# Cryopreservation of *Limnoperna fortunei* (golden mussel) sperm with polyethylene glycol

**Background.** Insufficient quantities of freshly harvested *Limnoperna fortunei* gametes and embryos constrain reproduction research in the laboratory. Cryopreservation would allow the accumulation and storage of gametes when they are available. The lack of available cryopreservation protocol for *Limnoperna fortunei* in the literature led our research group to undertake a study to establish which cryoprotective agents can be might be most useful for cryopreservation of this species' sperm. **Methods.** 2%, 5%, and 10% concentration of ethylene glycol, dimethyl sulfoxide, glycerol, and polyethylene glycol 4000 as well as 0.2 M glucose, sucrose, and trehalose (mixed into the 10% concentration of the aforementioned agents) were tested in a 1:1 ratio with sperm, in 0.25 ml straws, frozen in liquid nitrogen. **Results.** After 48 hours the best survival rate was in the samples with 10% polyethylene glycol 4000, 36.1%, which also resulted in viable sperm after 7 and 15 days.

# Cryopreservation of *Limnoperna fortunei* (golden mussel) sperm with polyethylene glycol

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## Abstract

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**Results.** After 48 hours the best survival rate was in the samples with 10% polyethylene glycol 4000, 36.1%, which also resulted in viable sperm after 7 and 15 days.

Key words: *Limnoperna fortunei*, cryopreservation, sperm, polyethylene glycol, liquid nitrogen, thawing



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## Introduction

*Limnoperna fortunei* (golden mussel) is notorious for its high reproductive capacity in nature (Darrigran, Damborenea & Penchaszadeh, 1998), and one could expect that it would be easy to reproduce them in the laboratory. Different research groups have tried to do it (personal communications), but only Cataldo et al. (2005) has reported successfully reproducing *L. fortunei* in the laboratory.

*L. fortunei* does not exhibit external sexual dimorphism, thus gender identification is possible only by histological analysis after sacrificing the animal. The male:female ratio, theoretically 50:50 (Invasive Species Compendium, 2017), is uncertain. Dei Tos, Quagio-Grassiotto & Mazzoni (2016) reported that the ratio of reproductively active males to reproductively active females they encountered got as high as 3:1, depending on season. There are no available data about fertility or fecundity for this species and no data on embryo survival rates. Cataldo et al. (2005) monitored larval mortality during development under laboratory conditions and found that it can reach up to 90%. These uncertainties make it extremely difficult to plan for laboratory experiments involving genetic manipulation that require gametes or embryos.

Cryopreservation would seem to be an attractive and feasible strategy to accumulate and stock gametes and embryos until the quantity necessary for an experiment or series of experiments is attained. Cryoprotective agents (CPAs) that prevent the crystallization of intracellular water during the freezing process, however, may be especially toxic to gametes and embryos. Thus, it is imperative to find the ideal concentration of each cryoprotective agent that is sufficient to prevent intracellular water crystallization during freezing, while minimizing toxicity (Leung 1991, Renard 1991). It has been known for 25 years that CPA toxicity can be reduced by adding sugars, which do not enter the cell but protect its membrane enhancing post-thawing viability (De Leeuw et al., 1993). Using a myriad of cryoprotective agents and techniques, research groups have been successful in the cryopreservation of gametes and embryos of different mollusk species with varying rates survival (Smith et al., 2001; Adams et al., 2009; Paredes et al., 2012; Smith et al., 2012; Suquet et al., 2014; Wang et al., 2014; Adams et al., 2015). As no consensual cryoprotective protocol for *L. fortunei* sperm currently exists, the objective of the present study was to systematically evaluate which CPA would best preserve sperm material until its further use.



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## Material and Methods

The mussels were collected at the Chavantes reservoir on the Paranapanema river, in southeastern Brazil. They were kept in the laboratory in 10 l aquariums with filtered tap water, aeration and filtration, at 22° C, and fed with Sera® Coraliquid, 25 µl/l three times a week.

Before being sacrificed, the mussels were briefly cleaned with 70 % alcohol. After being opened inside a laminar flow hood, the male gonads were identified using a Nikon SMZ660 stereo microscope and transferred to a sterile Petri dish containing Opti-MEM™ I Reduced Serum Medium (Gibco). The gonads were carefully torn with a sterile surgical blade and stimulated with a sterile Pasteur pipette for 30 min to mechanically release the sperm. Next, sperm were filtered using a 20 µm net; viable sperm were distinguished from non-viable one with 0.4% trypan blue (Sigma Aldrich) mixed 1:1 and counted in a Neubauer chamber under the light microscope (LABMED Lx300).

