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Toxicity tests of cryoprotecting agents for *Mytilus galloprovincialis* (Lamark, 1819) early developmental stages

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ABSTRACT

Global aquaculture production of blue mussel has increased over last years. This work reaffirms the great potential of cryopreservation technique on mussel industry and overcome economic barriers a cause of a traditional and rudimentary management and continue growing. The aim of this work is to set some preliminary basis attending to toxicity of cryoprotecting agents (CPAs) on different development stages of *Mytilus galloprovincialis* as a start point to develop a stable cryopreservation protocol. Toxicity tests were carried out by using common CPAs (dimethyl-sulfoxide (Me_2SO), glycerol, (GLY), propylene glycol (PG) and ethylene glycol (EG)) in a range from 0.5 to 3 M on fertilized egg, trochophore larva, and D-larva of *Mytilus galloprovincialis*. Results evidenced more resistance of older development stages to toxicity. Of all CPAs tested, toxicity testing highlights PG or EG as suitable CPAs for cryopreservation of early development stages; whereas D-larva was unaffected by any of the CPAs tested. Preliminary cryopreservation trials were developed to obtain information into cell cryoprotection. Further research should be focused on membrane permeability and other parameters, such as the balance between toxicity and cryoprotective effect of CPAs.

1. Introduction

Mussels are among the most farmed species in the aquaculture industry. In fact, 116,262 tons were produced worldwide in 2014 [54]. Spain is the second higher producer of mussels (preceded by China); more than 100,000 tons are consumed annually. Despite the demand, the Spanish production has not increased during last years a cause of biological, technological and socioeconomic factors.

Aquaculture industry, biological, biotechnological sciences and agriculture requires preservation of biological material in a stable state to get it at the time of need. In these lines, cryopreservation is accepted as preferred technique for achieving long-term storage [36].

Mollusc aquaculture has been intensively developed over the last 60 years [20]; although an increasing of the research effort is necessary to improve aquaculture techniques to maintain the competitive development of the industry and a long-term sustainable production. In recent years, many studies about cryopreservation applied to marine organisms have been published, most of them concerning fish species. However, there has been research focussed on the improvement of mollusc cryopreservation procedures, mainly species included in the *Crassostrea* genus, due their economic importance [5,32].

Aquaculture production will benefit from the development of successful cryopreservation protocols for marine organisms [2–4,21,23,33,35]. It ensures stable and sustainable shellfish resources and decreases mortality due associated risks such as; diseases caused by specific parasites or virus such as, *Crassostrea gigas* with Ostereid herpes virus 1 (OsHV-1), *Mytilus* sp. with *Edotia doellojuradoi* and *Nepinnotheres novaezelandiae* or *M. intestinalis* with *Marteilia* sp. [31] or natural/anthropologic disruptive events. According to FAO in 2018, the predation and diseases caused by parasites, such as *Mytilicola intestinalis* or *Marteilia* sp. do not currently affect the global mussel production. However, as a precaution there is an exhaustive monitoring program to evaluate mussel mortality and state of water quality to limit the mussel mortality due diseases or water pollution [54].

Moreover, the development of cryopreservation techniques could be used to provide a reference back-up copy of a family for selective breeding, enabling parental crosses on demand and enhancing breeding design flexibility. In hatcheries, progenies without seasonal limitations could be produced and genetic obstacles could be overcame. The storage of genetic material would surpass the lifetime of the donors and a repeated supply of gametes from specific individuals would be possible.

In these terms, there is published research that had tried to improve and establish sperm cryopreservation protocols, in contrast, there are few studies about oocytes, early embryos and larval cryopreservation

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(Table 2), due to the difficulty of cryopreserving early development stages or complex larvae [21,33,35,38,50,51].

A recent review of mollusc cryopreservation bibliography had been carried out recently in lab. Results determined that Propylene Glycol (PG), Dimethyl-sulfoxide (Me_2SO), Ethylene Glycol (EG) and Glycerol (GLY) were the most used cryoprotecting agents CPAs in marine invertebrates, those were therefore selected for this experiment at a wide concentration range between 0.5 and 3 M [32]. The aim of this project was to test their toxicity to different mussel development stages (fertilized egg, trochophore larvae, D- larvae of 48 and 72 h post-fertilization) to determine the suitable CPA and optimal concentration with minimal toxic effects as a start point to review the preliminary protocol developed by Paredes et al. [35].

