

1 **Comparative cryopreservation study of trochophore larvae from two**  
2 **species of bivalves: Pacific oyster (*Crassostrea gigas*) and Blue mussel**  
3 **(*Mytilus galloprovincialis*).**

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34 **Abstract**

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36 Oysters and mussels are among the most farmed species in aquaculture industry around  
37 the world. The aim of this study was to test the toxicity of cryoprotective agents to  
38 trochophore larvae from two different species of bivalves and develop an improved  
39 cryopreservation protocol to ensure greater efficiency in the development of  
40 cryopreserved trochophores (14 h old Oyster larvae and 20 h old Mussel larvae) to  
41 normal D-larvae for future developments of hatchery spat production. The  
42 cryopreservation protocol producing the best results for Oyster trochophores  
43 ( $60.0 \pm 6.7\%$  normal D-larvae) was obtained holding at  $0^{\circ}\text{C}$  for 5 min then cool at  $1^{\circ}\text{C}$   
44  $\text{min}^{-1}$  to  $-10^{\circ}\text{C}$  and hold for 5 min before cooling at  $0.5^{\circ}\text{C}$  to  $-35^{\circ}\text{C}$ , hold 5 min and  
45 then plunge into liquid nitrogen (LN), using 10% EG. For mussel experiments, no  
46 significant differences were found when cooling at  $0.5^{\circ}\text{C min}^{-1}$  or at  $1^{\circ}\text{C min}^{-1}$  for CPA  
47 combinations with 10% EG and at  $0.5^{\circ}\text{C min}^{-1}$  c. Using these combinations, around half  
48 of trochophores were able to develop to normal D-larvae post-thawing.

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50

51 **Keywords:** Cryopreservation, *Crassostrea gigas*, *Mytilus galloprovincialis*,  
52 Trochophore larvae.

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## 56 **1. Introduction**

57 Oysters and mussels are among the most farmed species in the aquaculture industry  
58 with around 2 million tonnes of mussels farmed and 4.5 million tonnes of oyster  
59 produced worldwide in 2010 (FAO 2010). The Pacific oyster, *Crassostrea gigas*, is  
60 among the most produced aquaculture species in New Zealand, with more than 230  
61 oyster farms in 2005 producing around 3300 tonnes of oysters (US\$20 million)  
62 according to FAO in 2010. Spain is also a big European oyster producer (4565 tonnes,  
63 data from ICEX 2010) but the most farmed bivalve species in Spain is the mussel,  
64 *Mytilus galloprovincialis*, which constituted 82% of Spain's marine aquaculture  
65 products in 2010 (FAO, 2010). Not long ago, the culture of those species, *C. gigas* and  
66 *M. galloprovincialis*, relied on the collection of natural seed because is a simple and  
67 cheap option, with *C. gigas* in New Zealand as well as in other countries as France or  
68 the US, this is no longer an option due to the Herpes-Virus infection. Diseases like  
69 Ostereid herpes virus 1 (OsHV-1) which has reduced the worldwide Oyster production -  
70 affecting several countries like France, Spain, New Zealand or the US- together with  
71 increasing environmental human pressure and weather unpredictability are sources of  
72 uncertainty and high economic risk for this economic activity. In response to the  
73 economic importance of these two bivalve species, there is an increasing interest in  
74 developing hatchery spat production to ensure a reliable, safe supply of on-growing  
75 biological healthy material and selective infection-resistant broodstock (Robert *et al.*  
76 1999, Adams *et al.* 2004).

77

78 Cryopreservation of shellfish embryos and larvae can improve aquaculture  
79 management, enhance breeding design flexibility and provides a reference family for  
80 selective breeding. Cryopreservation can enable enough spat supply in hatcheries,  
81 without the need to condition broodstock for out of season production. Moreover,  
82 cryopreservation techniques may help to manage the biological and commercial risks  
83 due to diseases or market changes. Indeed, cryopreservation of gametes is already  
84 widely used in many farmed aquatic species. Cryopreservation research has been carried  
85 out mainly with Pacific oysters (Chao *et al.* 1997, Lin *et al.* 1999, Tervit *et al.* 2005,  
86 Usuki *et al.* 2005, Adams *et al.* 2008), whilst much less effort has been devoted to  
87 mussels (Toledo *et al.* 1989, Adams *et al.* 2009, Wang *et al.* 2011).

