This is a peer reviewed version of an article published in Cryobiology, Volume 96, October 2020, Pages 37-44 The published version is available online at: https://doi.org/10.1016/j.cryobiol.2020.08.010

Development of a method to cryopreserve Greenshell mussel™ (*Perna canaliculus*) veliger larvae

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Abstract

Cryopreservation of larvae of Greenshell™ mussel *Perna canaliculus*, the most cultivated species in New Zealand, can provide flexibility for selective breeding programmes and enhance its global production. In this study, we set out to develop a reliable protocol for freezing D-stage larvae of Greenshell™ mussels that ensured long-term survival for successful rearing of thawed larvae in the hatchery. The effects of different combinations of cryoprotecting agents (CPA), varying CPA equilibration times, larval concentrations per straw as well as different larval development stages (48 h *vs* 72 h old) were evaluated by assessing the behavioural response (swimming activity, algal consumption), shell size and survival of larvae, up to 4 days post-thawing. The protocol yielding the best larval performances was a combination of the following CPA (final concentrations): 14% ethylene-glycol (EG) + 0.6M trehalose (TRE) + 1% polyvinyl-pyrrolidone (PVP), prepared with Milli-Q water. Stocking densities ranging from 50,000 to 150,000 larvae per straw (0.25 mL) and a 20 min equilibration time gave the best results, while no significant differences in fitness were found between larvae cryopreserved at 48h nor 72h-old. Using the improved cryopreservation protocol, over 50% of previously cryopreserved D-larvae were able to survive after 4 days of rearing, compared with 65% in the unfrozen control. More importantly, about one third of thawed larvae were able to swim and feed, and to potentially develop further. These findings contribute to enhance the selective breeding programmes for this species.

Keywords: *Perna canaliculus*, D-larvae, mussel, cryopreservation, fitness, selective breeding

1. Introduction

The Green-lipped or Greenshell™ mussel, *Perna canaliculus* (Gmelin, 1791), is the main species farmed and harvested in New Zealand, followed by the king salmon (*Oncorhynchus tshawytscha*), and the Pacific oyster (*Crassostrea gigas*). In 2017, Greenshell™ mussel farming produced over NZ$ 308 million in export revenue to 60 different countries, approximately 5% of the mussel global production [27, 32, 33].

The New Zealand mussel industry has been largely dependent on the supply of juveniles collected from the wild, which is unpredictable in timing and highly variable in quality. Recently, the industry has begun commercial production of hatchery spat at scale. The hatchery spat is produced from selectively bred mussels that have been chosen for their favourable production traits including fast growth. Indeed mussels within the selective breeding programme have been demonstrated to grow approximately 25-50% faster than wild mussels [34]. Cryopreservation is a powerful tool for selective breeding programmes. Cryopreservation can provide a library of families to go back to should breeding objectives change or be lost in adverse events. It also enables the creation of reference families to benchmark cohorts against.

The freezing of *P. canaliculus* sperm has proven to be relatively successful [22], whereas oocyte cryopreservation has demonstrated to be challenging with resulting embryos failing to develop into viable D-larvae [1]. The development of a cryopreservation protocol capable to produce seed from larval stages appears to be sensible as an alternative to separate cryopreservation of gametes. Thus, [14] evaluated the potential to cryopreserve trocophore larvae (16 – 20 h old) and found that long term viability of thawed larvae was poor, with only 2.8% of the thawed trocophores developing to competent pediveliger mussels. A more recent study by [21] examined the effects of cryopreservation on mussel larval development by comparing post-thaw larval performances of mussels frozen at trocophore (16 h old) and D-stage veliger (48 h old) larvae. Although the two life stages investigated yielded significantly low survivorship to the pediveliger stage (< 0.1%), authors showed that D-stage larvae were somewhat more resilient to cryopreservation than trocophore larvae, and could be a good life-stage candidate for cryobanking [21]. However, further optimisation of the methodology is required to improve pediveliger yields in order to implement larval cryopreservation in a selective breeding programme.

The development of a robust and reproducible freezing procedure requires the study of several factors including selection of a suitable cryoprotecting agent (CPA) according to its balance between toxic effect (yielding cell injuries) and cryoprotection efficiency which is cell-type dependent [4, 6, 10]. Prior work, focusing on the cryopreservation of *P. canaliculus* gametes [1, 22, 23], recommended the use of ethylene glycol (EG) and trehalose (TRE) for oocyte cryopreservation and dimethyl sulfoxide (Me_2SO) in combination with TRE for sperm cryopreservation. For the cryopreservation of larval stages of Greenshell™ mussels, only one CPA combination has been tested, namely 10% EG + 0.4M TRE (Final), overall yielding poor post-thaw survival [14, 21].

The aim of this work was to develop a reliable method to cryopreserve D-larvae of the commercially important Greenshell mussels. Through several experiments, we tested the effects of different CPA combinations, equilibration times, stocking densities (i.e. larvae per straw), as well as different larval developmental stages, on thawed *P. canaliculus*. Larval fitness was assessed following thawing, and the basis for larval cryopreservation implementation in selective breeding for this species was established.

2. Materials and methods

2.1 Gamete collection and handling

Adult Greenshell™ mussels (*Perna canaliculus*) were obtained from a commercial farm in the Marlborough Sound (South Island, New Zealand), and transferred to the Cawthron Aquaculture Park in Nelson, New Zealand. Animals were maintained for several months in a flow-through system under ambient seawater conditions and fed *ad libitum* by artificially fertilised eutrophic ponds. During their natural reproductive season, fifty sexed broodstocks were induced to spawn using thermal cycling procedure according to [1]. A total of 26 females and 16 males were selected. Spawning individuals were put into separate plastic containers to collect sperm and oocytes, gametes were microscopically checked for viability (oocytes) and motility (sperm) and stored for 1h at 4°C prior to fertilisation. Two pools of gametes, each consisting of 9 females and 4 males, were created to ensure genetic diversity. Eggs were washed through a 70 µm mesh to remove any gonad debris and pooled in a 1 L beaker for counting. Pooled sperm for each run was washed through a 43 µm mesh and sperm density was determined. Fertilization was carried out in 15 L of 1 µm-filtered, UV-treated seawater (FSW) previously treated with 12 µM Ethylene diamine tetraacetic acid (EDTA) to reach 5,000 oocytes per mL. A small volume of sperm was added to the oocyte suspension to obtain a sperm: egg ratio of 200:1, and a 20 min contact period was allowed with gentle agitation. Fertilisation success was then confirmed by counting oocytes exhibiting a first polar body. Embryos were then transferred into 170 L tanks and incubated in EDTA-treated and pH adjusted (8.35) FSW at 17°C (±1), to reach a final density of 10 x 10^6 embryos/tank [11, 17].

Forty-eight hours post-fertilization (hpf), swimming D-larvae were collected by draining each incubation tank through a 43 µm screen. Larvae were delicately washed on the 43 µm screen, collected with a disposable plastic pipette and concentrated into 50 mL Falcon tubes at an appropriate density required for experiments. Alternatively, swimming larvae were maintained an additional 24 h in the incubation tank and collected at 72 hpf as described previously.

