoa are sensitive to cooling rate. In cryopreservation trial, the two-step freezing protocol had a fast ($-2^\circ\text{C min}^{-1}$) and a low ($-0.5^\circ\text{C min}^{-1}$) cooling rate. After 1 day of storage in liquid nitrogen, ASV mean (\pm SD) percentage was $50.7 (\pm 1.5)$. Glycerol had no difference between concentrations 5 and 10% ($P > 0.05$) for the different times of storage. However there was significant difference ($P > 0.05$) in ASV for 30 days of storage in liquid nitrogen (Table 1).

Table 1. Mean apparent sperm viability (ASV) of *Litopenaeus schmitti* sperm cryopreserved in 5% or 10% glycerol for 1 day, 15 and 30 days. ^{a-b} Within a column, means without a common superscript differed ($P < 0.05$).

Cryoprotectant	Time (days)		
	1	15	30
Glycerol 5%	52.34 ± 1.5^a	42.92 ± 0.6^{ab}	17.16 ± 0.8^b
Glycerol 10%	49.06 ± 1.6^a	42.64 ± 0.4^{ab}	15.36 ± 1.1^b

DISCUSSION

In the present study, the freezing method developed by SALAZAR *et al.* (2008) was successfully tested for cryopreservation of *L. schmitti* sperm mass. Because glycerol had low toxic effect to shrimp sperm, it was subsequently used at a concentration of 5% and 10% in our cryopreservation trials. However, in the toxicity tests it was not found a significant difference between DMSO and glycerol which can be also observed to *Scylla serrata* and *L. vannamei* (JEYALACTUMIE and SUBRAMONIAM, 1989; SALAZAR *et al.*, 2008). Glycerol at 10 and 20% had high ASV (AKARASANON *et al.*, 2004) in *Macrobrachium rosenbergii* sperm. The ASV of *L. schmitti* sperm in the present study ranged from 78.37% to 97.42%, demonstrating the low toxicity of 5 and 10% DMSO and glycerol. Also contributed to glycerol selection it be a product easily found in the market and has less commercial value compared to other cryoprotectants. Furthermore, cryopreservation studies of penaeid shrimps semen had

satisfactory results using glycerol as cryoprotectants (ANCHORDOGUY *et al.*, 1988; SALAZAR *et al.*, 2008).

Glycerol 10% was tested as cryoprotectant for the spermatophores of *M. rosenbergii* in seven different exposure times of 0, 10, 15, 30, 60, 120 and 180 minutes. It was concluded that the greater exposure time increased the volume and weight of the spermatophore, indicating continuous penetration of cryoprotectant over time (CHOW *et al.*, 1985). Such characteristic implies in the increase of the cryoprotectant toxicity to sperm cells and the effect can vary with different cryoprotectants, for i.e. the toxicity damage can also be observed in low concentrations but with long exposure to cryoprotectant. This fact was not observed in the current study, because used low concentration of cryoprotectants (5 and 10%), same that time (10 and 30 minutes). In *Penaeus monodon* sperm ASV gradually declined over time when exposed to 5% DMSO and the higher concentrations (10, 15 and 20%) had the greater mortality (BART *et al.*, 2006; VUTHIPHANDCHAI

et al., 2007). The same pattern was observed in *Penaeus merguensis* sperm exposed to crescent equilibration times (5, 15, 30 and 60 min) and concentrations (5, 10, 15 and 20%) of different cryoprotectants (MEMON *et al.*, 2012).

In *L. vannamei* spermatophore cryopreserved for 35 days there was a significant decrease in ASV over time, being more prominent from Day 14 (NIMRAT *et al.*, 2006). It was also observed in the present study with *L. schmitti* sperm mass, in which ASV declined in approximately 27% between Day 15 and Day 30. However, during cryopreservation the metabolism of spermatozoa should be suspended, maintaining the biological characteristics and cell viability for a long period. But this is not observed in most of the shrimp sperm cryopreservation trials.

Fast freezing procedures can cause substantial damages to the cell and high sperm mortality suggesting that gradual cooling protocols must lead to better outcomes. The positive effect of gradual cooling protocols can be observed for sperm cryopreservation in *M. rosenbergii*, *Sicyonia ingentis*, *S. serrata*, *L. vannamei*, *P. monodon* and *P. merguensis* (CHOW *et al.*, 1985; AKARASANON *et al.*, 2004; ANCHORDOGUY *et al.*, 1988; JEYALACTUMIE and SUBRAMONIAM, 1989; VUTHIPHANDCHAI *et al.*, 2007, MEMON *et al.*, 2012). In addition, the final temperature during cryopreservation can also be an important element to guarantee satisfactory ASV rates. In *P. monodon* spermatophores cryopreservation, better results were with a gradual freezing and final temperatures of -30 and -196°C (NIMRAT *et al.*, 2006; VUTHIPHANDCHAI *et al.*, 2007). In *L. vannamei* sperm mass gradual cooling protocol and final temperature of (-196°C) resulted in satisfactory ASV rates (SALAZAR *et al.*, 2008). However, in the present study the protocol suggested by SALAZAR *et al.* (2008), but using glycerol instead of methanol, had also satisfactory ASV rates for up 15 days of cryopreservation. This result is probably due to the close relation between species considered in both studies, but the ASV decline between Day 15 and Day 30 remains unenlightened.