2. Materials and methods

2.1. Gamete collection and handling

Mature blue mussels (*Mytilus galloprovincialis*, Lamark 1819) were obtained from the wild in the south margin of Ria de Vigo (Galicia, NW Spain) and deposited in PVC tanks with Filtered Sea Water (FSW 0.22 μm + UVA) at 18 °C. Mussels were spawned by thermal cycling. Gametes from a male and a female were collected and transferred into FSW separately, in order to minimize genetic variability [16,45]. Oocyte quality and maturity were examined focusing on their shape and colour before fertilization, sperm was checked for motility. A small volume of sperm was added to the oocyte suspension (approximately a rate of 20:1) and a 15 min contact period was allowed before evaluation of the percentage of fertilization by counting oocytes with formation of the first polar body. A fraction was destined for a toxicity tests immediately while the rest of the sample was incubated up to 72 h at 18 °C. Periodically, a fraction of that sample was retrieved to collect trochophores (18–20 h post-fertilization), D-larvae (48 h) and finally D-larvae (72 h old).

2.2. Toxicity tests

Cryoprotectant dilutions were prepared, in FSW (0.22 μm + UVA) (Sigma Aldrich chemicals (St Louis, MO, USA)). Toxicity trials were undertaken using different concentrations of PG, GLY, Me_2SO and EG ranging from 0.5 to 3 M (0.5, 1, 1.5, 2 and 3 M). Three replicates were assayed for each CPA concentration and control trials. Cryoprotectant solutions were added 1:1 to a FSW mL with 100–200 mussel individuals in one step. In this case, preliminary research pointed out that the addition of CPAs in a single step is not lethal. Although there are no mathematical calculations for CPA permeation in mussel oocytes, embryos or larvae, empirical data seems to indicate that 15 min-exposure is time enough to reach osmotic equilibration [33,35]. After exposure and equilibration, samples were diluted with FSW 1:1 and filtered using a 40 μm membrane filter and rinsed with FSW to remove the CPA. Then, they were incubated in 20 mL of clean FSW at 18 °C until D-larval stage was reached or 48 h post-exposure. After that, cells were fixed with formalin.

Percentage of abnormal D-larvae was calculated as an indicator of toxicity of individual CPAs. This parameter was obtained after examining n = 100 cells for each replicate and treatment, under the microscope Nikon ECLIPSE 2000-5 and using Nikon NIS Elements-D software, version 4.13. This value was selected to determine the NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) levels.

2.3. Cryopreservation experiment

A preliminary cryopreservation experiment was carried out following toxicity tests and previous research [35,39] on 48 and 72 h-old D-larvae of *M. galloprovincialis*. Cryoprotecting solutions 10% EG in combination or not with 0.4 M TRE were added in one step 1:1 to 1 mL of FSW with D-larvae. Moreover, 15 min for equilibration were allowed and samples were loaded into 0.25 mL straws. 3 replicates were assayed for each treatment and control trials. Cooling was carried out using a Freeze Control System (Cryologic Pty Ltd), as described: hold at 4 °C for 2 min, then freezing -1 °C/min to −12 °C when seeding, hold at −12 °C for 2 min, cooling -1 °C/min to −35 °C. Samples were plunged into LN and then they were thawed by immersion in a water bath at 35 °C for 6 s. They were incubated into clean FSW at 18 °C for 24 h. Finally, samples were fixed in formalin for % normal D-larvae count.

2.4. Larval abnormality criteria

The discrimination between normal D-larvae and abnormal D-larvae was determined under microscope attending to previous work focused on shell larval morphology and guidelines from other experts in the shell abnormalities and abnormally developing larvae of related mollusc in ecotoxicological larval bioassays [14,35,40,49]. Typical larval abnormalities found ranged from: delayed development (trochophores), deviations from the D-larvae shell shape like indented margins or hinge deformations (concave or convex hinges) or presence of clear protruding mantle.