88

89 The cryopreservation of sperm is quite-well defined for most bivalve species (e.g.  
90 Adams *et al.* 2004, Di Matteo *et al.* 2009). On the contrary, oocytes and early embryos  
91 are more difficult to cryopreserve. The cryopreservation of trochophore larvae can be  
92 considered as an alternative to separately cryopreserving oocytes and sperm, since  
93 previous studies with the oysters *C. gigas* and *C. rhizophorae*, and with the mussel *M.*  
94 *edulis* have shown that the more advanced the developmental stage is, the more resistant  
95 cells are to cryoprotectant toxicity, and the higher the post-thaw survival results (Toledo  
96 *et al.* 1989). These studies together with our experiments on Greenshell™ mussel  
97 trochophores (Paredes *et al.* 2012) lead us to select the trochophore larval stage for  
98 further detailed cryopreservation studies. The aim of this study was to test the toxicity  
99 of cryoprotective agents to trochophore larvae from two different species of bivalves  
100 and develop an improved cryopreservation protocol to ensure greater efficiency in the  
101 development of cryopreserved trochophores to normal D-larvae.

102

## 103 **2. Materials and Methods**

### 104 **2.1. Gamete collection and handling**

105 Mature ripe oysters (*Crassostrea gigas*) were obtained from oyster farms in Northland,  
106 New Zealand, and maintained during the natural spawning season at the Cawthron  
107 Aquaculture Parkin Nelson. Sperm and oocytes were obtained by “strip spawning”. The  
108 oysters were opened and a small sample of gonad tissue was examined microscopically  
109 to determine sex. Oocytes were collected by lacerating the gonad wall with the tip of a  
110 pipette and gently scraping and washing the gonad contents into 1 L beakers filled with  
111 1 µm filtered seawater (FSW). Oocytes were maintained at 4°C to minimise any loss of  
112 viability associated with aging. Sperm were collected into plastic containers in the same  
113 manner, but held undiluted at 4°C prior to experiments. Oyster oocytes and sperm (500  
114 sperm/egg) were mixed during a contact time of approximately 15 to 20 min, before  
115 being transferred into tanks containing ~150 L of FSW and 1 mg L<sup>-1</sup>  
116 ethylenediaminetetraacetic acid (EDTA), at a temperature of 22°C and density of  
117 15×10<sup>6</sup> oocytes/tank.

118

119 Mature blue mussels (*Mytilus galloprovincialis*) were obtained from the wild in the  
120 south margin of Ria de Vigo (Galicia, NW Spain) and were maintained at the Estación  
121 Científica Mariña de Toralla (ECIMAT). Thermal cycling was used to induce mussels  
122 to spawn as described by Bellas *et al.* (2005). Oocytes were examined for maturity

123 based on their shape and colour, and sperm were visually assessed for vigorous motility  
124 after activation with FSW. Mussel sperm and oocytes were allowed a contact time of 15  
125 to 20 min before being transferred into tanks containing 30 L of 1  $\mu\text{m}$  filtered seawater  
126 (FSW) and 1  $\text{mg L}^{-1}$  ethylenediaminetetraacetic acid (EDTA), at a temperature of 20°C  
127 and a density of  $1 \times 10^6$  oocytes/tank.

128 After 14 h for oysters) and 20 h for mussels, trochophores of both species were  
129 collected by gently siphoning the contents of the tanks through a 15  $\mu\text{m}$  screen semi-  
130 submerged in order to avoid larvae being physically damaged. Trochophores were then  
131 gently swirled on the screen, concentrated and placed into 50 mL falcon tubes for  
132 experiments. For each experiment, three replicate runs were carried out. Oocytes and  
133 sperm were pooled from three individuals per pool and three female pools were used for  
134 each experiment.

135

## 136 **2.2. Experimental reagents**

137 CPA combinations of ethylene glycol (EG) and trehalose (TRE), obtained from Sigma-  
138 Aldrich chemicals (St Louis, MO, USA), were prepared in Milli-Q water. In addition,  
139 polyvinylpyrrolidone (PVP) obtained from Sigma-Aldrich was used in oyster  
140 experiments. Bovine serum albumin (BSA) was used during thawing (GIBCO  
141 Invitrogen and Sigma-Aldrich).