CONCLUSION

We inferred that glycerol can be used as cryoprotectant for cryopreservation of *L. schmitti*

sperm mass in concentrations of 5 and 10% in an exposure time of 10 minutes. The *L. schmitti* sperm mass can be cryopreserved in liquid nitrogen for up to 15 days following the protocol of gradual cooling suggested by SALAZAR *et al.* (2008).

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REFERENCES

- AKARASANON, K.; DAMRONGPHOL, P.; POOLSANGUAN, W. 2004 Long-term cryopreservation of spermatophore of the giant freshwater prawn, *Macrobrachium rosenbergii* (de Man). *Aquaculture Research*, 35: 1415-1420.
- ANCHORDOGUY, T.; CROWE, J.H.; GRIFFIN, F.J.; CLARK JR, W.H. 1988 Cryopreservation of sperm from the marine shrimp *Sicyonia ingentis*. *Cryobiology*, 25(3):238-243.
- ARCE, S.M.; MOSS, S.M.; ARGUE, B.J. 1999 Artificial insemination and spawning of pacific white shrimp *Litopenaeus vannamei*: implications for a selective breeding program. *UJNR Technical Report*, 28: 5-8.
- BART, A.N.; CHOOSUK, S.; THAKUR, D.P. 2006 Spermatophore cryopreservation and artificial insemination of black tiger shrimp, *Penaeus monodon* (Fabricius). *Aquaculture Research*, 37: 523-528.
- CHOW, S.; TAM, Y.; OGASAWARA, Y. 1985 Cryopreservation of spermatophore of the fresh water shrimp, *Macrobrachium rosenbergii*. *Biological Bulletin*, 168: 471-475.
- GWO, J.C. 2000 Cryopresevation of aquatic invertebrate semen: a review. *Aquaculture Research*, 31: 259-271.
- JAR, L.P. 2005 Fisiología y calidad reproductiva de machos de camarón blanco *Litopenaeus schmitti* en condiciones de cautiverio. La Paz. 150p (Tese de Doutorado. Centro de Investigaciones Biológicas del Noroeste, S.C.). Available at: <<http://hdl.handle.net/1834/1544>> Access on: 17 feb. 2009.

- JEYALACTUMIE, C. and SUBRAMONIAM, T. 1989 Cryopreservation of spermatophores and seminal plasma of the edible crab *Scylla serrata*. *Biological Bulletin*, 177: 247-253.
- LEZCANO, M.; GRANJA, C.; SALAZAR, M. 2004 The use of flow cytometry in the evaluation of cell viability of cryopreserved sperm of the marine shrimp (*Litopenaeus vannamei*). *Cryobiology*, 48: 349-356.
- MEMON, A.J.; TALPUR, A.D.; KHAN, M.I.; FARIDDUDIN, M.O.; SAFIAH, J.; ABOLMUNAFI, A.B.; IKHWANUDDIN, M. 2012 Optimization of spermatophores cryopreservation protocol of banana shrimp (*Penaeus merguensis*) (De Man, 1888). *Journal of Animal and Veterinary Advances*, 11(10): 1688-1704.
- NIMRAT, S.; SIRIBOONLAMOM, S.; ZHANG, S.; XU, Y.; VUTHIPHANDCHAI, V. 2006 Chilled storage of white shrimp (*L. vannamei*) spermatophores. *Aquaculture*, 261: 944-951.
- SALAZAR, M.; LEZCANO, M.; GRANJA, C. 2008 Protocol for cryopreservation of *Penaeus vannamei* sperm cells. In: CABRITA, E.; ROBLES, V.; HERRÁEZ, P. *Methods in Reproductive Aquaculture*. Marine and Freshwater Species. Boca Raton: FL, CRC Press. p.505-508.
- VUTHIPHANDCHAI, V.; BOONPRASERT, P.; NIMRAT, S. 2005 Effects of cryoprotectant toxicity and temperature sensitivity on the embryos of black tiger shrimp (*Penaeus monodon*). *Aquaculture*, 246: 275-284.
- VUTHIPHANDCHAI, V.; NIMRAT, S.; KOTCHARAT, S.; BART, A.N. 2007 Development of a cryopreservation protocol for long-term storage of black tiger shrimp (*Penaeus monodon*) spermatophores. *Theriogenology*, 68: 1192-1199.