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1 # Long term survival of cryopreserved mussel larvae (*Mytilus* 2 *galloprovinciallis*)

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9 **Abstract**

10 Due to the economic importance of the mussel *Mytilus galloprovincialis* in the Spanish aquaculture, 11 there is a growing interest in developing alternative methods to ensure the supply of good-quality 12 biological material and to minimize the uncertainty and variability that might be a source of 13 economic risk for this sector. The aim of this work was to develop a successful cryopreservation 14 protocol for *Mytilus galloprovincialis* larvae and study long-term effects by assessing survival and 15 development post cryobanking. We evaluated the effect of previously studied cryoprotecting agent, 16 Ethylene glycol (EG) for cryopreservation of trochophore larvae and D-veliger larvae 48h and 72h 17 old, following an existing preliminary protocol for *M. galloprovincialis* mussel trochophores. The 18 protocol selected for cryopreservation consisted on holding at 4ºC for 2 min, then cooling at 1ºC/min 19 to -12ºC, holding for 5 min, then cooling at -35ºC with a rate of 1ºC/min, then plunging into liquid 20 nitrogen for storage. Thawing took place by immersion in 35ºC water bath. With this protocol the 21 initial percentage of short-term developed a D-larvae (48 hours incubation post-thaw) was 22 48.9 ± 7.6% with 10% EG + 0.2 M Trehalose (TRE). This initial test only studied the trochophore 23 larvae until they reached the D-stage (48h old); in the present experiment experiments, we replicated 24 this study but also carried out a long-term larval rearing with cryopreserved trochophore larvae in 25 comparison to fresh larvae. This experiment allowed a comparative post-freezing analysis of both 26 larval development and survival. Larval settlement was also characterized after 13 days. The data 27 revealed that until settling, the survival of the cryopreserved larvae was slightly lower than the 2.8% 28 reported in the larval rearing for Greenshell™ mussel. Over time, there was an initial difference in 29 size of cryopreserved larvae when compared to controls, but from day 17 onwards the size between 30 both types of larvae began to stabilize. Attending to settlement, we obtained a 64% of success of 31 cryopreserved larvae respect to the control.

32 *Keywords: Mytilus galloprovincialis, trochophore larvae, cryopreservation, larval rearing.*

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33 **1. Introduction**

34 *Mytilus galloprovincialis* (Lamarck, 1819) is one of the most cultivated mollusc species worldwide
35 due to its great economic value and growing demand over last years (Di Matteo *et al.,* 2009; Wang
36 *et al.*, 2014). The mussel global production in 2014 was 1,901 million tons and represented 12% of
37 total mollusc production (FAO 2018). Moreover, its economic value amounted to 4,070 million
38 dollars (OESA, 2017, FAO 2018). The EU inner market of mussel is shy below 600,000 tonnes with
39 a low import rate and most of EU´s mussels come from Spain (FAO 2018), where mussels represent
40 annually 81% of their total national aquaculture production. Globally, Spain is the third mussel
41 producer, behing China and Chile (FAO, 2018). The latests Spanish figures show that in 2017,
42 273,517 tons were obtained from mussel harvesting, from a total production of 345,635 tons of total
43 molluscs (APROMAR, 2018). Most of Spanish mussels come from the northwest, 95.7% of all
44 Spanish´s production is concentrated in the region of Galiciawith 3386 mussel rafts. Mussel rafts are
45 usually family owned and the mussel farming business remains quite traditional but sustaining a
46 high socio-economic impact, generating 11000 direct jobs and and stimate that for each one of the
47 latter 2.5 indirect jobs are generated in the area.

48 Several factors can affect the larval development and/or culture, such as seasonal variations, food
49 availability, temperature or even population densities during feeding (FAO, 2017). Although not
50 only environmental factors can be the cause of a poor season. The production can also be affected by
51 other reasons such as the presence of parasites or punctual pollution events (FAO, 2017; Day et al.,
52 2007; Paredes et al., 2013). Due to the great importance of mussels in the mollusc aquaculture
53 industry, there is a big interest in the development of production methods for inland seed production,
54 to ensure the continuous good-quality supply of biological material. In these lines, selective breeding
55 programmes have been carried out to enhance farming and cryopreservation techniques, which have
56 been demonstrated useful for achieving selective breeding programs (Di Matteo et al., 2009; Paredes
57 et al., 2012). The cryopreserved sperm of livestock has become a billion-dollar global industry,
58 while cryopreservation of aquatic species remains a research activity with little commercial
59 application despite reports of successful protocols for many organisms. In the field of molluscs,
60 sperm cryopreservation has been widely studied (Paredes, 2014) but there are also positive reports
61 for different types of larvae. The initial approach of local aquaculture, still very traditionally
62 managed, to the global markets has been successful. For example, the average EU members increase
63 their annual consumption of mussels from 200 gr to 4 kg (FAO, 2018).