2.5. Statistical analysis

Statistical analysis for toxicity tests were conducted using the SPSS 15.0 version statistical software. Differences in the percentages of abnormal larvae among treatments were analyzed by one-way of variance (ANOVA) followed by the Dunnett's test to calculate the NOEC and LOEC levels. For analysis, the percentage of abnormal D-larvae data was first arcsine-transformed to achieve normality [13]. Statistical tests were performed according to Newman [25] and Sokal and Rohlf [44] when required. Cryopreservation data was evaluated by ANOVA-II. A p-value less than 5% was considered significant.

3. Results

Fig. 1 shows the toxicity of a range of CPA concentrations (PG, EG, GLY and ME_2SO), for different early development stages (15 min of exposure, 20 °C). Ethylene glycol was the least toxic CPA at the fertilized oocyte stage, followed by Propylene glycol. Percentage of abnormal D-larvae increased using the rest of CPAs, specially GLY or Me_2SO. In some cases, samples couldn't grow at all and/or develop to D-larvae despite of 48 h of incubation, when they otherwise-when healthy-should have been able to reach D-larval stage at normal conditions [37,40]. Other portion of the samples had irregular shell morphology, not the typical D shape. Mortality increased considerably when high concentrations of CPA were added to cells. Significant differences were observed for fertilized eggs at all tested CPA concentrations in comparison to controls, except for 0.5 M PG and 0.5 M EG.

Trochophore larvae (18–20 h post-fertilization, 18-20 °C) showed more resistance than the earlier development stage when exposed to CPAs; in fact, the percentage of abnormality was really low when concentrations of CPA were within the 0.5–2 M range. Significant perturbations in development were only found when using GLY and Me_2SO 3 M (near to 100% abnormal D-larvae). Trochophore larvae exposed to PG and EG did not differ from controls. In this case, PG and EG were the least toxic CPAs.

When exposing D-larvae to increasing concentrations of CPAs, there was not any expected increase in the percentage of abnormality with increasing concentration of any of the CPAs selected.

The NOEC and LOEC levels corresponding to fertilized egg, trochophore larvae and D-larvae for each treatment are showed in Table 1: fertilized egg is the most sensitive stage and toxic effects were evidenced even with the lowest CPAs concentrations tested, except when PG or EG were used. NOEC and LOEC levels of PG and EG were 0.5 M

