Cryobiology 65 (2012) 256–262

Contents lists available at SciVerse ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/ycryo

Cryopreservation of Greenshell™ mussel (*Perna canaliculus*) trochophore larvae ☆

E. Paredes [a,*], S.L. Adams [b], H.R. Tervit [b], J.F. Smith [b], L.T. McGowan [c], S.L. Gale [b], J.R. Morrish [b], E. Watts [b]

[a] Departamento de Ecoloxía e Bioloxía Animal, Universidade de Vigo, Estrada Colwxio Universitario s/n, 36310 Vigo, Galicia, Spain
[b] Cawthron Institute, Private Bag 2, 98 Halifax Street East, Nelson, New Zealand
[c] AgResearch, Private Bag 3123, Hamilton, New Zealand

ARTICLE INFO

Article history:
Received 20 March 2012
Accepted 24 July 2012
Available online 1 August 2012

Keywords:
Cryopreservation
Perna canaliculus
Trochophores
Greenshell™ mussel

ABSTRACT

The Greenshell™ mussel (*Perna canaliculus*) is the main shellfish species farmed in New Zealand. The aim of this study was to evaluate the effects of cryoprotectant concentration, loading and unloading strategy as well as freezing and thawing method in order to develop a protocol for cryopreservation of trochophore larvae (16–20 h old). Toxicity tests showed that levels of 10–15% ethylene glycol (EG) were not toxic to larvae and could be loaded and unloaded in a single step. Through cryopreservation experiments, we designed a cryopreservation protocol that enabled 40–60% of trochophores to develop to D-larvae when normalized to controls. The protocol involved: holding at 0 °C for 5 min, then cooling at 1 °C min^{-1} to −10 °C, holding for a further 5 min, then cooling at 0.5 °C min^{-1} to −35 °C followed by a 5 min hold and then plunging into liquid nitrogen. A final larval rearing experiment of 18 days was conducted to assess the ability of these frozen larvae to develop further. Results showed that only 2.8% of the frozen trochophores were able to develop to competent pediveligers.

Introduction

The Greenshell™ mussel (*Perna canaliculus*) is the main shellfish species farmed in New Zealand. Over 33 000 tonnes are presently exported ($NZ 152 million) annually [28]. The mussel industry currently obtains spat (juveniles) to on-grow from two wild populations. However, there is increased interest in moving to hatchery spat production to ensure a reliable supply for on-growing and a selective breeding programme for this species is also well underway [11].

Cryopreservation can be a powerful tool in hatchery production and selective breeding. In hatcheries, cryopreservation can enable a year-round spat supply without the need to condition broodstock for out of season production. In selective breeding, it can manage the biological and commercial risk associated with such programs (e.g. disease, market changes) as well as giving breeders more flexibility in their breeding design (e.g. enabling back crosses between different generations, even beyond the lifespan of donors, providing offspring from a given cross over multiple seasons). Indeed, cryopreservation of gametes is already widely used in animal husbandry of many farmed species including finfish such as salmon. In aquatic species, sperm cryopreservation protocols have been successfully developed for a number of invertebrates and fish species [4,12] but eggs and early embryos have proven to be much more challenging [1,2,8,27].

Previous work on Greenshell™ mussel oocyte cryopreservation [3,13] suggests that oocytes are especially difficult to cryopreserve. Although reasonable fertilization rates were obtained post-thawing, fertilized oocytes failed to develop into normal D shaped veliger larvae (30–48-h-post fertilization). At best only 2% developed to this stage. Mussel sperm cryopreservation also remains problematic with large variation in post-thaw fertility [2,20,21]. As an alternative to separately cryopreserving oocytes and sperm, this study investigates the feasibility of cryopreserving Greenshell™ mussel larvae at the trochophore stage. Previous studies on oysters (*Crassostrea gigas* and *Crassostrea rhizophorae*) have shown that the more advanced the developmental stage, the more resistant cells are to cryoprotectant (CPA) toxicity and the higher the survival after freezing [10,14]. Toledo et al. [24] successfully froze larvae of the blue mussel, *Mytilus edulis*, at the trochophore stage. Wang et al. [26] froze larvae of the blue mussel, *Mytilus galloprovincialis* at D-stage and were able to rear approximately 1% to pediveliger stage. These studies together with our preliminary unpublished experiments on different larval stages lead us to select the trochophore larval stage for detailed cryopreservation studies. The aim of this study was to evaluate the effects of CPA concentration, loading and unloading method, and different freezing and thawing strategies. The ability of frozen trochophores to develop to viable D-larvae and beyond was assessed.