64 Taking into account the increased challenges, the industry is facing irregular production due to:
65 pollution events, disseases, increasing production costs. Without forgetting about the long term
66 sources of uncertainty like global warming: ocean acidification, increasing coastal population,
67 expanding market demands orecosystem over exploitation. It is time to rely more on all the

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68 knowledge and resources technology can offer to the sector in order to allow the sector some
69 independence from unforeseen circumstances that can affect the mussel industry at a local or global
70 level. As in the case of livestock, cryopreservation is apowerful biotechnology for marine farming as
71 it has proven to be for inland farming.

72 Cryopreservation allows to store genetic information in a stable state at -196ºC in Liquid Nitrogen
73 (Dennison et al., 2000), proving to be an interesting tool for aquaculture management (Paredes et al.,
74 2013). Cryobiology not only allows biological storage, but from the industrial point of view it helps
75 to synchronize the gametes of both sexes from different seasons, decrease the expenses on
76 maintaining broodstock all year round and provides seed supply throughout the year (Smith et al.,
77 2012; Smith et al., 2001). In a suitable scale, cryopreservation could store genetic biodiversity for
78 seed production of species such as *M. galloprovincialis* (Paredes et al., 2013; Wang et al., 2014).

79 Lanan 1971 on *C. gigas* sperm was a Pioneer study on applying cryopreservation to marine
80 organisms and molluscs. Most publications in the field of marine cryopreservation (±100) deal with
81 molluscs, and among them, oysters are the best studied due to their global economic importance.
82 *Crassostrea gigas*, followed by *C. virginica* are the most popular (Paredes 2015). Regarding
83 mussels, sperm cryopreservation is the most reported followed by larvae due to the difficulty of
84 cryopreserving oocytes successfully, described protocols and cryopreservation information for *M.*
85 *galloprovincialis* can be found in few reports like Di Mateo et al. 2009, Wang et al 2011, Paredes et
86 al. 2013, Wang et al. 2014 or Heres et al. 2019.

87 The aim of this study was to test and improve the preliminary cryopreservation protocol for *M.*
88 *galloprovincialis* larvae developed by Paredes et al. (2013) that had only addressed mussel survival
89 48 hours post-thaw and study the long-term survival post-cryopreservation. In addition, we tested the
90 capacity of this preliminary protocol to produce seed and alternatively look for other larval stages
91 that could potentially resist cryopreservation better.

92

93 **2. Material and Methods**

94 **2.1 Gamete Collection and Cryopreservation Methods**

95 Mature blue mussels (*M. galloprovincialis*, Lamark 1819) were obtained from the wild in the south
96 margin of Ria de Vigo (Galicia, NW Spain) and deposited in PVC tanks with Filtered Sea Water -
97 35-37‰ (FSW 0.22 µm + UVA) at 18ºC. Mussel spawning was induced by thermal cycling into a
98 20L tray and during the process, actively spawning individuals were transferred to 250 mL beakers.
99 Gametes from a male and a female were collected and transferred into FSW separately, in order to
100 minimize genetic variability (Stebbing et al., 1980; Klöckner et al., 1985). Oocyte quality and

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101 maturity were examined focusing on their shape and colour before fertilization, sperm was checked
102 for motility. A small volume of sperm was added to the oocyte suspension (approximately a rate of
103 20:1) and a 15 minutes contact period was allowed before the evaluation of the fertilization
104 percentage. The cell batches were incubated up to 72h at 18ºC. At 18-20h post-fertilization, the
105 fertilized eggs had developed to trochophores, at this point a subsample was retrieved for
106 cryopreservation experiments. The rest of the sample continued to be incubated to 48 and 72h-old D-
107 larvae, at both this points in time (48 and 72h) subsamples were also collected for subsequent
108 cryopreservation trials to evaluate, in this case, the short-term cryopreservation effects on each
109 development stage. In another spawning event a pool composed by two females and a pool of three
110 males were collected to carry out another set of cryopreservation experiments with trochophore
111 larvae (18-20h post-fertilization) followed by a complete larval rearing to study long-term effects of
112 cryopreservation and settlement.

113 The protocol selected for cryopreservation (Cryologic Controlled-rate freezer) consisted on holding
114 at 4ºC for 2 min, then cooling at 1ºC/min to -12ºC, holding for 5 min for seeding check, then cooling
115 at -35ºC with a rate of 1ºC/min, then plunging into liquid nitrogen for storage. Thawing of the 0.25
116 mL cryopreservation straws took place by immersion in 35ºC water bath for 6 seconds.
117 Addition/dilution of the cryoprotecting agents was done in a single (1:1) step at room temperature
118 (18-20ºC).