Seven cryoprotective agents (CPA) were tested: ethylene glycol (**EG**), Sigma Aldrich 102466; dimethyl sulfoxide (**DMSO**), Sigma Aldrich D2650; glycerol (**Glyc**), Sigma Aldrich G2025; polyethylene glycol 4000 (**PEG**), Sigma Aldrich 95904; glucose (**Glc**), Sigma Aldrich 7021; sucrose (**Suc**), Acros 419760010; and trehalose (**Tre**), Sigma Aldrich T0167. All solutions were prepared with Opti-MEM™ I Reduced Serum Medium (Gibco) and sterilized using a 0.22 µm filter (Kasvi). The solutions were stored at 4 ˚C for up to one week before the use.

The screening of the CPAs was conducted during a pre-test and five independent experiments. In the pre-test EG, DMSO, Glyc and PEG were evaluated in solutions with a final concentration of 2.5%, 5%, and 10% (v/v (volume/volume), except for PEG, in which case was w/v (weight/volume)). In the first independent experiment, CPAs from the pre-test were used at a final concentration of 10%, while in the second independent experiment, each of these four CPAs (10% final concentration) were mixed with a second CPA: Glc, Suc or Tre (0.2 Molar in the final concentration). The third, fourth and fifth independent experiments were designed to determine sperm viability (survival) rates (in 10% PEG) over a longer time period.

Cryoprotective agents were mixed with sperm in a 1:1 ratio (by volume) in previously cooled sterile microtubes. The sperm concentration varied between 1 and 5 x $10^7$ cells ml/1. Each mixture of sample and CPA was transferred to three 0.25 ml “EcoStraw clean” straws (Minitube). Once filled, the straws were sealed using polyvinyl alcohol (Minitube). The tubes were exposed to liquid nitrogen vapor (three centimeters above the liquid surface) for 10



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minutes, and then plunged into a liquid nitrogen tank. The sperm survival rate was measured by thawing each triplicate of straws at room temperature, mixing them 1:1 ratio with 0.4% trypan blue (Sigma Aldrich), and then counting them using the Neubauer chamber on the light microscope (LABMED Lx300). The counting was done after 48 hours for the pre-test and experiments one to three; after 7 days for the experiment four; and after 15 days for experiment five. The survival rate results presented are the median percentage for each triplicate of straws.

**Results**

In the pre-test, the sperm survival occurred when cryoprotective agents were used at the 10% concentration (data not shown). The results obtained during the experiment one and two are presented in Table 1.

Table 1.

The best survival rate was 36.1% for samples with 10% PEG, while the survival rate with other CPAs was less than 2%. In the second experiment, addition of sugars resulted in increase of the survival rate for some CPAs. Addition of trehalose increased the survival of sperm in EG up to 12.6%. Glucose increased the survival rate with DMSO (5.8%) and glycerol (22%). When sucrose was used, it increased the survival rate in samples with DMSO (4.7%) and glycerol (8.1%).

Results for the third, fourth and fifth independent experiment show change of the survival percentage in samples with PEG for 48 hours, 7 and 15 days and they are given in Table 2.

Table 2

The survival rate after 48 hours of 35.6% continued the trend recorded in the second experiment. During the period of 7 and 15 days, the decline in the percentage of viable sperm was: 22.3% after 7 days and 13.5% after 15 days. Nevertheless, this percentage was higher than most of the results with the other CPAs.

**Discussion**

After screening seven cryoprotective agents, we succeeded in achieving a survival rate exceeding 30%, using polyethylene glycol. Despite being one of the least studied compounds, the reason for its success may be its non-permeable nature, which allows reversible leakage of solution



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through the cell membrane subject to osmotic pressure (Meryman, 1971). PEG was also used by Ghirardini et al. (2001) for cryopreservation of sea urchin (*Paracentrotus lividus*) gametes. The authors reported minimal toxicity to sperm and no effect on egg fertilization capacity in concentrations below 200 mg/l. Suquet et al. (2016) also found a higher motility (35%) in post-thawing samples of great scallop (*Pecten maximus*) sperm when using 15% or 20% concentrations of PEG (dilution ratio of PEG to sperm was 3:1) compared to DMSO. Riesco et al. (2016) managed to have more than 85% of viable *Crassostrea angulata* sperm using 10% PEG (in final concentration) with no significant difference to fresh control samples in motility and viability.