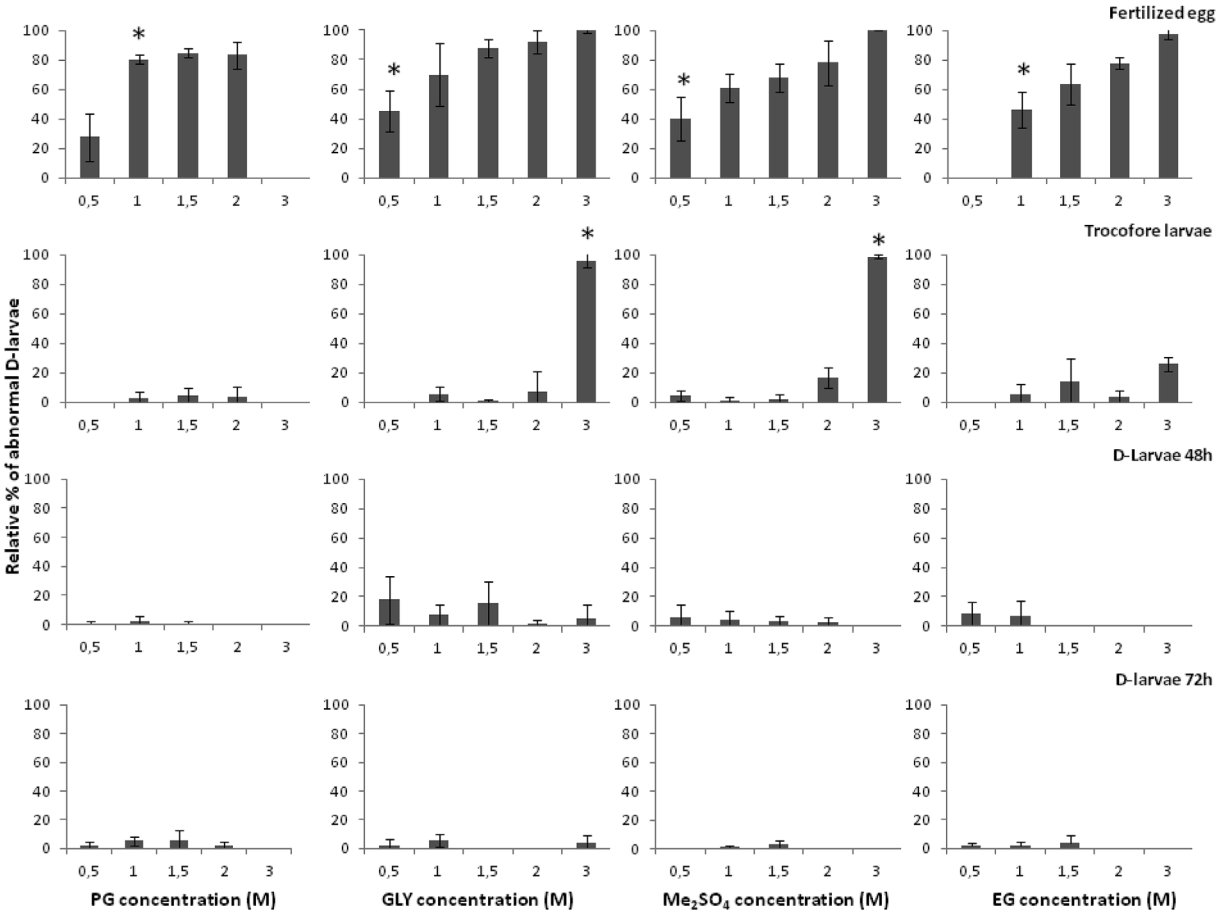

Fig. 1. Percentage of abnormal D-larvae developed 48 h after 15 min exposure to increasing concentrations of PG, GLY, Me_2SO and EG (M) in fertilized egg, trochophore larvae, 48 h old D-larvae and 72 h old D-larvae. Most common abnormalities were D-larvae with protruding mantle, convex hinge, cupped D-larvae and underdeveloped larvae (trochophores). Toxicity was assessed as the average percentage of total abnormal D-larvae in reference to the control after 48 h post exposure (n = 100, 3 replicates per treatment). Different letters show statistical differences with $p^{<}0.05$.

Table 1
NOEC (No Observed Effect Concentration) and LOEC (Lowest Observed Effect Concentration) for the four development stages (Fertilized egg, Trochophore, D-larvae48 and D-larvae72) and cryoprotecting agents (CPA) tested (n = 3, $p < 0.05$).

CPA	NOEC (M)				LOEC (M)			
	Fert. egg	Troch.	D-48	D-72	Fert. egg	Troch.	D-48	D-72
PG	0.5	–	–	–	1	–	–	–
GLY	< 0.5	2	–	–	0.5	3	–	–
EG	0.5	–	–	–	1	–	–	–
ME_2SO	< 0.5	2	–	–	0.5	3	–	–

and 1 M respectively. The higher tolerance of trochophore larvae was evident, with higher but similar NOEC and LOEC values for GLY and Me_2SO (2 M and 3 M respectively). PG and EG did not affect significantly the development to D-larvae at all. NOEC and LOEC levels were not determined for D-larvae.

4. Discussion

There is an increasing interest in the development of hatchery spat production to ensure a sustainable resource for the mussel industry, here is where cryopreservation represents as a way to develop a more competitive and efficient aquaculture sector. By providing a safe repository for improved resistant lines to diseases or demanded/marketable phenotypes, protection on supply in the event of natural disasters; guaranteeing a supply of gametes for spat production outside the natural breeding season; and by reducing the costs associated with conditioning broodstock [21].

There are few published data about mussel cryopreservation in spite of its economic value inside aquaculture industry (Table 2). Sperm and oocyte cryopreservation is presented as preferred way to enhance mussel aquaculture, although cryopreservation of larval stages could be an alternative for the actual impossibility of cryopreserving marine invertebrate oocytes [12,21,32] with good outcome: for example, trochophore larvae of *M. edulis* evidenced more resistance than early developmental stages to cryopreservation [46].