119 **2.2 Cryoprotecting Reagents**

120 The cryoprotecting solutions consisted of different combinations of Ethylene-Glycol (EG) and
121 Trehalose (TRE), chemicals obtained from Sigma Aldrich chemicals (St Louis, MO, USA). All of
122 them were prepared in Filtered Sea Water (FSW, 0.22 µm + UVA). The cryoprotecting solutions of
123 Ethylene-Glycol (10-15% v/v) were always prepared at double the final concentration required in the
124 experiments (During exposure to cryoprotectants there is a 1:1 dilution step). Cryoprotecting agents
125 were selected according to results from Heres et al., 2019.

126 **2.3 Larval Rearing**

127 Pre-cryopreservation incubation was made in two 150 L tanks at 18-20 ºC ($0.04x10^6$ /litre). After 20
128 h under these conditions, the larval development was checked and cells ability to reach the
129 trochophore larvae stage was evaluated (motility, normal development). Trochophore larvae were
130 carefully collected by gently siphoning the contents of the tanks through a 40µm screen semi-
131 submerged in order to avoid larvae being mechanically damaged. Trochophores were then gently
132 swirled on the screen, concentrated into 30 mL tubes for experiments.

133 As on prior occasions, the cryoprotective agent was added 1:1 in a single step, to a FSW with the
134 sample. After 15 min of equilibration, the 0.25 mL straws were loaded, sealed and introduced in the
135 controlled-rate freezer.

136 Taking into account prior reports of 50% survival to the cryopreservation process (Paredes et al.
137 2013), twice as many cells were cryopreserved in comparison to control density (1million/tank).
138 Therefore, if 50% of cryopreserved larvae survived, as expected after 48 hours consideration
139 preliminary data (Paredes et al., 2013), both cryopreserved and controls would have the same density
140 at the start of the larval rearing. During 33 days a larval rearing of the mussel *M. galloprovincialis*
141 was carried out from cryopreserved trochophore larvae (18-20h) to seed, the tanks were repeatedly
142 sampled throughout the incubation, usually twice a week, in order to check parameters such as
143 survival, density or larvae growth. The constant aeration necessary for the larvae was provided by
144 glass tubes and the cultivation temperature was maintained at 18-20 °C in tanks of 150 L. Feeding
145 consisted 60 to 100 equivalentes (mix of *Tisochrysis lutea, Rhodomonas lens, Chaetoceros*
146 *neogracile, Phaeodactylum tricornutum* and *Tetraselmis suecica*), as described in Mueller-Feuga et
147 al. 2003.

148 From day 22 onwards, the larvae stopped being in suspension in the water column and were placed
149 in settlement drums (150 micron mesh). On the last day of culture (day 33), all juvenile mussels that
150 had been fixed to the drums were collected and fixed with formalin in labelled containers. In the
151 subsequent analysis, the total number of larvae fixed was calculated and the average size of each
152 treatment were measured (n=35 for each tank).

153 **2.4 Cryopreservation Survival Alongside Larval Age**

154 In order to compare survival 48 hours post cryopreservation at different development stages,
155 trochophore larvae, D-lavae 48 hours old and D-larvae 72 hours old were cryopreserved, using the
156 same protocol and same cryoprotecting agent concentration. In the case of trochophores, the
157 guideline for survival was the percentage of metamorphosed larvae to D-larvae stage after 48 hours.
158 In the case of larvae that had been cryopreserved in the D-larvae stage, survival criteria was based
159 on feeding the larvae for 48 hours and counting the number of normal D-larvae that had fed (clear
160 coloration can be seen through the proto-shell).

161 **2.5 Larval Abnormality Criteria**

162 The discrimination between normal D-larvae and abnormal D-larvae was determined under
163 microscope attending to previous work focused on shell larval morphology and guidelines from
164 other experts in the shell abnormalities and abnormally developing larvae of related mollusc in
165 ecotoxicological larval bioassays (His et al., 1997, Paredes et al., 2013, Rusk, 2012, Ventura et al.,

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166 2016). Typical larval abnormalities found ranged from: delayed development (trochophores),
167 deviations from the D-larvae shell shape like indented margins or hinge deformations (concave or
168 convex hinges) or presence of clear protruding mantle.

169 **2.6 Data and Statistical Analysis**

170 Filtered Sea Water parameters were monitored with a multiparametric probe, cooling/warming
171 during cryopreservation were controlled by either a Cryologic controlled-rate freezer or a K-type
172 Hanna thermocouple. The data were analysed by one-way analysis of variance (ANOVA) followed
173 by Dunnett or Bonferrony post-hoc tests, using the SPSS 15.0, with $p<0.05$. Prior to the statistical
174 analysis, both homogeneity of variance and normality were tested and when necessary data were
175 first arcsine-transformed to achieve normality (Hayes, 1991). Statistical tests were performed
176 according to Newman, 1995 and Sokal and Rohlf, 1995.