For *L. fortunei*, sperm survival rates in batches cryopreserved with 10% PEG did decline after 7 and 15 days of storage in liquid nitrogen, dropping to 22.3% and 13.5%, respectively, which was still higher than the yields with other cryoprotectants. The reason for decline might be the relatively low concentration of CPA used. In the case of non-permeable cryoprotectants, this concentration might not be sufficient for long term storage. None of the lower cryoprotective agent concentrations (2.5% or 5%) tested prevented ice formation, leading to zero sperm survival. The addition of two sugars did not improve PEG's cytoprotection of sperm. Indeed, the addition of glucose or trehalose appears to have increased PEG toxicity, leading to zero or low survival rates. Sucrose, on the other hand, exhibited results comparable to PEG alone. At this time, we cannot offer explanation for these phenomena. Other than PEG, after thawing, no other CPA, even in lower concentrations or enhanced with sugar, provided a concentration of viable sperm needed for further manipulation in research.

Results in the literature for the cryopreservation of other species are so different it is hard to take and guidance from them. Matteo et al. (2009) got up to 70% fertilization rate when using 7% EG as CPA for *Mytilus galloprovincialis* sperm. On the other hand, Smith et al. (2012) did not have success in fertilization when using 12% EG for cryopreservation of Greenshell™ mussel. While Matteo et al. (2009) and Fahy (2010) pointed out DMSO's toxic characteristics, Smiths et al. (2012) successfully used it in combination with trehalose for cryopreservation of Greenshell™ mussel (*Perna canaliculus*) sperm.

The high variability among replicates in our results, as well as the 85% recovery rates of Riesco et al. (2016) suggest that there is a room for further protocol optimization. One way to improve PEG cryoprotective efficiency might be to combine it with some of permeable cryoprotectants.



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Dong et al. (2006) tried this approach and got consistently high post-thawing sperm mobility with 6% PEG with 4% polypropylene glycol and 6% PEG with 4% DMSO. As suggested by Souquet et al. (2016), bovine serum may be an option to reduce post-thawing toxic effects. These approaches, as well as increasing the concentration of PEG when used alone, are just several alternatives worthy of investigation in pursuit of the optimal cryopreservation protocol for *L. fortunei* sperm.

## Conclusion

We successfully cryopreserved *Limnoperna fortunei* sperm using non-permeable polyethylene glycol (PEG), recovering more than 30% viable sperm after thawing. While we are aware that viability alone is not an indicative of fertility, this study is an important step towards obtaining a reproducible cryopreservation protocol to accumulate a sufficient quantity of gametes for development and genetic modification studies.

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Table 1. *Limnoperna fortunei* sperm survival rates after cryopreservation with different cryoprotective agents.

| Time (h) | Cryoprotective agent 1 (volume/volume - v/v, weight/volume - w/v) | Cryoprotective agent 2 (0.2M) | Median (max-min) Survival Rate (%) |
|---|---|---|---|
| 48 | ethylene glycol (10% v/v) | none | 1.3 (1.5 - 0.0) |
| | | glucose | 0.0 (0.0 - 0.0) |
| | | sucrose | 1.8 (5.8 - 0.0) |
| | | trehalose | 12.6 (14.7 - 1.0) |
| 48 | dimethyl sulfoxide (10% v/v) | none | 1.1 (9.5 - 0.6) |
| | | glucose | 5.8 (11.2 - 0.3) |
| | | sucrose | 4.7 (13.7 - 0.0) |
| | | trehalose | 0.3 (0.8 - 0.0) |
| 48 | glycerol (10% v/v) | none | 0.0 (0.0 - 0.0) |
| | | glucose | 22.0 (37.0 - 15.5) |
| | | sucrose | 8.1 (8.3 - 6.1) |
| | | trehalose | 0.0 (0.0 - 0.0) |
| 48 | polyethylene glycol (10% w/v) | none | 36.1 (47.0 - 19.1) |
| | | glucose | 0.0 (0.0 - 0.0) |
| | | sucrose | 26.0 (37.4 - 15.2) |
| | | trehalose | 1.3 (3.3 - 1.3) |



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Table 2. *Limnoperna fortunei* sperm survival rates after cryopreservation in 10% polyethylene glycol for 48 hours, 7 days, and 15 days.

| Time | Cryoprotective agent 10% (weight/volume) | Median (max-min) Survival Rate (%) |
|---|---|---|
| 48 h | polyethylene glycol | 35.6 (43.8 - 31.0) |
| 7 days | polyethylene glycol | 22.3 (26.2 - 6.7) |
| 15 days | polyethylene glycol | 13.5 (15.8 - 10.0) |



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