2.2 Cryoprotecting reagents

Cryoprotecting agent (CPA) solutions were prepared at twice the desired final concentration. Different concentrations of ethylene-glycol (EG) were used in combination with increasing concentrations of trehalose (TRE) and 1% polyvinyl-pyrrolidone (PVP) (Sigma Aldrich, Chemicals). For CPA solution preparation, 2M TRE and 20% PVP stock solutions were prepared in either FSW or in deionized Milli-Q water then diluted to the appropriate working solution. In addition, bovine serum albumin (BSA) (Albumax I Lipid Rich BSA, GIBCO Invitrogen, New Zealand Limited, Auckland, NZ) made up in FSW was used during thawing of the larvae.

2.3 Cryopreservation and thawing procedures

The CPA solutions were added at a ratio of 1:1 in a single step to 1 mL of concentrated D-larvae solution in Kimble® tubes, capped and continuously agitated during equilibration time before freezing. Mussel larvae + CPA were then loaded into 0.25 mL straws (IMV Technologies, France) and sealed with PVC powder. Following 25 min of equilibration (17 ± 1°C), straws were placed into a controlled rate freezer (Freeze Control System Cryologic Pty Ltd, Mt Waverley, Australia). For each experiment, a handling control treatment, consisting of larvae diluted 1:1 with FSW + 12µM EDTA, was concurrently loaded in straws.

Straws containing CPA were slow cooled using the following protocol: hold at 0°C for 5 min, then cool at 1°C/min to -10°, hold at -10°C for 5 min (seeding), then cool at 0.5°/min to -35°C, hold for 5 min and

then plunge into liquid nitrogen (LN). Seeding at -10 °C was carried out manually by dipping forceps into LN and then touching the top of each straw in the freezer with the lid removed. Following 1 h in LN, straws were thawed individually by immersion in a 28°C water bath for a few sec until the ice in the straw had visibly melted. Straws were unloaded in well plates and diluted twice, first 1:1 with 1 mL FSW + 12 µM EDTA + 0.1 % (w/v) BSA. After 10 min, a further 0.5 mL FSW + 12 µM EDTA + 0.1 % (w/v) BSA was added, allowing for a 15 min equilibration. Handling control straws were unloaded in well plates 25 min after FSW dilution and loading following the same procedure for thawed straws.

2.4 Larval rearing and sampling

Post-thawed larvae were cultured in a flow-through larval rearing system for 4 days at 18°C ±1. Larval rearing tanks consisted of 2.5 L polycarbonate containers (Cawthron Ultra Dense Larval rearing system, or CUDL) previously described in [18]. Filtered seawater was continuously mixed in a 100 L header tank with hatchery-cultured microalgae (*Chaetoceros calcitrans*), delivered to the header tank by computer-controlled pneumatic pumps at specific rates. From here, the algae-enriched seawater was supplied to the larval rearing CUDLs by gravity through manifolds at a constant flow rate of 80 mL min^{-1}. The outflow of the CUDL was equipped with a nylon screen of appropriate aperture to retain the larvae in the containers, and aeration was supplied through a second glass tube, bubbling from the base of the CUDL.

Different larval fitness indicators (survival, swimming activity, feeding rate, shell size) were evaluated on day 1 post-thawing, and at the end of the experiment on day 4 post-thawing. More specifically, larval survival was assessed in each CUDL tank by counting the number of live larvae in 0.5 mL aliquots collected from the top of the CUDL. Dead larvae consisted of translucent shells with no internal organisation and opened valves. Percent recovery and final survival, based on the initial number of D-larvae stocked on day 0, were determined on day 1 and 4 post-thawing respectively. Swimming activity was evaluated from each tank by counting the number of resting larvae compared with the total number of live larvae one minute after swirling in a culture dish, in a non-fixed sample. 'Spinning' behaviour in some larvae was considered an abnormal swimming and included in the resting category. Additionally, the proportion of larvae (n=50) exhibiting a brown coloration (i.e. presence of algal food in the stomach), as well as shell lengths of 30 larvae were assessed from each CUDL, after fixation with 10% buffered formalin, using Olympus cellSens image analysis software, an Olympus CX41 inverted microscope and Olympus DP74 camera.

2.5 Experimental design

Three independent experiments were conducted to optimise our methodology for cryopreserving mussel larvae.

Experiment 1: effects of different combinations of CPA solutions in Milli-Q water or FSW

The cryoprotecting effects of varying concentrations of EG and TRE, prepared with either Milli-Q water or FSW, were investigated in this experiment. Forty-eight hour-old D-larvae were collected from each pool, and concentrated to reach a final concentration of approximately 1 x 10^6 per mL. The CPA addition, freezing rate and thawing procedures were as described previously. After thawing, 4 straws per treatment were added to each CUDL. Four additional straws diluted and loaded with larvae were used to estimate the average number of larvae per straw. This was determined to be 202,800 larvae per straw for run A, and 148,500 larvae per straw for run B. Four straws were unloaded into each CUDL, corresponding to 600,000 to 800,000 D-larvae per CUDL to be reared at a final density of ~280 larvae per mL.

Experiment 2: effects of increasing equilibration time and larval density per straw

Based on Experiment 1, the CPA combinations yielding the highest rates of viable larvae (14% EG + 0.6M TRE + 1% PVP and 16% EG + 0.6M TRE + 1% PVP in Milli-Q water) were selected. The aims of experiment 2 were to further investigate the combined effects of (1) increasing exposure times of larvae to CPA before freezing (i.e. equilibration time) and (2) larval density during freezing on the cryopreservation success. Forty-eight hour-old D-larvae were cryopreserved following exposure to CPA solutions for 20, 40 and 60 min (18 ± 1°C) using the cooling regime outlined in Section 2.3. Straws (0.25 mL) were loaded at three different stocking densities: low (~50,000), medium (~100,000) and high (200,000 larvae per straw). Handling controls were treated the same way, except they did not undergo the cryopreservation processes. Following thawing, appropriate numbers of straws were unloaded into each CUDL to reach similar tank densities (i.e., ~200 larvae per mL) and reared for 4 days following the procedure previously described in Section 2.4. Two replicated freezing runs, run A and B, were performed for Experiment 2.

Experiment 3: effects of D-larval stage on freezing tolerance

In this last experiment, the tolerance of 48 h and 72 h-old D-larvae to cryopreservation was evaluated through two replicated freezing runs (A and B). Both developmental stages were cryopreserved using the best protocols determined in Experiments 1 and 2. More specifically, 14% EG + 0.6M TRE + 1% PVP in Milli-Q water was chosen, using a 20 min equilibration time and a stocking density of 112,000 D-larvae per 0.25-mL straw. As described previously, thawed mussel larvae were stocked at 230 larvae/mL and reared in a flow-through system for 4 days.

2.6. Statistical analyses

All data are expressed as means ± (Standard Errors, SE) for assessment of larval performances. Statistical analyses were performed using the IBM SPSS (v15.0) software package. Prior to statistical analyses, all percentage data were arcsine square root transformed to improve normality. Normality of data distribution was tested using the Shapiro-Wilk test ($p > 0.05$) while homogeneity of variances was checked using the Levene's test ($p > 0.05$). Larval performances were compared between treatments by Kruskal-Wallis one-way ANOVA, followed by a multiple pairwise comparison using a Dunnett's T3 post-hoc test. Finally, Student's t-tests were conducted to compare unfrozen control vs cryopreserved larvae for each fitness parameters (Fig. 4). All statistical testing was assessed for significance at $p \leq 0.05$.

