# Gamete cryopreservation, an asset for a durable pearl farming in French Polynesia

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## ABSTRACT

Cryopreservation is a useful tool for genetic improvement which has been applied to several bivalve mollusk species. It would allow to keep the gametes of selected individuals and to preserve ex-situ the biodiversity of wild populations of the black-lip pearl oyster *Pinctada margaritifera* threatened of standardization by the seed transfers between the islands in French Polynesia. The ability to cryopreserve spermatozoa would bring about significant benefits to the cultured black pearl industry. Sperm freezing supposes the control of different steps: preparation of breeders, sperm collection, evaluation of sperm quality and the freezing process itself. Broodstock conditioning in hatchery allows to obtain gametes from the pearl oyster. Sperm quality is evaluated using a motility index and bioluminescence ATP measurements of spermatozoa. The following cryoprotectant additives (CPA) mixture were evaluated : (1) 0.41 M trehalose and 0, 0.45, 0.91, 1.36 M dimethyl sulfoxide ($Me_2SO$), (2) 0.7 M trehalose and 0.8 M $Me_2SO$ and (3) 0.10 M trehalose. Freezing was made in two steps, in nitrogen vapor followed by direct plunging of spermatozoa in liquid nitrogen. Total motility was best retained when spermatozoa were cryopreserved with 0.41 M trehalose and 0, 0.45, 0.91, 1.36 M $Me_2SO$ and 0.7 M trehalose and 0.8 M $Me_2SO$. Bioluminescence ATP measurements showed that spermatozoa conserved their energetic stock. Moreover, fertilization efficiency of thawed sperm was demonstrated using artificially matured oocytes, which allowed the development of embryos and D larvae.

## Keywords

Pearl oyster, *Pinctada margaritifera*, Cryopreservation, Spermatozoa, Fertilization.

## 1. INTRODUCTION

The black pearl oyster, *Pinctada margaritifera*, is used for pearl production. Production relies almost exclusively on the collection of wild spat, which makes the activity very dependent on natural resources. Recruitment is highly variable in space and time and so spat is very often transferred by the farmers between atolls or even between archipelagos in French Polynesia. Cryopreservation techniques for gamete could greatly assist in preserving black pearl oyster lineages in the pearl farming industry, and selective breeding for desirable traits in the future.

Spermatozoa cryopreservation is a useful tool for genetic improvement and have been applied to several bivalve mollusc [2][5]. Exploration of the development of gametes cryopreservation techniques from the black-lip pearl oyster *P.margaritifera* showed the significance of cryoprotectant additives (CPA) on spermatozoa motility [8]. Acosta-Salmón et al (2007) has evaluated the toxicity and the effects of the molar concentration of trehalose and dimethyl sulfoxide ($Me_2SO$) and the freezing protocol on motility of *P.margaritifera* spermatozoa. Previous work on cryopreserving black pearl oyster sperm has found that motility was retained when spermatozoa were cryopreserved in 0.45 M trehalose and 0, 0.64, 1.02 and 1.53 M dimethyl sulfoxide ($Me_2SO$) [1][8], but the fertility of these cryopreserved spermatozoa was unknown to date.

The present study is to carry on with this work to estimate the fertility of cryopreserved spermatozoa and to complete with the effect of some other molar concentration of CPA on spermatozoa motility.

## 2. MATERIALS AND METHODS

### *2.1 Collection of gametes*

*P. margaritifera* used in this study were from Vairao lagoon, Tahiti, French Polynesia. Oyster were transferred from the culture long-line to a conditioning place during 1 month to obtain mature oyster. Sperm end and eggs were obtained by "strip spawning".

Eggs were collected by lacerating the gonad wall with a scalpel and gently scraping and washing the gonad contents. Eggs were released into 100 ml seawater containing 2mM $NH_3$ to induce oocyte maturation [7][11].

Spermatozoa were manually stripped and collected with a Pasteur pipette., Spermatozoa were obtained from 3 oysters.

### *2.2 Experiments*

#### *2.2.1 Experiment 1: effect of CPA's on post-thaw motility of cryopreserved sperm*

We evaluated 6 CPA mixtures at the following in straw concentrations: (1) 0.41M trehalose (Sigma, France) alone and in combination with 0.45, 0.91, 1.36 M $Me_2SO$ (Sigma, France) , (2) 0.7 M trehalose and 0.8 M $Me_2SO$ and (3) 0.10 M trehalose alone. Cryoprotectant solutions were prepared in distilled water. The concentrated spermatozoa was diluted in the appropriate CPA on a 1:10 (v/v) then drawn by suction into 500 µl semen straws (IMV, France). Equilibration period, the time between sperm dilution and the start of cooling, was less than 10 min at room temperature (27 °C). The straws were left in liquid nitrogen (LN) steam during 10 min and then immersed in the LN. For thawing, the straws were immersed in seawater at room temperature (27°C) for 25s. Fresh and post-thaw spermatozoa were diluted with

seawater and DCSB4 solution [3] on a 5:5 ? (v/v) at a final dilution ratio 1:500. A 6 µl aliquot of the activated spermatozoa was transferred to a glass slide and motility was observed without a coverslip. Sperm motility was measured using the Motility Index by Christen and al. (1987).

### *2.2.2 Experiment 2: ATP content of sperm*

ATP content of the whole sperm was evaluated using bioluminescence (ATP lite Luminescence ATP detection Assay System; Perkin Elmer Life and Analytical Sciences B.V.) as following.

$$\text{ATP + D-Luciférine + } O_2 \xrightarrow[\text{Luciférase}]{Mg2+} \text{Oxy-luciférine + Ppi + AMP + } CO_2 \text{ + Photons}$$

Luminescence was read with a Spectrafluor Plus luminometer (TECAN Group Ltd, Maennedorf, Switzerland) [10].