142

## 143 **2.3. Toxicity tests**

144 Toxicity trials with mussel and oyster trochophores were undertaken using different  
145 concentrations of EG and TRE. In addition, PVP was also tested for toxicity with oyster  
146 embryos on the basis of previous experimental results (unpublished data).  
147 Cryoprotectant solutions were added 1:1 to a high concentrated suspension of  
148 trochophores in one step. Samples were allowed 15 min for equilibration and then were  
149 incubated in FSW at a density of 40 embryos  $\text{mL}^{-1}$ , for a further 24 h at 20-22°C until  
150 D-stage was reached. Larvae were then fixed by adding formalin (1% v/v final  
151 concentration).

152

153 The percentage of trochophores developing to normal D-larvae was calculated as an  
154 indicator of CPA toxicity and the No Observed Effect Concentration (NOEC) and  
155 Lowest Observed Effect Concentration (LOEC) were determined. The percentage of D-  
156 larvae was calculated in each experiment and at least three replicates were counted

157 under a light microscope for each treatment. The average count of the three replicates  
158 was normalized against the controls before being used in further data analysis.

159

#### 160 **2.4. Cryopreservation experiments**

161 Following the toxicity evaluation of the CPAs, cryopreservation experiments were  
162 carried-out using a Freeze Control System (Cryologic Pty Ltd). Cryoprotective agents  
163 were prepared at twice their final concentration (in Milli-Q water) and 1 mL of CPA  
164 was then added in a single step to 1 mL of trochophores suspension, allowing for 15  
165 min equilibration before freezing. Trochophores were then loaded into 0.25 mL plastic  
166 straws (IMV Technologies, France) and sealed with PVC powder. Two cooling rates  
167 were tested  $0.5^{\circ}\text{C min}^{-1}$  and  $1^{\circ}\text{C min}^{-1}$  as described: hold at  $0^{\circ}\text{C}$  for 5 min then cool at  
168  $1^{\circ}\text{C min}^{-1}$  to  $-10^{\circ}\text{C}$  and hold for 5 min before cooling at either  $0.5^{\circ}\text{C}$  or  $1^{\circ}\text{C}$  to  $-35^{\circ}\text{C}$ ,  
169 hold 5 min and then plunge into liquid nitrogen (LN). Seeding was always checked at  
170 the  $-10^{\circ}\text{C}$  hold and straws were manually seeded using a LN cooled cotton bud where  
171 necessary.

172

173 Straws were thawed in a  $28^{\circ}\text{C}$  water bath until the ice had melted. Trochophores were  
174 then diluted 1:1 with FSW containing 0.1% (w/v) BSA and left for 15 min before being  
175 diluted into 20 L buckets with FSW containing EDTA for a further 24 h incubation  
176 (density of  $\sim 300,000/\text{bucket}$ ) at  $20\text{-}22^{\circ}\text{C}$ , until D-larval stage was attained. D-larvae  
177 were collected on a  $45\ \mu\text{m}$  mesh screen and samples were fixed with formalin (1% v/v  
178 final concentration) for larval length measurement and % normal D-larvae count.

179

#### 180 **2.5 Effects of cryopreservation on larval development**

181 Finally, a mussel trochophores cryopreservation experiment was performed using 10%  
182 EG + 0.2M TRE. Thawed mussel larvae were then incubated for 48, 72 and 96 hours to  
183 determine whether the average D-larvae size changed significantly along the first days  
184 of incubation due to recovery from exposure to low temperatures.

185

#### 186 **2.6. Statistical analysis**

187 Statistical analyses of toxicity tests data were conducted using the SPSS version 5.0  
188 statistical software. Differences in the percentage of normal larvae and larval growth  
189 among each one of the treatments and control were analyzed by one-way analysis of  
190 variance (ANOVA), followed by the Dunnett's test for calculation of the CPA's No

191 observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration  
192 (LOEC). Cryopreservation data was analyzed using R 2.12.2 free software with the  
193 additive main effects model being used for ANOVA-II without replication (pool effect  
194 was introduced as another factor into the analysis). In all instances, a P-value of less  
195 than 5% was considered significant.