3. Results

3.1 Experiment 1: effects of different combinations of CPA solutions

Survival

Based on initial estimates of larval density, which ranged from ~150,000 to ~200,000 per straw, each tank was stocked with 600,000 and 800,000 thawed larvae for Run A and Run B, respectively. On the next day (i.e., day 1 post-thawing), numbers of recovered larvae were generally high with the majority of the tanks having over 72% live larvae. One exception was treatment '10% EG + 0.4 M TRE + SW' which had 37.9% (± 35.6) of recovered larvae on day 1 (data not shown). Following 4 days of larval

rearing, the handling control group showed relatively good survival, with 60.2% (± 5.3) of the larvae being alive (Fig. 1A). Overall, increasing concentrations of EG yielded the highest larval survival rates (> 25%), and these were not statistically different from the control/unfrozen larvae (ANOVA: $F_{20, 23}$ = 4.346, p > 0.05; Fig. 1A). The best survival was obtained using 16% EG + 0.4 M TRE + 1% PVP in Milli-Q (47.5% ± 0.2). On the contrary, the poorest survival was generally obtained in treatments using the lowest concentrations of EG, 8% and 10% EG, with rates below 17% (Fig. 1A). Apart from the '8% EG + 0.6M TRE + MQ' combination, significant differences were observed when comparing these treatments with the control (ANOVA: $F_{20, 23}$ = 4.346, p ≤ 0.05).

Fig. 1

Swimming activity

The swimming activity of thawed larvae was significantly lower (≤ 34%) compared with the controls (84.4% ± 4.3; Fig. 1B) (ANOVA: $F_{20,23}$ = 10.550, p ≤ 0.05). On the whole, larvae cryopreserved using the lowest EG concentrations were the least active after thawing (Fig. 1B). The use of SW appeared to be beneficial in larvae cryopreserved with 14 or 16% EG + 0.4M TRE + 1% PVP. However, when using 0.6M of TRE, swimming activity tended to be higher when CPA solutions were prepared in Milli-Q Water (Fig. 1B). Based on swimming assessment, the highest rate of actively swimming larvae (34.2% ± 15.8) was obtained using the CPA treatment '14% EG + 0.4M TRE in FSW' (Fig. 1B). However, this treatment had a very low survival (7.9% ± 7.3; Fig. 1A) and swimming activity could only be assessed on a limited number of live larvae, which can explain the high swimming rate and variability. In view of this, the CPA combination yielding the most active larvae (31.7% ± 3.1) was 16% EG + 0.4M TRE + 1% PVP + SW (Fig. 1B).

Algae consumption

As expected, cryopreservation resulted in a significant decrease in the percent of larval feeding activity compared to controls (ANOVA: $F_{20,23}$ = 10.109, p ≤ 0.05; Fig. 1C). In addition, larvae cryopreserved with low concentrations of EG appeared to consume less algae than those cryopreserved with higher EG concentrations although no significant differences were found. The use of 14% and 16% EG generally gave better feeding response in thawed larvae, with over 18% of the larvae exhibiting gut colouration (Fig. 1C). The CPA treatment '14% EG + 0.6M TRE + 1% PVP prepared in Milli-Q water yielded the highest feeding percentage after thawing (37% ± 3; Fig. 1C).

Shell size

Overall, cryopreserved larvae were significantly smaller (~ -10%) than unfrozen larvae from the handling control group (ANOVA: $F_{20,149}$ = 2,316, p ≤ 0.05; Fig. 1D). For instance, the mean shell length of control larvae was 96.2 µm (± 6.8) compared with 85.5 to 87.5 µm for larvae from the cryopreserved groups (Fig. 1D). No significant differences between cryopreserved treatments were found.

Overall, the CPA combinations 14% EG + 0.6M TRE + 1% PVP in Milli-Q and 16 % EG + 0.6 M TRE + 1% PVP in Milli-Q resulted in the best larval fitness in Experiment 1, and were thus selected for the next experiments.

3.2. Experiment 2: effects of increasing equilibration time and larval density per straw

Using the best two CPA combinations (14% EG + 0.6M TRE + 1% PVP and 16 % EG + 0.6 M TRE + 1% PVP, in Milli-Q water), CPA equilibration time prior to freezing and larval density per straw during cryopreservation were investigated through two replicated freezing runs.

Survival

Recovery of larvae one day post-thawing was generally high, ranging from 77% for '14% EG-60min-Med' to over 88% for all other treatments (Data not shown). Four days post-thawing, overall survival remained high, ranging from 30% (± 23.1) to 76.3% (± 23.7) in some treatments (Fig. 2A). Statistical comparisons of the cryopreserved treatments with their respective controls (i.e., low, medium, and high densities) did not reveal any significant differences (ANOVA: $F_{19,20}$ = 1,050, p ≤ 0.05) (Fig. 2A). Nonetheless, the highest survival (76.3% ± 23.7) was obtained when using 16% EG + 0.6 M TRE + 1% PVP in Milli-Q water as a CPA solution, combined with a stocking density of 100,000 D-larvae per straw and a 20 min equilibration time. The second best treatment, yielding a more reliable 70.9% (± 2) survival success, was 14% EG + 0.6M TRE + 1% PVP in Milli-Q water combined with a low stocking density (50,000 D-larvae per straw) and a 20 min equilibration time before freezing (Fig. 2A).

Fig. 2

Swimming activity

Thawed larvae were logically less active in terms of swimming behaviour compared with their unfrozen counterparts (71 – 80%) at day 4 post-thawing (Fig. 2B). Most cryopreserved treatments resulted in significantly more inactive and abnormally swimming larvae (ANOVA: $F_{20,23}$ = 10,550, $p \leq 0.05$), with spinning behaviour consistently observed. However, several treatments showed no statistical differences with the control (ANOVA: $F_{20,23}$ = 10,550, $p > 0.05$) (Fig. 2B). More specifically, the cryopreservation treatments '16%EG-60min-Med', '16%EG-20min-Low', and '14%EG-20min-Low' yielded relatively high larval swimming rate of 54.4% (± 30.1), 43.4% (± 8.1), and 36.4% (± 6.6), respectively (Fig. 2B). Neither the CPA combinations, nor larval density nor the equilibration time showed any significant effect on this fitness parameter.

Algae consumption

The proportion of larvae with food in their stomach was high (≥ 78%) in the control groups while most of the treatments tested resulted in thawed larvae with low feeding abilities when compared to their respective controls (ANOVA: $F_{17,18}$ = 11,381, p ≤ 0.05) (Fig. 2C). In addition, D-larvae cryopreserved at medium or high stocking densities were generally the most affected with the lowest feeding success although no significant differences were observed (Fig. 2C). One treatment, namely '14%EG-20min-Low' corresponding to 14% EG + 0.6M TRE +1% PVP combined with a low stocking density and 20 min equilibration, gave the highest rate of feeding larvae (65% ± 12.7) and was not significantly different from the control (ANOVA: $F_{17,18}$ = 11,381, p > 0.05) (Fig. 2C).

Shell size

The cryopreservation process yielded significant delays in the development of post-thawed larvae compared to unfrozen larvae with mean shell lengths ranging between 85.5 µm and 87.8 µm compared with 102 µm for the controls (ANOVA: $F_{20,441}$ = 46,669, $p \leq 0.05$) (Fig. 2D). This growth inhibition was less pronounced when *P. canaliculus* D-larvae were frozen in 14% EG + 0.6M TRE + 1% PVP combined with 20min of equilibration at a low stocking density, with mean length reaching 92.45 µm ± 0.81 (ANOVA: $F_{20,441}$ = 46,669, $p > 0.05$; Fig. 2D).