☆ *Statement of Funding:* Support for this research was provided by Vigo University by a research stay fellowship and also by the Ministry of Science and Innovation to Cawthron Institute (CAWX0802).

* Corresponding author. Fax: +34 986 81 25 56.
E-mail address: eparedes@uvigo.es (E. Paredes).

0011-2240/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cryobiol.2012.07.078

Materials and methods

Gamete collection and handling

Mature Greenshell™ (*P. canaliculus*) mussels in reproductive condition were obtained from farms in the Marlborough Sounds, New Zealand, and maintained at the Glenhaven Aquaculture Centre in Nelson before being used in experiments. Thermal cycling was used to induce mussels to spawn and gametes were collected as previously described [2,3]. Sperm concentration was determined using a Neubauer haemocytometer. Oocyte concentration was determined by counting 20 μL aliquots from a known dilution of each oocyte pool. Oocytes were fertilized at a concentration of 500 sperm per oocyte and 1000 oocytes mL^{-1}. Sperm and oocytes were frequently mixed during a contact time of approximately 15–20 min before being transferred into tanks containing ~150 L of 1 μm filtered seawater (FSW) and 1 mg L^{-1} ethylenediaminetetraacetic acid (EDTA) at a temperature of 20 °C and a density of 15×10^6 oocytes/tank. After 16–20 h, trochophores were collected by gently siphoning the contents of the tank through a 15 μm screen. The trochophores were then gently swirled on the screen to concentrate them and placed into 50 ml falcon tubes for experiments. For each experiment, three replicate runs were carried out. Oocytes and sperm were pooled from at least three individuals per pool and three female pools were used for each experiment.

Experimental reagents

For all the experiments, we used the CPA combination of ethylene glycol (EG) and trehalose (TRE) obtained from Sigma–Aldrich chemicals and prepared in Milli-Q water. Bovine serum albumin (BSA) was used during thawing (Albumax I Lipid Rich BSA, GIBCO Invitrogen, New Zealand Limited, Auckland, NZ).

Toxicity tests

Toxicity trials were undertaken using five different concentrations of EG (0, 10, 15, 20 and 25% v/v, final) and three different TRE concentrations (0, 0.2 and 0.4 M, final). Cryoprotectant solutions were added 1:1 to a concentrated suspension of trochophores in one step or five equimolar steps one min apart. Samples were allowed 15 min of equilibration time and then diluted in one or five steps, respectively. Trochophore larvae, at a density of 40 mL^{-1}, were incubated in multiwell culture plates in 4 mL FSW containing EDTA for a further ~24 h at 20 °C until D-stage was reached. Larvae were then fixed by adding 400 μL of 10% formalin. For each treatment 4 replicate wells were set up. The percentage of trochophores developing to normal D-larvae was then calculated as an indicator of CPA toxicity in order to determine the "No Observed Effect Concentration (NOEC)" and "Lowest Observed Effect Concentration (LOEC)" levels. Data were normalized to controls (controls used to normalize the data were trochophores that were collected and handled the same way as treated larvae but without any further manipulation, then incubated to D-larvae stage) prior to statistical analysis.