196

### 197 **3. Results**

#### 198 **3.1. Toxicity tests**

199 The NOEC for oyster trochophores was 15% EG while LOEC was 20% EG. There was  
200 no significant difference in toxicity between using EG in combination with TRE alone  
201 or with both TRE and PVP, as long as the EG concentration was less than 20% (Figure  
202 1). Two non-toxic levels of EG were selected to continue with cryopreservation  
203 experiments using oyster trochophores, 10% EG and 15% EG in combination with TRE  
204 and PVP.

205

206 The NOEC for mussel trochophores was also 15% EG and the LOEC was 20% EG.  
207 Below the LOEC level there was no significant difference in toxicity between using EG  
208 alone or in combination with TRE 0.2 M or 0.4 M. For mussel trochophores, 10% EG  
209 and 15% EG in combination with TRE were selected as non toxic levels of CPAs for  
210 further cryopreservation experiments (Figure 2).

211

#### 212 **3.2. Cryopreservation experiments**

213 Development of thawed oyster trochophores to D-larval stage was highest ( $60.0 \pm 6.7\%$ ;  
214 mean  $\pm$  SE) when trochophores were frozen in 10% EG and a cooling rate of  $0.5^\circ\text{C min}^{-1}$   
215 was used (Figure 3). The addition of TRE or TRE plus PVP did not improve D-larval  
216 yield from that obtained with EG alone. The size of D-larvae obtained from thawed  
217 oyster trochophores was significantly different to the size of unfrozen control larvae  
218 (10.3% smaller on average; data not shown).

219

220 For mussels, the percentage of D-larvae developing from thawed trochophores was  
221 highly variable between the replicate pools evaluated. Despite having taken into account  
222 pool variability in our statistical analysis no significant differences were found when  
223 cooling at  $0.5^\circ\text{C min}^{-1}$  or at  $1^\circ\text{C min}^{-1}$  for CPA combinations with 10% EG and at  $0.5$   
224  $^\circ\text{C min}^{-1}$  with 15% EG + 0.4M TRE (Figure 4).

225

226 Finally, the growth of D-larvae produced from thawed mussel trochophores was  
227 examined over a four day incubation period. Whereas unfrozen larvae increased in size  
228 over the incubation period, thawed larvae showed no significant increase in size (Figure  
229 5).

230

#### 231 **4. Discussion**

232 Our test species developmental stage was selected because trochophores had been  
233 widely documented to be more resistant to CPA toxicity (Chao *et al.* 1997, Gwo 1995,  
234 Nascimiento 2005) than other stages. We also choose to use this stage in our previous  
235 work on Greenshell™ mussels (*Perna canaliculus*; Paredes *et al.* 2012).

236

237 Cryoprotectant toxicity tests are an important step in optimizing a cryopreservation  
238 protocol for a given species or cell type. In this study, the CPA toxicity tests with  
239 bivalve trochophores yielded a NOEC of 15% EG and a LOEC of 20% EG for both  
240 species. Below this concentration there were no toxic effects of EG, whether it was  
241 used alone or in combination with TRE and/or PVP. These results are consistent with  
242 our previous studies on Greenshell™ mussel trochophores (Paredes *et al.* 2012).  
243 However, the response to CPA exposure of the same developmental stage in different  
244 species should not be assumed since CPA tolerance can be specific to a given species  
245 and/or cell type/developmental stage and toxicity studies may or may not take into  
246 account the osmotic tolerance limits and permeability of the particular cell that is of  
247 interest (Nascimiento *et al.* 2005) (Chao *et al.* 1997, Gwo 1995, Paniagua 2001, Tervit  
248 *et al.* 2005). Here, cryoprotectants were added in a single step and it is possible that  
249 cells could tolerate a higher cryoprotectant concentration if stepwise methods of  
250 addition and removal had been used. The variability among responses to CPAs found in  
251 literature confirm that different species and different developmental stages may behave  
252 in a different way when exposed to a CPA and cryopreserved, although our results also  
253 suggest that the same development stage of similar species might behave in a similar  
254 way due to shared characteristics on membranes composition, cell structure and  
255 permeability as well as similar response to CPAs toxicity (Paredes *et al.* 2012)

256

257 In the present study, the presence of TRE and/or PVP did not reduce or enhance EG  
258 toxicity below a concentration of 20% EG. Results of our cryopreservation experiments



259 showed that 10% EG alone yielded the best results for oyster trochophores with 60±6%  
260 developing to normal D-larvae when a cooling rate of 0.5°C min<sup>-1</sup> was used.