Building on these findings, the treatment 14% EG + 0.6M TRE + 1% PVP, combined with 20 min of exposure to CPA before freezing and low stocking density (50,000 larvae per straw) was selected for the next experiment.

3.3 Experiment 3: effects of D-larval stage on freezing tolerance

Survival

Recovery of viable larvae one day post-thawing was high for all treatment tested with over 91.5% of the larvae surviving the freezing process (Fig. 3A). At the end of the 4 days experiment, the percentage of surviving larvae did not differ considerably between the two larval stages used and, overall, statistical analysis did not show any significant effect of cryopreservation on survival (ANOVA, $F_{1,3}$ = 5.500, *p = 0.114*) (Fig. 3A). For example, 63.9% (± 3.2) of larvae frozen at 48h old were alive compared with 66.9% (± 2.4) for the unfrozen 48h old larvae (Fig. 3A). In fact, more larvae survived when cryopreserved at 72h old (67.1% ± 3.5) than unfrozen 72h old larvae (60.8% ± 2.2) (Fig. 3A).

Swimming activity

Assessment of the larval swimming activity showed no significant differences between frozen and unfrozen treatments (ANOVA: $F_{3,7}$ = 0.686, *p = 0.606*) (Fig. 3B). The rates of actively swimming larvae were higher in the control unfrozen 48 h and 72 h-old groups, with 57% (± 17.3) and 77% (± 19) respectively, than in the cryopreserved treatments. Swimming ability between the cryopreserved treatments was comparable (39.6% ± 9.7 and 37.5% ± 0.2, for larvae cryopreserved at 48 h and 72 h, respectively (Fig. 3B).

Fig. 3

Algal consumption

In agreement with our previous results, feeding ability was reduced in thawed larvae compared to unfrozen larvae (ANOVA: $F_{3,7}$ = 96.598, p < 0.001). More specifically, 99% (± 1) of the larvae from the control groups had algal food present in their stomachs whereas only 28% (± 2) thawed larvae frozen at 48h old (ANOVA: $t_{3,7}$ = 12.905, *p = 0.001)* and 41% (± 1) of thawed larvae frozen at 72h old stage (ANOVA: $t_{3,7}$ = 11.022, *p = 0.001*) had food present in their stomachs (Fig. 3C). No significant difference was noted between the feeding rates of larvae cryopreserved at 48h old when compared with larvae cryopreserved at 72h old (ANOVA: $t_{3,7}$ = 1.883, *p = 0.248*) (Fig. 3C).

Shell size

Assessment of larval shell lengths at day 4 post-thawing yielded similar results to the feeding assessment. Mean shell size of control 48 h-old larvae was 102.9 µm (± 3.5) while control 72 h-old larvae averaged 111.9 µm (± 3.6). Thawed larvae were smaller than their unfrozen counterparts (ANOVA: $F_{3,7}$ = 7.301, *p = 0.042)*, with mean shell sizes of averaging 87.7 µm (± 3) and 93.8 µm (± 3.9), respectively for thawed larvae frozen 48 h post fertilization and 72 h post fertilization, respectively (Fig. 3D). No significant advantage in terms of life stages used for cryopreservation was found.

3.4 Larval fitness overall comparison of unfrozen versus cryopreserved larvae

Across Experiments 1-3, the best post-thaw performance was obtained when larvae were cryopreserved at 48h-old stage using 14% ethylene-glycol (EG) + 0.6M trehalose (TRE) + 1% polyvinyl-

pyrrolidone (PVP) prepared with Milli-Q water (Final concentrations), and 20 min of equilibration time. Using this treatment, larval survival one day post-thawing did not differ between the unfrozen control and the cryopreserved group at 89.1% ± 3.9 and 89.2 % ± 5.3, respectively (t-test: t_{10} = -0.099, *p = 0.923*). Likewise, survival four days post-thawing was not significantly different between the unfrozen and cryopreserved larvae (64.9% ± 3.5 versus; 53.5% ± 4.5, respectively; t-test: t_{10} = 2.011, *p = 0.072*) (Fig. 4A).

Behavioural responses such as swimming and feeding abilities were negatively affected by cryopreservation: significantly less larvae (T-test: t_{10} = 4.714, *p < 0.001*) were swimming following cryopreservation (24.5% ± 6.6) compared with their unfrozen counterparts (71.1% ± 7), corresponding to ~ 35% of the unfrozen control (Fig. 4A). Similarly, cryopreservation resulted in significantly lower (t-test: t_{10} = 16.198, *p < 0.001*) rate of feeding larvae (27% ± 3.4) compared with unfrozen controls (97.5% ± 0.9), corresponding to ~ 28% of the control (Fig. 4A). Finally, growth was delayed after thawing (t-test: t_{10} = 4.421, *p = 0.0013*). Shell length measured at 4 days post thawing was smaller for thawed larvae (89.2 µm ± 0.4) compared to unfrozen larvae (99.1 µm ± 0.4; Fig. 4B).

Fig. 4

4. Discussion

The development of a reproducible and robust cryopreservation protocol requires detailed study of several parameters that evaluate fitness post-thaw and determine whether a particular cryoprotectant and preservation method is adequate for implementation [13]. In this study, we used survival, swimming activity, algae consumption, and shell length as indicators of larval fitness and quality to assess the success of cryopreservation. Differences between the measurements, for example in Experiment 2, highlight the importance of not just looking at survival information but other indicators of larval fitness as well, as they provide a much richer picture of which treatment is likely to result in the highest long term survival and therefore be the most useful.

Research on other mollusc species like Pacific oyster (*Crassostrea gigas*) or blue mussel (*Mytilus galloprovinciallis*) indicate that there is not strong inter-species variability in the response to cryopreservation. In general, EG is the most used CPA for larvae cryopreservation in combination with other non-permeating CPAs like TRE or polyvinylpyrollidone (PVP) [3, 6, 8, 9, 16, 19, 24, 25, 26, 28]. In this study, it was found that higher concentrations of EG, in combination with TRE at a higher concentration and PVP were generally better for survival than lower EG concentrations. At these higher concentrations, the media used for CPA preparation, Milli-Q water or seawater had little effect. Overall, survival was not significantly different from the control. Differences in swimming behaviour, feeding ability and shell length however were seen, suggesting that long term survival may be compromised by cryopreservation, even at these higher concentrations. It would be interesting to use some of these combinations in further studies to look at what proportion of larvae are able to develop and settle as spat and then refining the concentrations further. For example, it would also have merit to look at PVP concentration, before re-considering other CPAs such as Me_2SO which has generally yielded lower survival rates for shellfish [7, 29, 30, 31].

Parameters such as CPA equilibration time and the stocking density per straw had not previously been studied for this developmental stage of Greenshell™ mussel. In the case of the equilibration time, the 20 min of exposure seemed to be appropriate to offer proper cryoprotection while minimizing toxicity. An excessive prolongation of exposure time could yield cell injuries and increase mortality following cryopreservation [1, 13, 22]. However, equally, too shorter equilibration can result in insufficient CPA permeating the larvae to afford protection and therefore also compromise survival. In further studies,

the methods for adding and removing CPAs could be investigated to determine whether a more stepwise process to avoid osmotic shock is beneficial. Similarly, if older larvae are investigated it may be important to re-investigate this variable as different cell types may have varying permeation rates.

Stocking density is an important variable to consider in cryopreservation of aquaculture species where a single family cross can literally result in millions of offspring and large numbers of individuals must be taken through larval rearing (10,000s to 100,000s) and settlement for field on-growing and evaluations. For practicalities of implementing cryopreservation in aquaculture selective breeding, it is important to determine the highest optimal value for scale-up purposes. If cryopreservation is to be implemented in commercial spat production, outside of selective breeding, then a process that enables hundreds of million to billions of larvae to be frozen at one time is required.