## *2.3 Evaluation of fertility of fresh and cryopreserved spermatozoa*

Fertilization assays were used to evaluate the fertility of sperm before and after cryopreservation. Eggs were fertilized with fresh and cryopreserved spermatozoa at four sperm/egg ratio : 20 000/1, 40 000/1, 90 000/1 and 380 000/1. The fertilization rates were evaluated by counting normally cleaved eggs under stereomicroscope 4h after fertilization. Approximately 100 eggs in each well were examined microscopically to determine the percentage of fertilized eggs.

# 3. RESULTS

## *3.1 Effects of CPAs on post-thaw motility of cryopreserved sperm*

The total motility can be successful retained when cryopreserved *P. margaritifera* spermatozoa in 0.41 M trehalose and 0, 0.45, 0.91, 1.38 M $Me_2SO$ and was particularly best retained for spermatozoa cryopreserved in 0.7 M trehalose and 0.8 M $Me_2SO$ (Fig. 1).

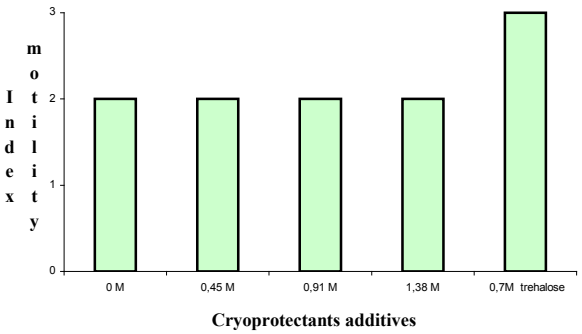


**Figure 1. Mean motility after thawing of *P. margaritifera* spermatozoa with each CPA: 0 M**: 0.41M trehalose; **0.45 M**: 0.41 M trehalose in 0.45 M $Me_2SO$, **0.91 M**: 0.41 M trehalose in 0.91 M $Me_2SO$, **1.38 M**: 0.41 M trehalose in 1.38 M $Me_2SO$, 0.8 M: **0.7 M** trehalose in 0.8 M $Me_2SO$**.**

## *3.2 ATP measurements*

Bioluminescence ATP measurements showed that spermatozoa conserved their energetic stock is changing sample to sample (Fig. 2).

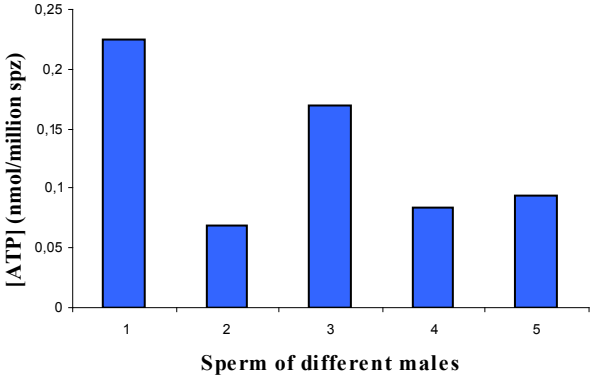


**Figure 2. Bioluminescence ATP measurements**

## *3.3 Fertilization essays*

Fertilization rates of more than 80% were obtained in both fresh and post-thaw spermatozoa with a ratio of 3.8 spermatozoa per egg (Fig. 3). Fertilization efficiency of thawed sperm (quel CPA ?) was demonstrated using artificially matured oocytes which allowed the development of embryos and D larvae.

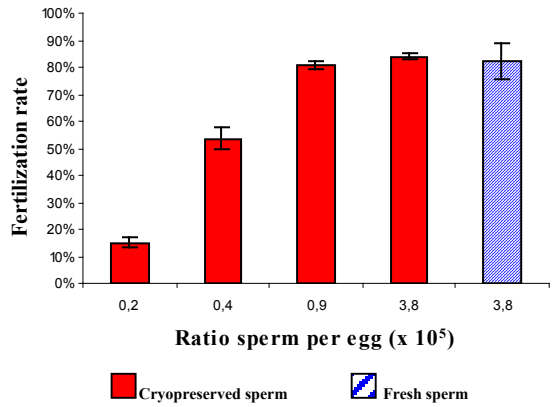


**Figure 3. Effects of spermatozoa density on fertilization rates using fresh and cryopreserved sperm.**

# 4. DISCUSSION

This study has confirmed the importance of CPA on motility and for the first time shown fertility of cryopreserved spermatozoa of *P. margaritifera.* Trehalose and $Me_2SO$ were confirmed to be the most effective CPA in preserving the motility and the fertilization ability of freezed *P. margaritifera* [1][8], and *Crassostrea gigas* [2] spermatozoa. We have shown that cryopreserving spermatozoa in 0.7 M trehalose and 0.8 M $Me_2SO$ coupled with a medium rapid cooling rate resulted in the best retention of motility. The higher concentrations of cryoprotectants help reduce damage due to cryoinjuries but also increase biological toxicity to

cells [9]. In this study, high fertilization rates were obtained by adding $15x10^8$ sperm $ml^{-1}$ to $2x10^3$ eggs $ml^{-1}$ . Embryos and D larvae development were obtained with low rates due to in-vitro maturation of oocytes. Fertilization test with natural mature oocytes are required. For pratical application in pearl oyster aquaculture, larger-volume cryopreservation procedures must be developed in future studies.

## 5. ACKNOWLEDGMENTS

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