261

262 The highest % normal D-larvae (over 80%) were achieved with one single pool, using  
263 10 % EG + 0.2 M TRE, and a cooling rate of 0.5°C min<sup>-1</sup>. In this case, considering this  
264 high outcome as an outlier and examining the results achieved for the other two pools  
265 and other treatments with no significant differences, namely 10% EG + 0.2 M TRE and  
266 10% EG +0.4 M TRE (1°C min<sup>-1</sup>), and 15% EG + 0.4 M TRE (0.5°C min<sup>-1</sup>), with  
267 average percentages of normal D-larvae of 48.9±7.6%, 47.5±11.2% and 48.8±17.5%,  
268 respectively, we obtained more probable consistent, results. Concluding, our protocol  
269 for mussel cryopreservation provides an outcome around 50% normal larvae.

270

271 As for oyster larvae, mussel D-larvae that were produced from thawed trochophores  
272 were found to be significantly smaller (17.9% smaller) than controls, and these size  
273 differences persisted over the 4 day incubation that with cryopreserved larvae showing  
274 no growth over this time. These results are consistent with Paredes *et al.* (2012), where  
275 observed size differences between frozen and fresh Greenshell<sup>TM</sup> mussel D-larvae that  
276 were maintained till settlement and with Wang *et al.* (2011) with *Mytilus*  
277 *galloprovincialis* where cryopreserved D-larvae remained smaller than controls during  
278 the first week of incubation following cryopreservation. However the incubation time  
279 was too short to derive any conclusions about the viability of the resulting larvae.

280

281 For both, oysters and mussels, results were very similar while freezing at 0.5 °C min<sup>-1</sup> or  
282 1 °C min<sup>-1</sup> with 10% EG, and no significant differences among cooling temperatures  
283 were found. However, when using 15% EG, better results were obtained at 0.5°Cmin<sup>-1</sup>.  
284 Survival of cryopreserved and thawed bivalve trochophores was 60.0±6.7% for oysters  
285 and 48.9±7.6% for mussels.

286

287 In conclusion, a successful freezing protocol for both bivalve species trochophore larvae  
288 (*M. galloprovincialis* and *C. gigas*) was developed with specific adaptations for the  
289 different bivalve requirements. Further investigations to increase the percentage of  
290 normal D-larvae resulting from cryopreservation, and cryopreserved larval growth and  
291 health along incubation to spat, would ensure the future of application of

292 cryopreservation techniques as useful tools to guarantee a reliable supply of on-growing  
293 biological material in developing hatchery spat production.

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295

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306

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380 **Figure captions**

381

382

383 Figure 1. - Percentage of D-larvae developing from oyster trochophores following  
384 exposure to increasing concentrations of EG in combination to TRE (M) and/or 1%  
385 PVP. The FSW bar represents a handling control. All data has been normalized to the  
386 controls. Mean  $\pm$  Standard Error (SE).

387

388 Figure 2. - Percentage of D-larvae developed from Mussel trochophores following  
389 exposure to increasing concentrations of EG in combination to TRE (M). All data has  
390 been normalized to the controls. Mean  $\pm$  Standard Error (SE).

391

392 Figure 3.- Percentage of Pacific oyster trochophores developing to D-larvae following  
393 cryopreservation at 0.5 °C min<sup>-1</sup> and 1 °C min<sup>-1</sup> in 10 or 15% ethylene glycol (EG) alone  
394 or in combination with 0.2 M trehalose (TRE) with or without 1% polyvinylpyrrolidone  
395 (PVP). All data has been normalized to the controls. Mean  $\pm$  Standard Error (SE).

396

397 Figure 4.- Percentage of Mussel D-larvae following cryopreservation at 0.5 °C min<sup>-1</sup>  
398 and 1 °C min<sup>-1</sup> in 10 and 15% EG in combination to TRE(M). All data has been  
399 normalized to the controls. Mean  $\pm$  Standard Error (SE).

400

401 Figure 5. - D larvae size for unfrozen controls and frozen trochophores at 48, 72 and  
402 96 incubation hours post fertilization. Mean  $\pm$  Standard Error (SE).