When cryopreserving a large amount of larvae at once, there is a possibility that the CPA molecules do not penetrate the larvae/cells evenly, therefore some of them do not achieve enough concentration of permeating CPA before cryopreservation and are not adequately protected. In this study, larval density per straw did not affect survival but the swimming ability of larvae was higher in low density treatments and one treatment in particular at a low density also had feeding rates that were not significantly different from control larvae (14% EG, 0.6M TRE, 1% PVP, 20 min equilibration). This result agrees with that of [12] who found that larval survival of the Eastern oyster, decreased as larval density increased but that study evaluated a much wider range of density (25 larvae/mL -100 000/mL). It may be worth considering whether larger volume straws or cryovials could be used to achieve the same number of larvae per unit at a lower density and increase survival. Although shifting to larger straws or cryovials increases inefficiencies of cryo storage; this may be worthwhile, particularly if larval performance can be further improved. Similarly, it may be worth investigating lower densities per straw for the same reason – so that the total number of larvae (as opposed to percentage) of larvae that are recovered post-thawing is higher. Other authors have observed the opposite effect with higher densities beneficial for some species [15] it is not known what is driving density effects but likely that there are a number of factors at play such as oxidative processes, handling, CPA permeation and that some of these may be better at high density at some at lower so there maybe trade-offs at play.

The final part of this study investigated whether larval performance post-thawing could be improved by freezing larvae at a later stage. It is well known from many previous studies that D-larvae are more resilient to freezing when compared with earlier developmental stages such as trochophore [6, 19, 20, 21, 25]. Although, [8] reported that Pacific oysters cryopreserved from a late D-larvae stage (i.e. 30 hpf) did not perform as well as larvae cryopreserved at earlier stages. We speculated that older mussel D-larvae that we had not yet fed may be more resilient to cryopreservation because they had a lower lipid content [2, 5]. However, in this study we saw no differences between the two stages of D-larvae evaluated, although 72 h Ds had slightly higher feeding rates. Previous research on Greenshell™ mussel larvae has demonstrated that stages earlier than those tested here appear more sensitive [21]. Further studies could consider whether larvae older than those tested here are better candidates for cryopreservation, despite their increased complexity of internal structures.

Although, we compared the best cryopreservation method across all experiments with unfrozen larvae, survival post-thawing was not significantly different from control larvae. However, swimming, feeding and growth were all less than what was observed for the controls.

Further research should be focused on the study of the long term effects of this cryopreservation protocol and its capability to produce mussel juveniles as well as the variability that can be expected amongst families. This is the next step.

Acknowledgements

Authors would like to acknowledge the Consellería de Educación, Universidad y Formación Profesional da Xunta de Galicia (Spain) for funding P.H. research stay at Cawthron and the New Zealand Ministry for Business, Innovation and Employment for funding the research (Shellfish Aquaculture; CAWX1801)

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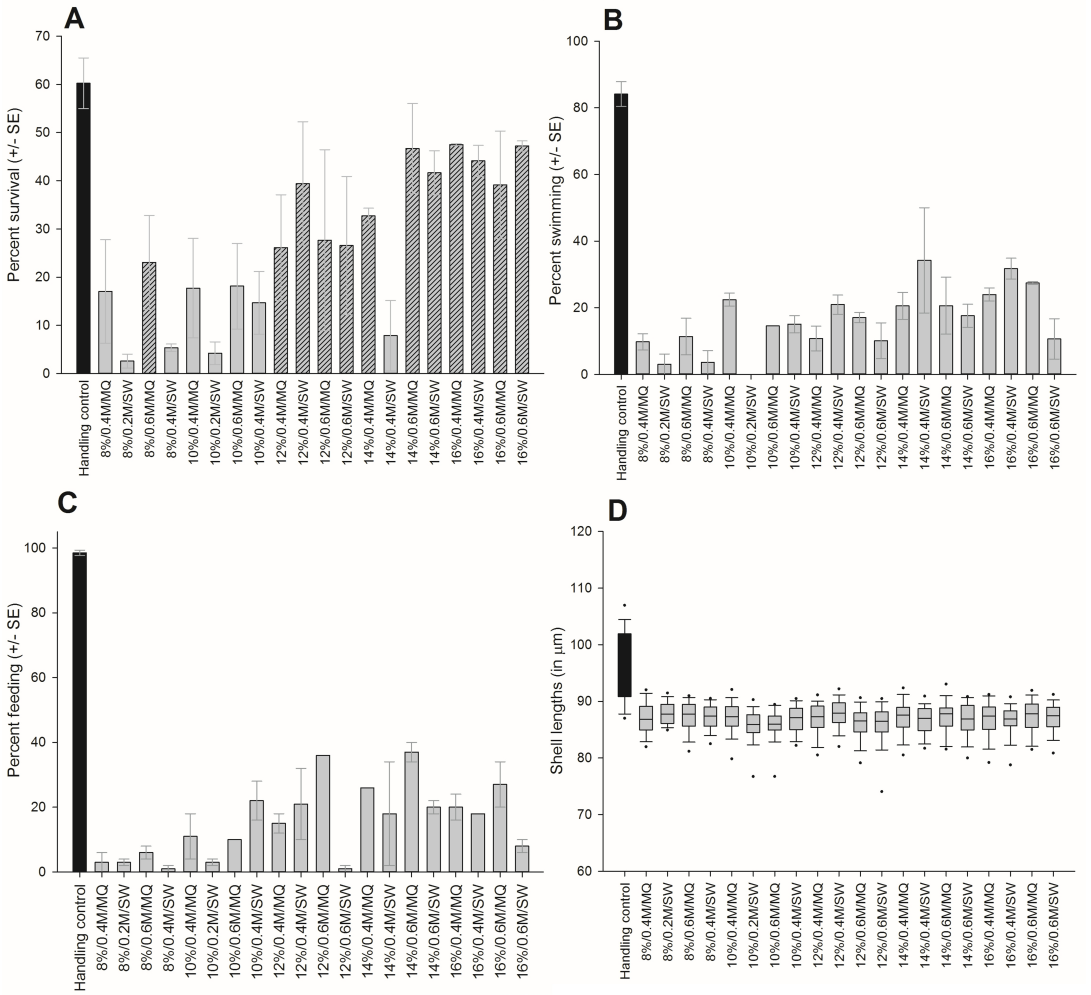
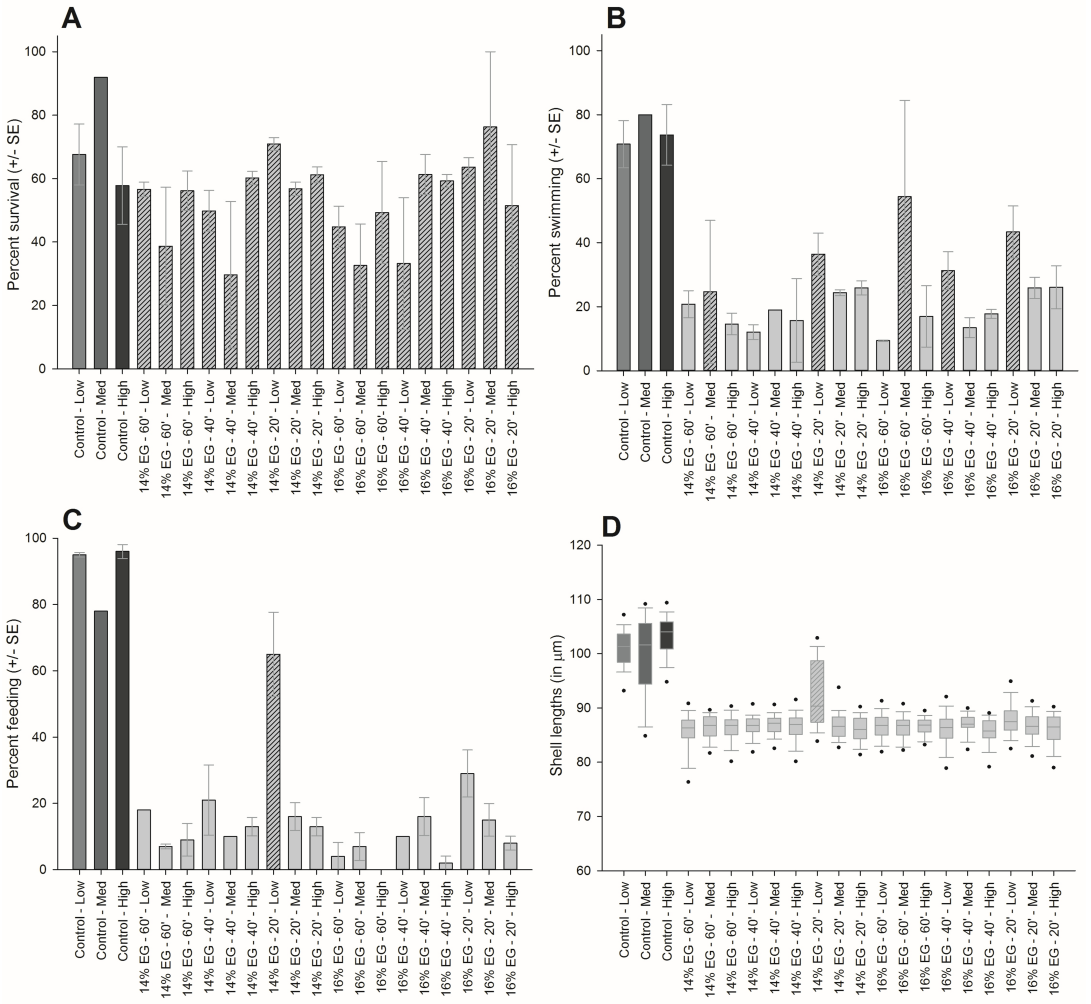
Figure 1. Fitness parameters were evaluated 4 days post-thawing on *Perna canaliculus* larvae and included: (A) survivorship, (B) swimming activity, (C) feeding efficiency, and (D) shell lengths of larvae. Endpoints were assessed on larvae previously unfrozen (handling control, black bars) and cryopreserved (grey bars) at the D-stage (48h-old) with different CPA combinations of EG (Ethylene-Glycol), TRE (Trehalose), 1% PVP (Polyvinylpyrrolidinone (w/v)) prepared in Milli Q water (MQ) or filtered Seawater (SW). Results are expressed as mean percentages (± SE, n = 2) or as box plots for the distribution of shell sizes measured from 60 larvae and expressed in µm (D). Line inside the box is the median value; box ends are the lower and upper quartiles (i.e. 25 and 75% of the population), lower and upper whiskers represent values outside the middle 50% quantile, and individual points the 5th and 95th percentiles. Bars/boxes presented in plain grey indicate a significant difference ($p \leq 0.05$) with the control (black), while dashed grey bars indicate no significant difference with the control (ANOVA, Dunnett's post-hoc test).