177 **3. Results**

178 The effects of cryopreservation beyond 48 hours post-thawing are shown in figure 1 as larval density
179 for 22 days (Cryoprotecting agent used 10% EG + 0.4M Trehalose). There was a steep drop in larval
180 survival for the first 12 days post-thaw. From that point onwards, survival stabilized at 0.17% of the
181 initial amount of larvae, meanwhile in the case of controls the survival in average was 28.13%. This
182 represented a survival of cryopreserved trochophore larvae less than 1% of the controls.

183 Regarding larval fitness, both larval normality (morphological assessment) and size (growth
184 indicator) were within typical parameters. In the case of larval normality (Fig. 2), it was found that
185 normality remained constant through the incubation without significant differences during the first
186 days, progressively increasing up to day 17 when no difference was found longer with controls. This
187 high number of normal larvae coincided with the point in time where (Fig. 1) survival of the
188 cryopreserved larvae equilibrated.

189 The length difference between cryopreserved and control D-larvae (Fig. 3) was 15% on day zero
190 (corresponds with day 5 post-fertilization and by this time all larvae had undergone metamorphosis
191 to D-veliger larvae) and almost 4% on day 17 (Fig. 3, Table 1). Larval settlement was calculated as a
192 last quality measure of the larval rearing, it is a crucial step in larval development, settlement in
193 controls was higher than in the cryopreserved treatments only by 36% (Fig. 4).

194 Table 1. Average larval size (μm) during 17 days, measured from normal D larval rearing from
195 control and cryopreserved larvae. Mean ± SD, n=35 per tank, 3 tanks per treatment.

Days	Control larvae	Cryopreserved larvae

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0	110,5±1,7	93,6±0,9
2	115,9±0,7	87,4±1,3
4	135,4±1,4	100,3±2,9
7	166,5±11,8	135,3±16,7
10	201,4±5,5	139,9±29,5
11	211,2±15,2	156,0±12,0
17	287,6±11,4	276,3±11,4

196

197 When comparing post-thaw normal development of larvae alongside different larval stages and
198 concentrations of cryoprotecting agents. There was a clear variability when comparing larval stages.
199 Ethylene glycol 10% and 10%+0.4M Trehalose was the best option for Trochophore larvae,
200 meanwhile D-larvae (48h) seems to be severely affected by cryopreservation with at least 75%
201 abnormality. On the opposite side of the spectrum was D-larvae (72h) which shows survivals over
202 75% normal larvae in all treatments (Fig. 5). For D larvae of 48 and 72 hours old, the best results
203 were obtained using a concentration of EG 15% (96.3 ± 3.51%) and EG 10% (6.42 ± 3.10%)
204 respectively.

205 **Discussion**

206 Worldwide mussel aquaculture depends mostly on the harvesting of mussel seed from natural
207 environment, despite the fact that this seems quite cheap and convenient, it implies that the supply of
208 mussel individuals for rearing might be limited due to the seasonal availability and other unexpected
209 circumstances, such as water quality parameters. Conditions that in the context of climate change
210 and raising coastal population might be highly unreliable (Sea Grant 2007, Kamermans and Capelle
211 2019) are increasingly unpredictable both short and long-term.

212 However, cryopreservation could ensure a sustainable source of seed and provide a repository of
213 interesting biological material and genetic crosses or selected mussel lines whose genetic properties
214 provide suitable generations for hatchery production (Adams et al. 2009, Smith et al. 2001). Mussels
215 (germ cells, embryos and larvae) are also a very interesting and increasingly popular model
216 organism for marine research; therefore, this study has also important implications for marine basic
217 research. In the present study, we have developed a long-term evaluation of cryopreserved
218 trochophore larvae from the spawning of the reproductors to cryopreservation, thawing, larval
219 rearing and settlement for the first time using *M. galloprovincialis.*

220 The survival of cryopreserved trochophore larvae in the first 48 hours post-thaw had been reported
221 to be around 50% by Paredes et al., (2013). In this current set of experiments, the previously
222 mentioned protocol was used to test the ability to produce seed from the cryopreserved trochophores.
223 Clear differences were located among treatments of cryopreserved larvae in comparison to controls
224 within a few hours post-thaw, even despite the initial correction on density (cryopreserved larval
225 rearing started at double the control density to compensate for the before mentioned expected
226 mortality of 50% in the first 48 hours). The important drop in survival found in the first 10-12 days
227 had also been observed in another mussel species, *Perna canaliculus* (Paredes et al., 2012, Rusk,
228 2012). The achieved 1% survival after 22 days is also in the line with the survival obtained with *P.*
229 *canaliculus* cryopreserved trochophores, which was 2.8% and 0.03% respectively (Paredes et al.,
230 2012, Rusk, 2012).