Cryopreservation experiments

Following the toxicity assessment of the CPAs, cryopreservation experiments were undertaken with trochophores using a Freeze Control System (Cryologic Pty Ltd., Mt Waverley, Australia). As in toxicity experiments, CPAs were prepared at twice their final concentration and 1 mL of CPA was then added in a single step to 1 mL of trochophores in suspension for ~15 min equilibration before freezing. Unless otherwise specified, the diluted trochophores were then loaded into 0.25 mL plastic straws (IMV Technologies, France) and sealed with PVC powder.

Experiment 1: effect of cooling rate

The effect of cooling rate was evaluated using EG 10% in combination with TRE 0.2 M (final). Trochophores were frozen using two different cooling regimes: hold at 0 °C for 5 min then cool at 1 °C min^{-1} to −10 °C and hold for 5 min before cooling at either: (1) 0.5 °C min^{-1}; or (2) 1 °C min^{-1} to −35 °C, hold for 5 min and then plunge into liquid nitrogen. Seeding was always checked at the minus −10 °C hold.

In this experiment, the effect of thawing with BSA in the seawater was also investigated. Straws were thawed in a 28 °C water bath until the ice melted. Straws were then cut and the trochophores released into 1 mL of FSW alone or containing 0.1% BSA (w/v). The trochophores were then left for 15 min before being transferred into buckets containing 15 L of FSW and 1 mg L^{-1} EDTA for a further ~24 h at 20 °C to allow them to develop to the D-larval stage. Buckets were drained through a 45 μm mesh screen and samples fixed with 10% formalin for larvae measurement and % normal D-larvae count. For determination of percentage D-larvae, larvae were suspended in a known volume of FSW and at least three aliquots were taken and counted under a light microscope for each treatment. The average count of the three aliquots was normalized against the controls (using handled controls as 100% D-larvae) before being used in statistical data analysis.

Experiment 2: effect of varying EG and TRE concentrations

Based on the results of the previous experiments, a second cryopreservation trial was performed to explore in detail which of the non-toxic CPA combinations yielded the best results. Concentrations of 10–15% EG in combination with 0.2–0.4 M TRE were used to assess which CPA concentration gave the highest post-thaw survival rate when a cooling rate of 0.5 °C min^{-1} was used. Thawing and incubation was performed as described above. BSA was used during thawing.

Experiment 3: effect of freezing container type and volume

The effect of container type and volume was investigated using two concentrations of CPA (10% EG + 0.4 M TRE and 15% EG + 0.2 M TRE). Two different containers with different volumes were tested: 0.25 and 0.5 mL straws were compared to 2 and 4 mL cryovials. The CPA and trochophore concentration was kept the same for all volumes and the thawing and incubating procedures whereas described for Experiment 1. BSA was used during thawing.

Experiment 4: effect of cryopreservation on development beyond D-larval stage

The effect of cryopreservation on development beyond the D-larval stage was investigated. Gametes from five separate male and female pairs were used to make individual crosses.

Based on the previous experiments, 10% EG + 0.4 M TRE (final) was used as the CPA. The CPA was added to trochophores in a single step and 0.25 ml straws then loaded for freezing. Straws were frozen in controlled rate freezers that were programmed to cool by holding at 0 °C for 5 min then cooling at 1 °C min^{-1} to −10 °C and holding for 5 min before cooling at 0.5 °C min^{-1} to −35 °C followed by a 5 min hold and then plunging into liquid nitrogen.

Larvae were thawed in the presence of BSA and reared through to the D-larval stage in tanks containing ~150 L FSW and 1 mg L^{-1} EDTA at 20 °C. Once the D-larvae stage was reached, larvae were drained onto a 45 μm mesh screen and the percentage of trochophores reaching D-larval stage determined. For each cross, the same number of D-larvae were transferred for the unfrozen control and cryopreserved treatment to the Cawthron Ultra-Density Larval System (CUDLS) for on-growing (44 000–151 000 larvae per CUDL)