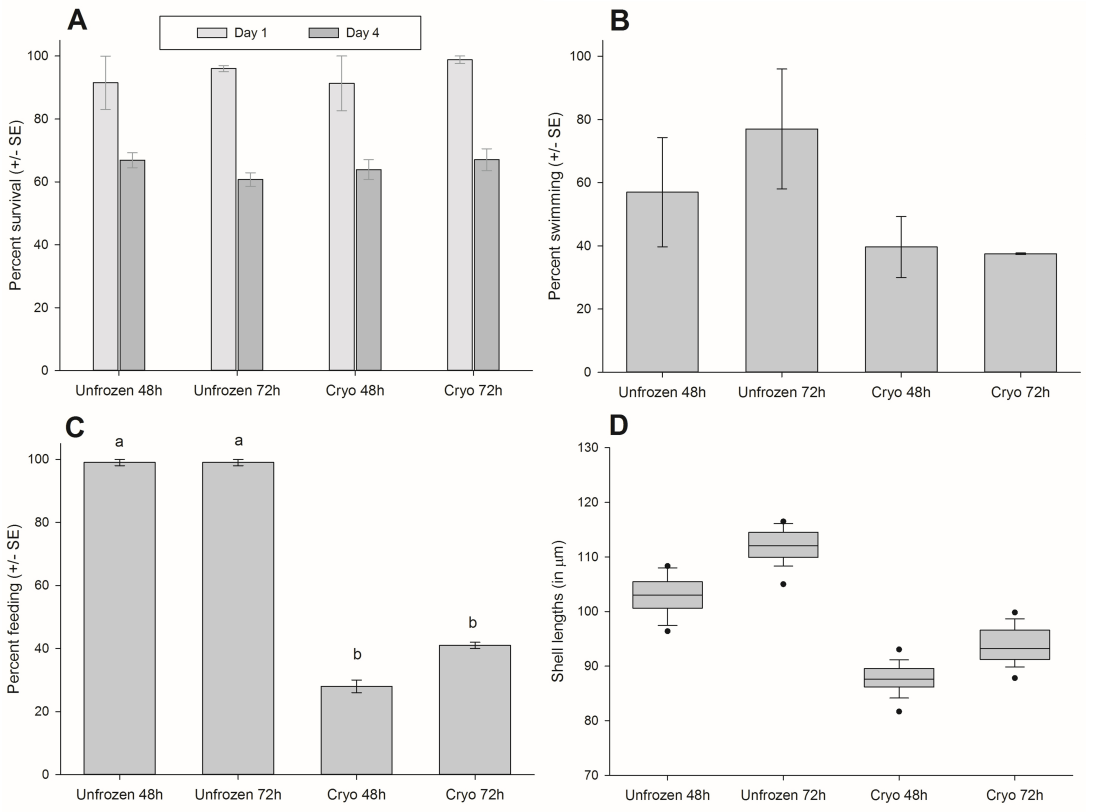
Figure 2. Fitness parameters were evaluated 4 days post-thawing on *Perna canaliculus* larvae and included: (A) survivorship, (B) swimming activity, (C) feeding efficiency, and (D) shell lengths of larvae. Endpoints were assessed on larvae previously unfrozen (control, dark bars) and cryopreserved (grey bars) at the D-stage (48h-old) at three stocking densities - Low (~50,000 per straw), Medium (100,000 per straw), High (200,000 per straw) - with 14% EG + 0.6M TRE + 1% PVP and 16% + 0.6M TRE + 1% PVP [Final concentrations of Ethylene-Glycol (EG), Trehalose (TRE), Polyvinylpyrrolidinone (PVP)] prepared in Milli-Q water, with varying equilibration times: 20', 40', and 60'. Results are expressed as mean percentages (± SE, n = 2) or as box plots for the distribution of shell sizes measured from 60 larvae and expressed in µm (D). Line inside the box is the median value; box ends are the lower and upper quartiles (i.e. 25 and 75% of the population), lower and upper whiskers represent values outside the middle 50% quantile, and individual points the 5th and 95th percentiles. Bars/boxes presented in plain grey indicate a significant difference ($p \leq 0.05$) with the respective control (dark), while dashed grey bars/boxes indicate no significant difference with their corresponding control treatment (ANOVA: Dunnett's post-hoc test). No thawed larvae were stocked in one replicate tank of the 'Control-Med' treatment resulting in no variance for this group.