231 Fitness indicators showed a high percentage of normality of the metamorphosed D-larvae but also
232 showed an evolving difference in size of those D-larvae along the rearing period, from 15%
233 difference with controls at the beguinning to a 4% difference at the end of the 22 days incubation
234 period. Trochophore larvae are lecitotrophic and therefore they do not feed, the metamorphosis to D-
235 larvae depends on the energy accumulated in the remaining yolk. Cryopreserved trochophore larvae
236 do their metamorphosis to D-veliger larvae and two interesting effects can be seen in 48 hours: first,
237 there is a small percentage of abnormal larvae. This includes those larvae that do not undergo
238 metamorphosis and therefore remain as trochophores and those that have morphological defects
239 including indented margins or hinge deformations in the shell (concave or convex hinges) or
240 presence of clear protruding mantle. This low percentage of abnormality reflects that
241 cryopreservation in this case does not interfere with metamorphosis and therefore, the percentage of
242 D-larvae post metamorphosis cannot be used as an indicator of long-term cryopreservation success,
243 although it can be very useful as an early quality indicator.

244 Second, there is a difference in size between cryopreserved larvae and controls. This could be
245 explained as a growth delay due to low temperature exposure and metabolic reactivation post-thaw
246 but together with the survival data, it might indicate sublethal damage caused during
247 cryopreservation. The stresses and micro-damages caused during the process of cryopreservation
248 might use up part of the energy otherwise used for metamorphosis and growth and deviate it to deal
249 with Reactive Oxygen Species (ROS) or repair structural micro-damages (Odintsova et al., 2017).

250 It seems clear that most of those larvae can indeed grow and feed and no direct relation was found
251 between mortality during larval rearing and initial D-larvae size (although this might need an in
252 depth study in order to understand underlying mechanisms). This finding about reduced size is in
253 agreement with prior studies; cryopreserved trochophores of *Crassostrea gigas* showed a 10% and

254 *Perna canaliculus* D-larvae showed a20% delay in size when compared to controls after 48h
255 incubation post-thaw (Paredes et al. 2012, Rusk 2012, Paredes et al 2013, Heres et al. 2019).

256 The development of a suitable cryopreservation protocol depends on the targeted species and cell
257 type, meanwhile the prior has been suggested to be not as determinant in the case of Molluscs as it
258 seems to be for other fila like Echinodermata (Paredes et al., 2013), the latter is a widespread
259 agreement in the cryobiology community and has been proved many times, including in this work
260 (Adams et al., 2009, Odintsova and Boroda, 2012, Paredes and Bellas, 2009, Tiersch et al., 2011,
261 Rusk, 2012, Wang et al., 2011).

262 The high survival obtained with the D-larvae of 72h-old is quite surprising taking into account that
263 after 72 hours the mussel protoconch is quite developed. Prior studies with mollusc´s D-larvae from
264 Rusk, 2012 (48h old larvae *P. canaliculus*) and Wang et al., 2011 (30h old larvae of *M.*
265 *galloprovinciallis*) obtained respectively high (50-60%) post-thaw survival but after a larval rearing
266 a 0.1% and 1% survival was obtained. Results shown in figure 5 for D-larvae of 48 h-old are not
267 promising at all with our protocol; meanwhile the 72h-old D-larvae yielded the best survival
268 numbers obtained up to date. This results point towards an interesting direction for future research,
269 pondering the differences among the protocols, the larval age, development characteristics in order
270 to understand why this variability happens. It would also be in order to carry out a complete larval
271 rearing with the 72hour-old D-larvae in order to assess survival along incubation and settlement and
272 study in depth the possible correlations between growth and survival post-thaw.

273 Settlement is the final fitness test. Paredes et al., 2012, Rusk, 2012 and Wang, 2011 reported a high
274 number of competent larvae among those few that had survived the larval rearing process but there
275 was no actual data of settlement. We obtained a 64% settlement in comparison to control larvae.
276 This is extremely important because, even after 22 days of larval rearing, settlement is significantly
277 lower than controls, therefore there are some long-term repercussions on the cryopreservation
278 process. When addressing the survival of a cryopreservation test, is important that survival is
279 reported, but also that is clear how powerful the experimental ending point is, there are weaker
280 survival clues: movement post-thaw, division, intact morphology. On the other side, there are
281 stronger points of survival assessment: metamorphosis, growth, presence/absence of abnormalities,
282 feeding. On top of that, it seems that above all, it is important that not only one point of assessment
283 be used to report survival post cryopreservation.