403

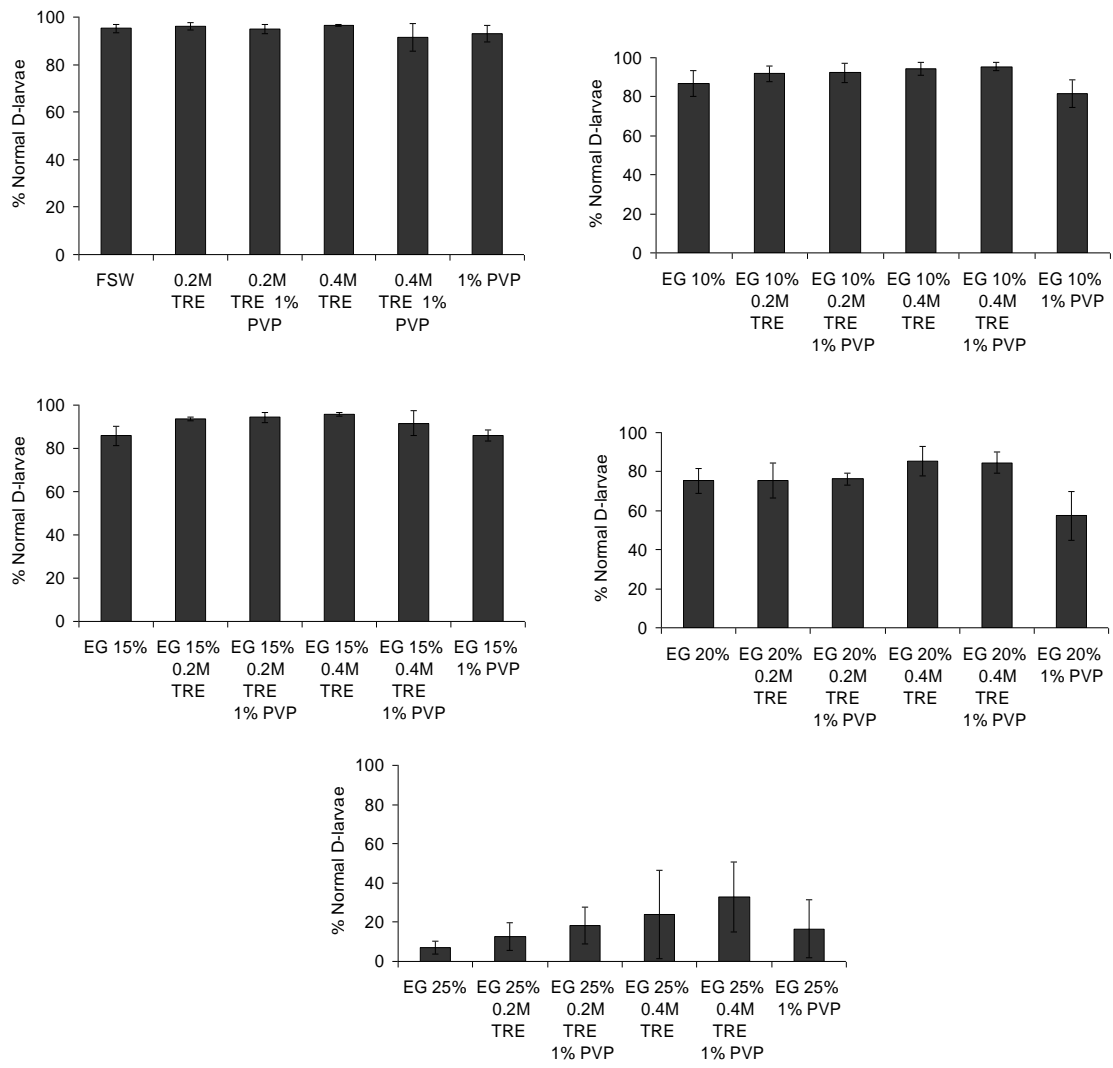


Figure 1

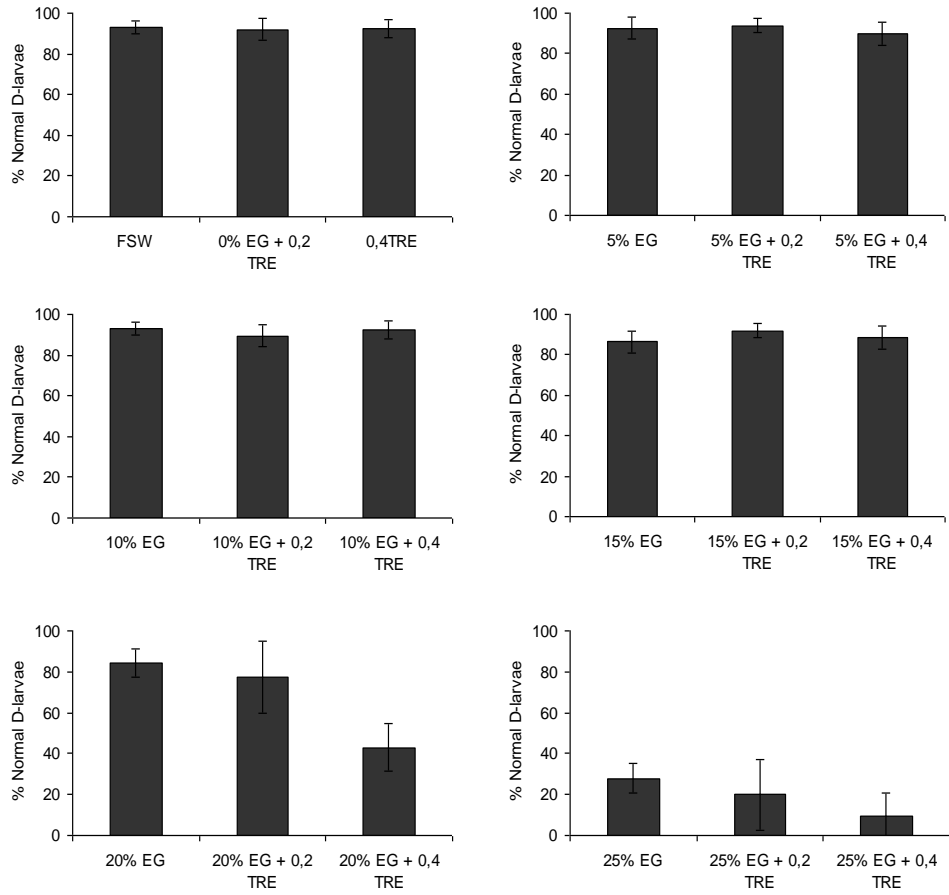