One of the critical steps for a successful cryopreservation is the initial selection of the proper cryoprotecting agents and useful concentrations. Several intracellular effects caused by specific CPAs are well known: Me_2SO affects to ion transporters and inhibits catalase and peroxide enzymes [18,30,42]; EG and PG decrease the polarity of the aqueous phase and damage the membrane [8,18,34]. In despite of the negative effects, CPAs are essential to protect the cell during cooling process. There have been also successful combinations of a permeating CPA and a non-permeating CPA (sugar or polymer) reported for marine invertebrate cryopreservation [1,12,21,32]. Focusing on *Mytilus* genus, permeating CPAs commonly used are Me_2SO, EG, PG, and GLY; and sometimes, in combination with non-permeable CPAs, such as trehalose (TRE) or sucrose (SUC) (Table 2). The CPA addition procedure represents also a potential step to enhance mortality due to osmotic shock. In contrast to other marine invertebrate species studied, such us sea urchin, where adding CPA's solutions following a gradual stepwise

Table 2
Previous *Mytilus* cryopreservation protocols published from 1989 to 2018. Survival assessment results shown are the highest ones reported in each study. Cryoprotecting agents (CPAs) acronyms: Me_2SO: dimethyl sulfoxide, EG: ethylene glycol, PG: propylene glycol, Gly: glycine, G: glycerol, AceT: acetamide, SUC: sucrose, TRE: trehalose, PVP: polyvinyl pyrrolidone, FIC: ficoll.

Species	Cell type	References	Protocol	Survival assessment
Mytilus galloprovincialis	Sperm	[9]	EG 7% (v/v). Three cooling periods: 0,25°C/min from 20 to 2°C; -1°C/minto −15°C; -6°C/min-1 to −80°C. Warming rate 60 °C/min. Thawing temperature 25°C.	90% Fertilization rate. 65%Motility. 60%Fertilization (10:1)
		[22]	Me_2SO 8% + GLY 0.8%. Non-programmable freezing technique. Sperm cryopreserved at 7.8 cm above the LN surface. Thawed in a 60 °C water bath.	95% Fertilization rate
	Oocytes	[20]	7.5% FIC and 10% EG and post-thaw CPA removal medium consisting of 9% sucrose. The D-larval was determined 40–48 h post-fertilization and calculated as the percentage of oocytes that develop into D-larvae.	14% D-larvae. Shell lengths: control: 377.6 ± 2.0 μm, n = 200; treatment: 364.4 ± 1.7 μm, n = 300 (P N 0.05).
	Larvae	[51]	Me_2SO 5% (v/v). Two step cooling rate 1.5:0.4 °C/min. Warming rate 2664 °C/min	85% Moving cilia, 55% Swimming motion, 8% Food intake after 21
		[35]	EG 10% (v/v) + TRE 0.2 M. Cooling rate 1 °C/min Thawing in water bath 28 °C 6 s	50% D-veliger stage (48 h. post-thaw)
Mytilus edulis	Embryo	[46]	Me_2SO 1.5 M. Two step cooling rate 5:0.5 °C/min. Thawing in water bath 18 °C	48.8% Survival
Mytilus trossulus	Embryo	[7]	Me_2SO 10% (v/v) + TRE 1.5% (w/v) + Vitamins + lipids. Cooling rate 7 °C/min. Thawing in water bath 20–22 °C	35% Viable cells
	Larvae	[27]	Me_2SO 6% (v/v) + 40 mM TRE + 0.15% (w/v) Antioxidants. Cooling rate 7 °C/min^1. Thaw in water bath 10–15 °C	40% Survival
	Mantle, gills, muscles	[28]	Me_2SO 10% + TRE 3–30 mg/ml. Samples (somatic and larval tissues of bivalve molluscs and from embryos of sea urchins) frozen to −196 °C by two-step freezing.	60–95% Cell viability and RNA synthetic activity. After freeze-thaw. The embryonic cells of *M. trossulus* protected Me_2SO + trehalose + mussel lipid extract had the best intensive RNA synthesis. Cells were destroyed.
Perna canaliculus	Sperm	[2]	Me_2SO 12% (v/v) + TRE 0.25 M. Cooling rate 5 °C min 1. Thawing in water bath 28 °C	60% Fertilization rate
		[43]	Me_2SO 12% + TRE 0.2 M 1:1 or 1:3. Freezing method NPM. Freezing 3 cm above LN for 10 min 18 °C	80% Fertilization (160,000:1)
	Oocytes	[3]	EG 10% (v/v) + TRE 0.4 M. Two step cooling ramp 1:0.5 °C/min. Thawing in water bath 28 [a]C	1% D-yield larvae
	Larvae	[3]	EG 10% (v/v) + TRE 0.2 M. Cooling rate 1 °C/min. Thawing in 28 °C 6 s	60% D larvae 48 h, good motility