284 It is clear that this protocol can right now be used to produce juveniles from cryopreserved larvae, in
285 enough quantities that could be used either for research purposes or for small scale aquaculture
286 proceeding like selective breeding (Nguyen et al., 2012) all year-round. Mussels are incredibly
287 fertile organisms and the cryopreservation process still allows us to increase the density of larvae
288 during the cryopreservation process to increase the final number of larvae settled. On the other side,

289 there are some factors worth studying in order to understand the drastic decline in survival along
290 larval rearing and it´s connection to larval abnormality levels and size. These studies should be
291 accompanied by measurements of the ROS, apoptotic processes and other parameters that could help
292 us understand the stresses these larvae are under during the process of cryopreservation in order to
293 try to improve their fitness. Finally, we already knew that trochophore larvae were very delicate and
294 our preliminary results about D-larvae has allowed us to identify a larval stage that seems to be quite
295 resistant to cryopreservation.

296

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395

396 **Captions**

397 Figure 1. Percentage of survival (± SD) of *Mytilus galloprovincialis* larvae after cryopreservation
398 during a larval rearing of 22 days (1 million larvae/tank, n=3 tanks per treatment, except squares
399 where n=2)). The open cycle represents the theoric 50% survival from cryopreserved trochophore to
400 D larvae that has been obtained by Paredes et al. 2013 and fits with our survival data.

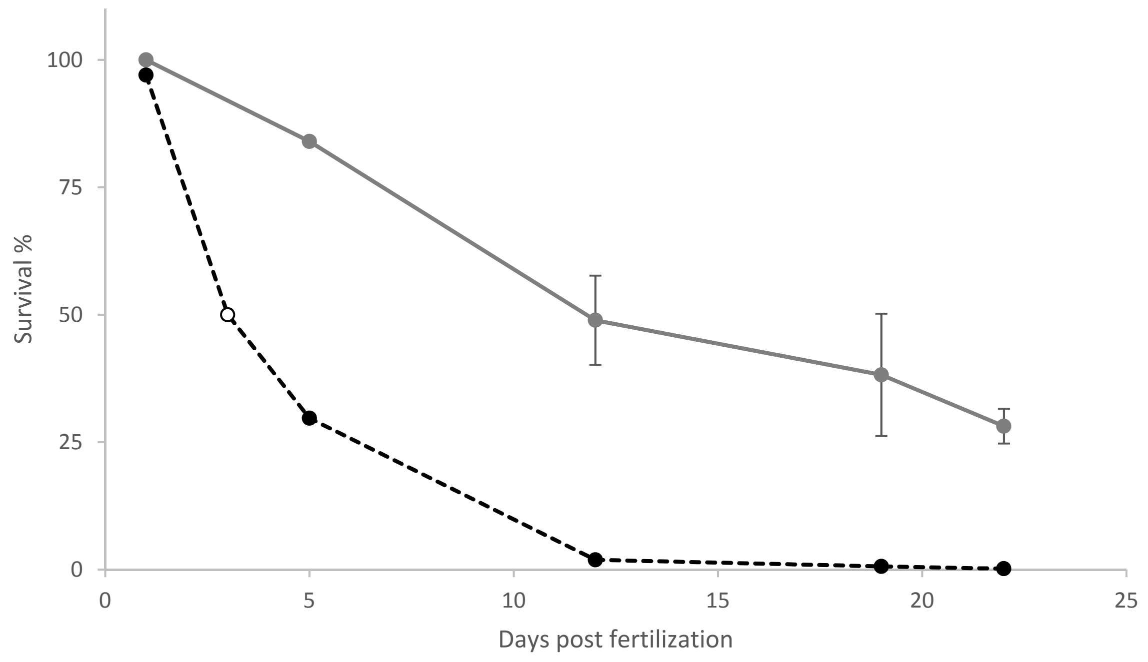
401 Figure 2. Percentage of normal D-veliger larvae for the two treatments after cryopreservation at the
402 trochophore larvae stage. Typical larval abnormalities found ranged from: delayed development
403 (trochophores), deviations from the D-larvae shell shape like indented margins or hinge
404 deformations (concave or convex hinges) or presence of clear protruding mantle. The asterisk
405 symbol indicate statistical differences $P < 0.05$. Mean ± SD

406 Figure 3. Average growth (µm) during 17 days of normal D larval rearing from control and
407 cryopreserved larvae of the trochophore larvae cryopreservation. The asterisk symbol indicate
408 statistical differences $P < 0.05$. Mean ± SD