Figure 2

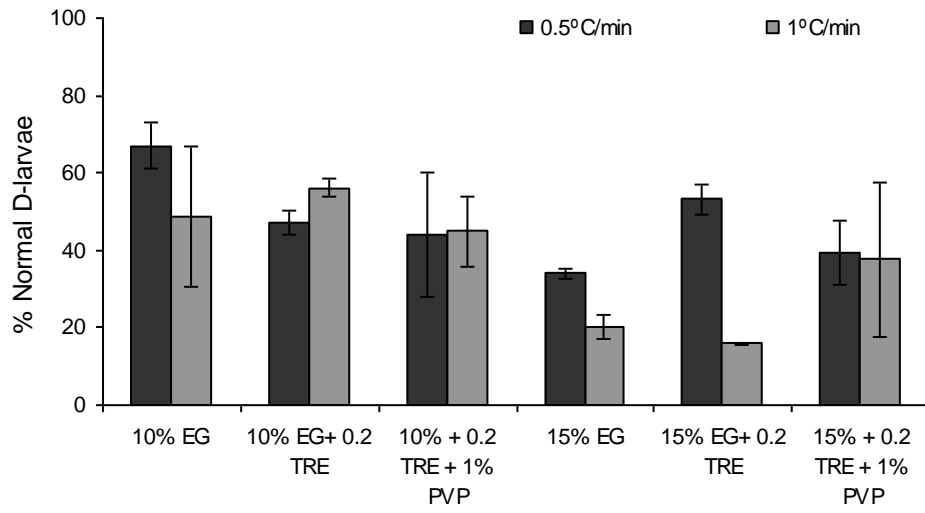


Figure 3