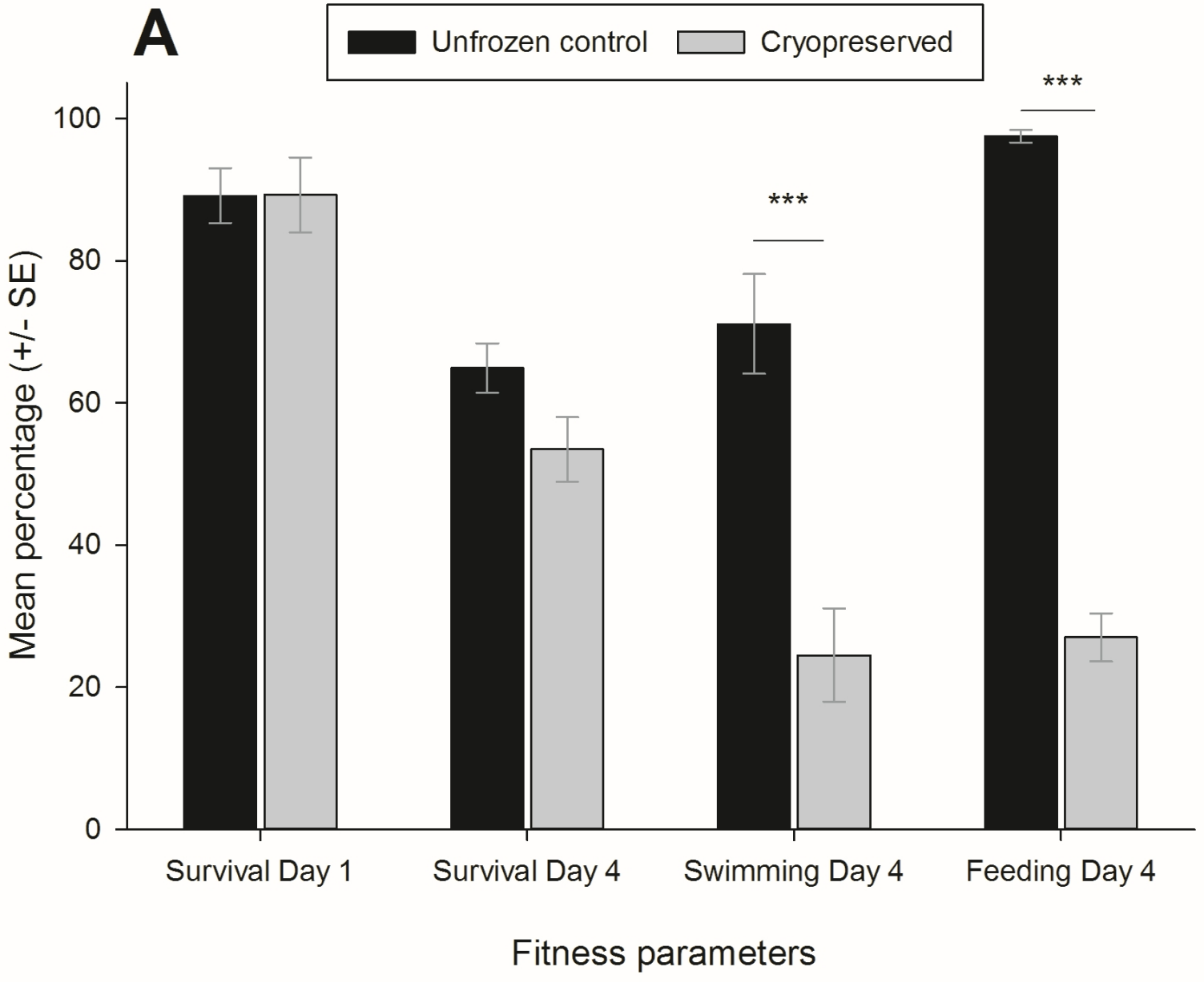
Figure 3. Fitness parameters were assessed on *Perna canaliculus* larvae derived from unfrozen and cryopreserved 48 and 72h-old D-larvae, and included: (A) survival on day 1 (light grey) and day 4 (dark grey) post-thawing, (B) swimming activity, (C) feeding efficiency, and (D) shell lengths of larvae 4 days post-thawing. Cryopreservation procedure consisted of a combination of 14% EG + 0.6M TRE + 1% PVP [Final concentrations of Ethylene-Glycol (EG), Trehalose (TRE), Polyvinylpyrrolidinone (PVP)] prepared in Milli-Q water], a 20min of equilibration time and a stocking density of ~100,000 larvae per straw. Results are expressed as mean percentages (± SE, n = 2) or as box plots for the distribution of shell sizes measured from 60 larvae and expressed in µm (D). Line inside the box is the median value; box ends are the lower and upper quartiles (i.e. 25 and 75% of the population), lower and upper whiskers represent values outside the middle 50% quantile, and individual points the 5th and 95th percentiles. Different letters indicate a significant difference between treatments (ANOVA, Dunnett's post-hoc test, $p \leq 0.05$).