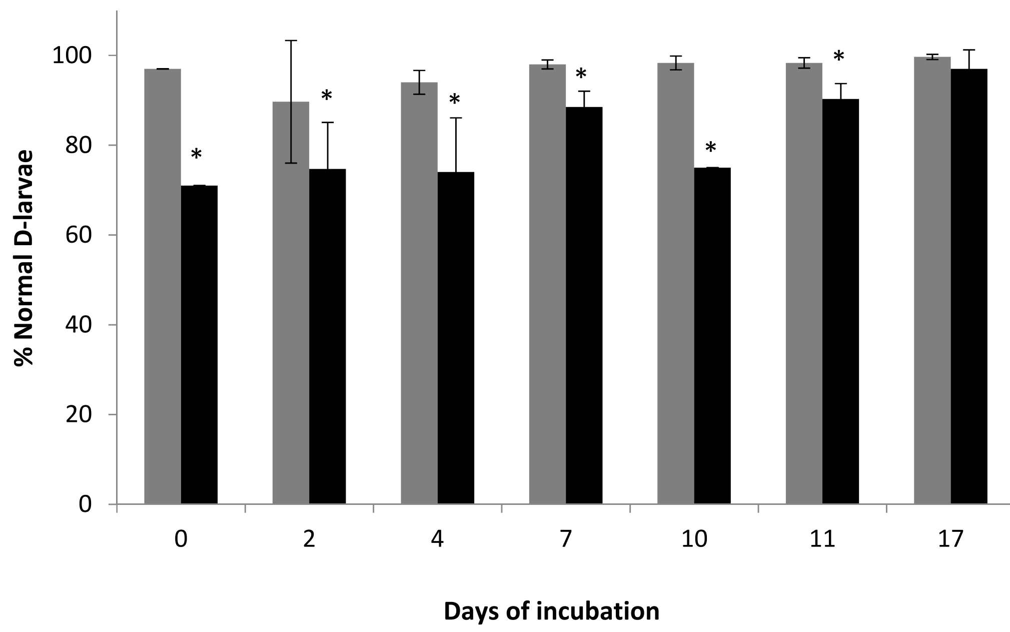
409 Figure 4. Number of settled larvae for both types of treatment. Mean ± SD (n=3)

https://doi.org/10.1016/j.aquaculture.2019.734326

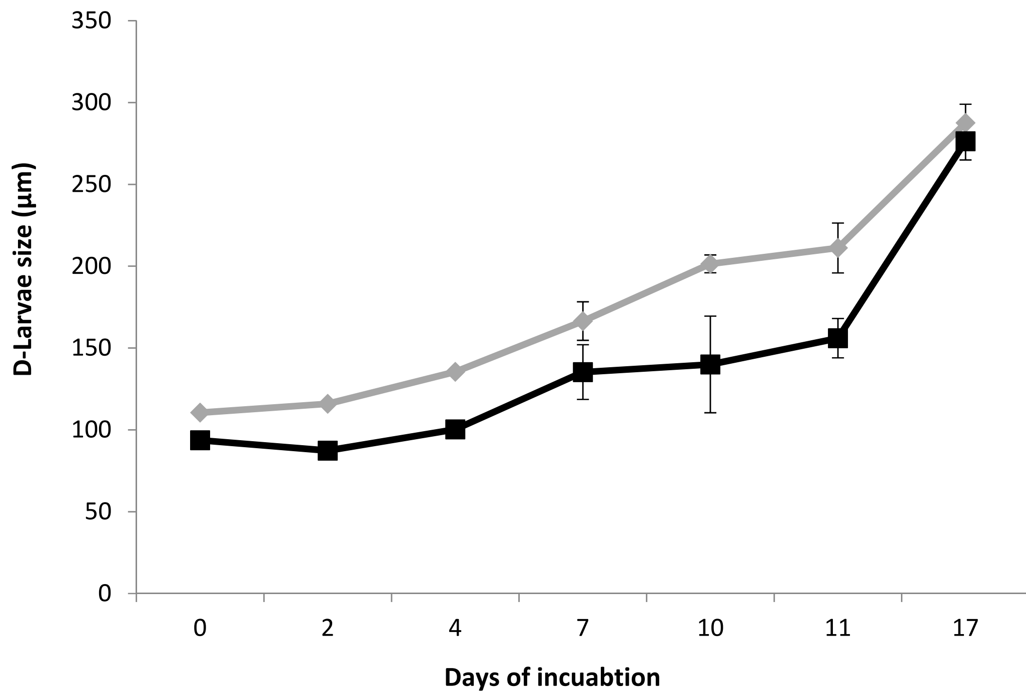
410 Figure 5. Percentage of normal D-veliger larvae after 48 hours post-thaw obtained after
411 cryopreservation of 24 h (dashed) trochophores and 48h (black) and 72h (grey) old D-veliger larvae.
412 Data has been normalized regarding to their corresponding controls. Mean (n=4) ± SD.