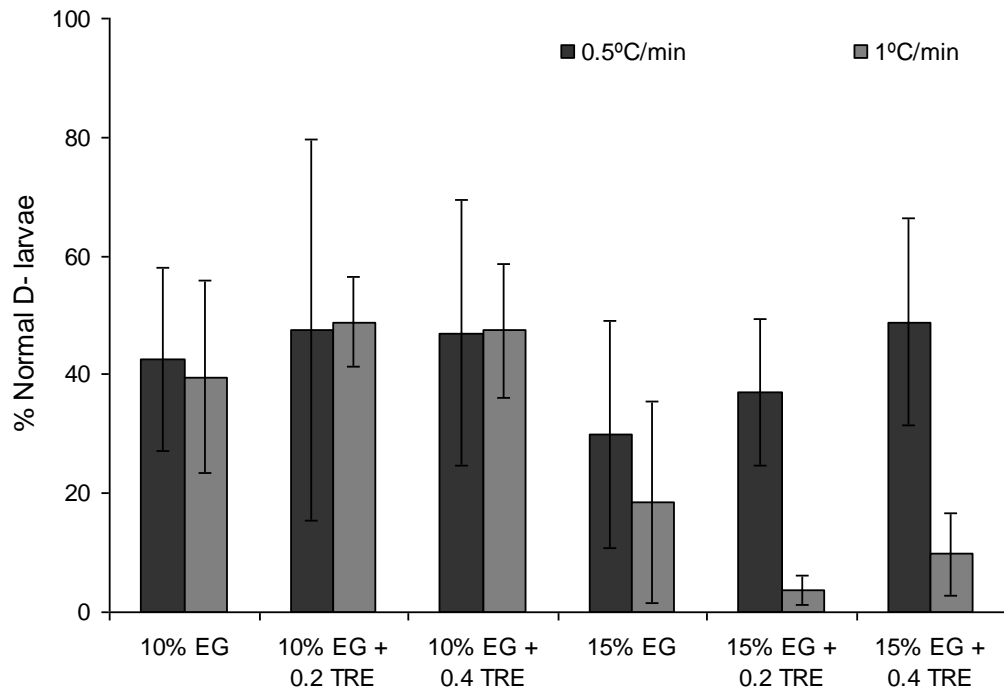


Figure 4



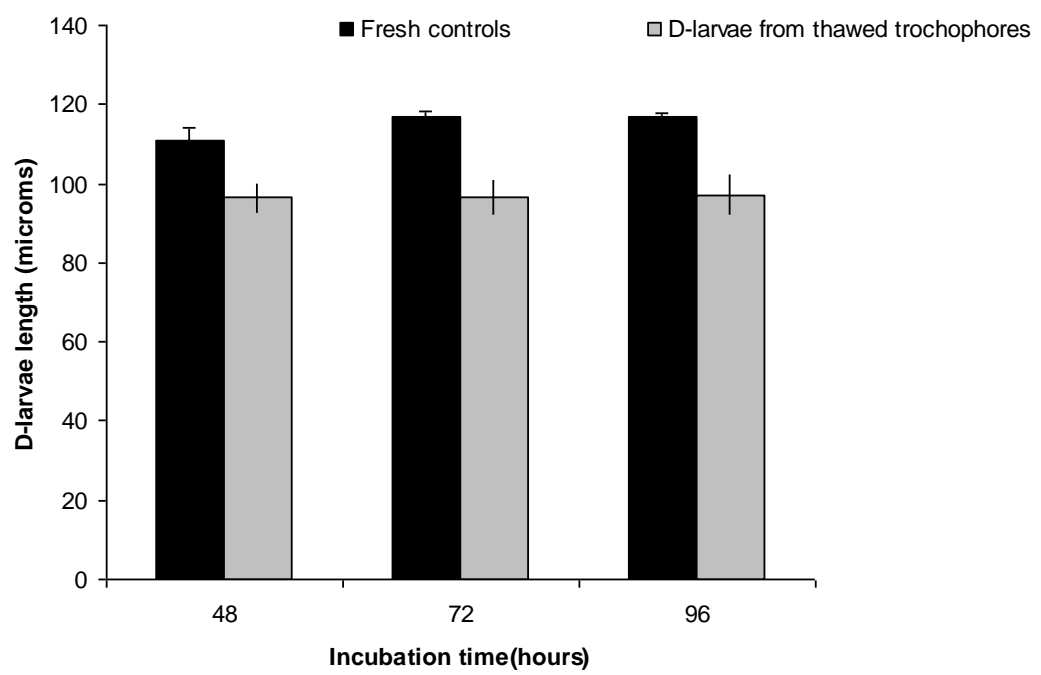


Figure 5