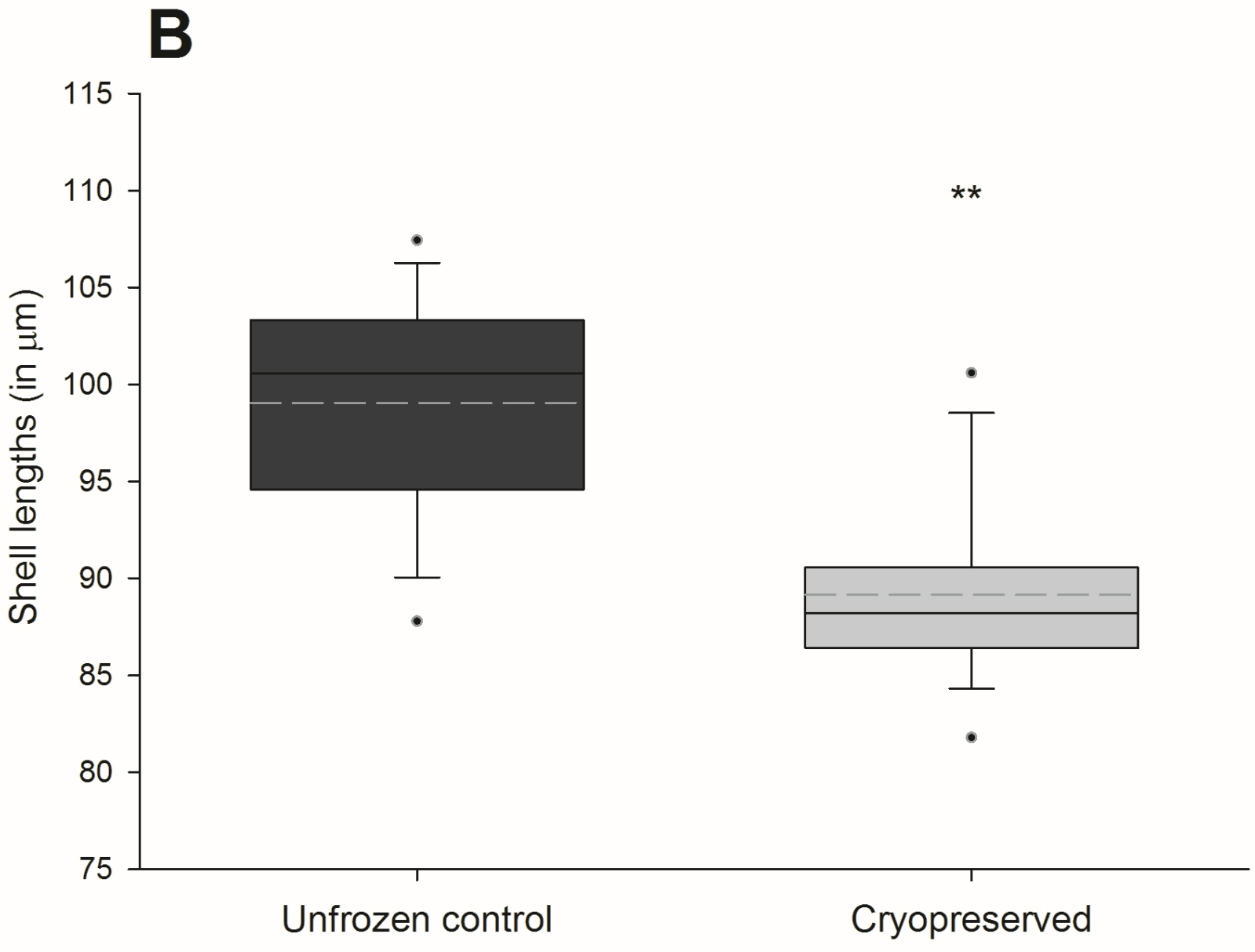
Figure 4. Summary of the different larval fitness parameters (A) and shell lengths on day 4 (B) from the three experiments. Fitness parameters were assessed on *Perna canaliculus* larvae derived from unfrozen (black) and cryopreserved (grey) 48h-old D-larvae using the best cryopreservation protocol: 14% EG + 0.6M TRE + 1% PVP [Final concentrations of Ethylene-Glycol (EG), Trehalose (TRE), Polyvinylpyrrolidinone (PVP)] prepared in Milli-Q water] combined with a 20min equilibration time and a stocking density of 50,000 – 150,000 larvae per straw. Results are expressed as mean percentages (± SE, n = 6) for (A) or as box plots for the distribution of shell sizes measured from 180 larvae and expressed in µm (B). Line inside the box is the median value, and dash line is the mean value; box ends are the lower and upper quartiles (i.e. 25 and 75% of the population), lower and upper whiskers represent values outside the middle 50% quantile, and individual points the 5^{th} and 95^{th} percentiles. Asterisks indicate a significant difference between 'unfrozen control' and 'cryopreserved' treatments at $p \leq 0.001$ (***) or $p \leq 0.01$ (**) using a Student's t-test.


A
Percent survival (+/- SE)
0
10
20
30
40
50
60
70
Handling control
8%/0.4M/MQ
8%/0.2M/SW
8%/0.6M/MQ
8%/0.4M/SW
10%/0.4M/MQ
10%/0.2M/SW
10%/0.6M/MQ
10%/0.4M/SW
12%/0.4M/MQ
12%/0.4M/SW
12%/0.6M/MQ
12%/0.6M/SW
14%/0.4M/MQ
14%/0.4M/SW
14%/0.6M/MQ
14%/0.6M/SW
16%/0.4M/MQ
16%/0.4M/SW
16%/0.6M/MQ
16%/0.6M/SW
B
Percent swimming (+/- SE)
0
20
40
60
80
100
Handling control
8%/0.4M/MQ
8%/0.2M/SW
8%/0.6M/MQ
8%/0.4M/SW
10%/0.4M/MQ
10%/0.2M/SW
10%/0.6M/MQ
10%/0.4M/SW
12%/0.4M/MQ
12%/0.4M/SW
12%/0.6M/MQ
12%/0.6M/SW
14%/0.4M/MQ
14%/0.4M/SW
14%/0.6M/MQ
14%/0.6M/SW
16%/0.4M/MQ
16%/0.4M/SW
16%/0.6M/MQ
16%/0.6M/SW
C
Percent feeding (+/- SE)
0
20
40
60
80
100
Handling control
8%/0.4M/MQ
8%/0.2M/SW
8%/0.6M/MQ
8%/0.4M/SW
10%/0.4M/MQ
10%/0.2M/SW
10%/0.6M/MQ
10%/0.4M/SW
12%/0.4M/MQ
12%/0.4M/SW
12%/0.6M/MQ
12%/0.6M/SW
14%/0.4M/MQ
14%/0.4M/SW
14%/0.6M/MQ
14%/0.6M/SW
16%/0.4M/MQ
16%/0.4M/SW
16%/0.6M/MQ
16%/0.6M/SW
D
Shell lengths (in µm)
60
70
80
90
100
110
120
Handling control
8%/0.4M/MQ
8%/0.2M/SW
8%/0.6M/MQ
8%/0.4M/SW
10%/0.4M/MQ
10%/0.2M/SW
10%/0.6M/MQ
10%/0.4M/SW
12%/0.4M/MQ
12%/0.4M/SW
12%/0.6M/MQ
12%/0.6M/SW
14%/0.4M/MQ
14%/0.4M/SW
14%/0.6M/MQ
14%/0.6M/SW
16%/0.4M/MQ
16%/0.4M/SW
16%/0.6M/MQ
16%/0.6M/SW


A
Percent survival (+/- SE)
B
Percent swimming (+/- SE)
C
Percent feeding (+/- SE)
D
Shell lengths (in µm)
Control - Low
Control - Med
Control - High
14% EG - 60' - Low
14% EG - 60' - Med
14% EG - 60' - High
14% EG - 40' - Low
14% EG - 40' - Med
14% EG - 40' - High
14% EG - 20' - Low
14% EG - 20' - Med
14% EG - 20' - High
16% EG - 60' - Low
16% EG - 60' - Med
16% EG - 60' - High
16% EG - 40' - Low
16% EG - 40' - Med
16% EG - 40' - High
16% EG - 20' - Low
16% EG - 20' - Med
16% EG - 20' - High


A
Day 1
Day 4
Percent survival (+/- SE)
0
20
40
60
80
100
Unfrozen 48h
Unfrozen 72h
Cryo 48h
Cryo 72h
B
Percent swimming (+/- SE)
0
20
40
60
80
100
Unfrozen 48h
Unfrozen 72h
Cryo 48h
Cryo 72h
C
Percent feeding (+/- SE)
0
20
40
60
80
100
a
a
b
b
Unfrozen 48h
Unfrozen 72h
Cryo 48h
Cryo 72h
D
Shell lengths (in µm)
70
80
90
100
110
120
130
Unfrozen 48h
Unfrozen 72h
Cryo 48h
Cryo 72h


A
Unfrozen control
Cryopreserved
***
***
100
80
60
40
20
0
Mean percentage (+/- SE)
Survival Day 1
Survival Day 4
Swimming Day 4
Feeding Day 4
Fitness parameters


B
115
110
105
100
95
90
85
80
75
Shell lengths (in µm)
**
Unfrozen control
Cryopreserved