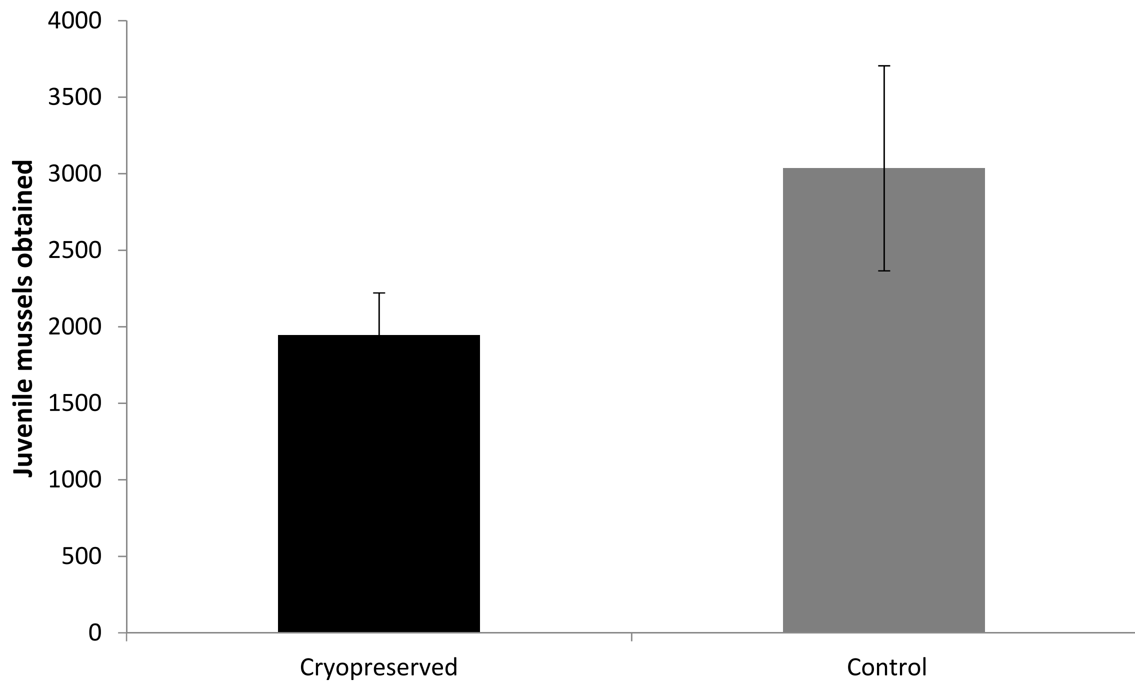


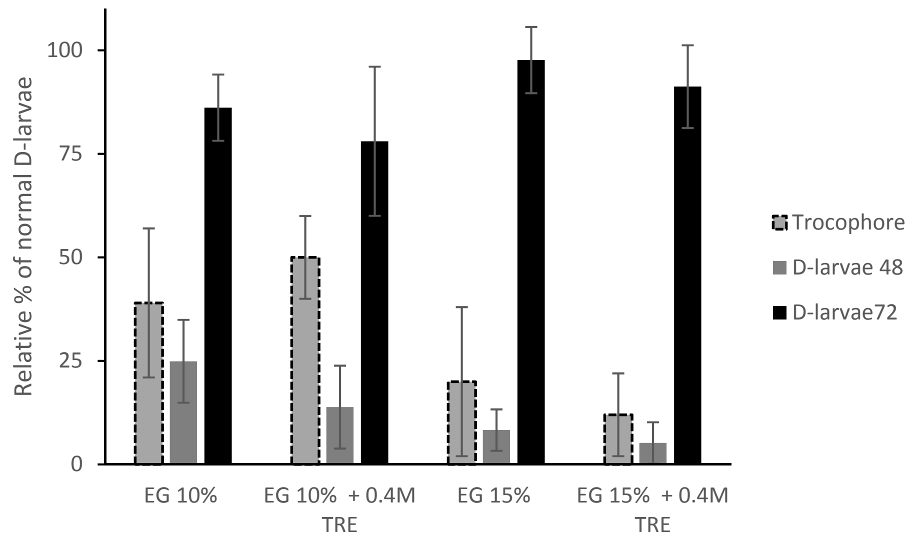
100
75
50
25
0
Survival %
0
5
10
15
20
25
Days post fertilization



% Normal D-larvae
100
80
60
40
20
0
*
*
*
*
*
*
0
2
4
7
10
11
17
Days of incubation


350
300
250
200
150
100
50
0
D-Larvae size (µm)
0
2
4
7
10
11
17
Days of incuabtion


Juvenile mussels obtained
4000
3500
3000
2500
2000
1500
1000
500
0
Cryopreserved
Control


Relative % of normal D-larvae
100
75
50
25
0
EG 10%
EG 10% + 0.4M TRE
EG 15%
EG 15% + 0.4M TRE
Trocophore
D-larvae 48
D-larvae72