protocol is really beneficial to reduce toxicity and osmotic stress, which could lead to cell damage. Previous research evidenced that one-step addition is not detrimental for mussel individuals [33,35].

There are few studies that have analyzed cryopreservation of embryos from related species of *M. galloprovincialis*, such us *M. edulis* and *M. trossulus*. Toledo et al. [46] obtained 27.99 ± 4.99% survival cryopreserving *M. edulis* embryos with Me_2SO 1.5 M; Kostetsky et al. [17] used a combination of Me_2SO 10% (1.28 M) with TRE 1.5%, vitamin E 0.5% and lipids 0.15% to cryopreserve embryos and trochophore larvae of *M. trossulus* and yielded 35 ± 0.3% larval survival. All of the prior references have used Me_2SO, but previous extensive toxicity tests were not carried out to determinate the suitable CPA for mussel embryos in most of the cases.

Among the cryoprotecting agents tested on *M. galloprovincialis* fertilized eggs during our experiments, GLY showed the highest toxicity after 15 min of exposure, followed by Me_2SO, EG and PG. Therefore, similar concentrations of Me_2SO that had been reported for other cells had produced in fertilized eggs almost 70% abnormal D-larvae post exposure. Therefore, Me_2SO, which is highly toxic, could apparently be replaced by EG or PG for embryo cryopreservation to avoid additional mortality due to toxicity, although there is not much information about the cryoprotection of these compounds or the permeability to mussel oocytes and fertilized oocytes. This is in agreement with prior data. On other species of mussel, such as (*Perna canaliculus*) Adams et al. [5] obtained the best post-thaw fertilization percentage (36.7 ± 10.9%) using EG (9–10%) + TRE (0.2–0.4 M) for oocytes.

In the case of trochophore larvae, there were not significant differences in toxicity with PG and EG in all of their concentrations tested and controls; whereas GLY and Me_2SO showed statistical differences with control assessments only at 3 M. Previous cryopreservation experiments of *M. galloprovincialis* trochophore larvae support these results: Paredes et al. [35] determined that EG 10% or 15% were good concentrations of this CPA for cryopreservation, obtaining the highest percentage of D-larvae (over 50% after 24 h post-thaw). Similar survival percentages (about 50%) were yielded by Toledo et al. [46] who evaluated the resistance of trochophore larvae against freezing using Me_2SO 1.5 M. Focusing on other studies in mussels, EG 10% + TRE 0.2 M was demonstrated as useful combination to cryopreserve trochophore larvae of *P. canaliculus* [33]. The high tolerance of trochophore larvae to CPAs, specially EG or PG point out towards the possibility of increasing concentration in order to achieve higher survivals.

Toxicity tests of D-larvae hardly showed any toxic effect up to 3 M for all the CPAs tested. There are two possible explanations for this finding. It could be that older larvae were more resistant to CPA toxicity than younger cells. This agrees with published research on marine toxicity studies, these studies report that older organisms are not as sensitive to the presence of toxics in the ocean as young embryo-larval stages [48] and therefore these early development stages are chosen for ecotoxicological bioassays due to their high sensitivity [15,47]. A mollusc cryopreservation literature shows different responses to toxicity tests and cryopreservation among species, but it should be mentioned that Chao et al. [6]; Gwo [11]; Lin and Chao [19]; Sansone et al. [41] agree with obtained results in terms of toxicity tolerance among development stages, showing more resistance to toxicity in later development stages than earlier development stages. D-larvae of *M. galloprovincialis* evidenced high resistance for cryopreservation with all of the CPAs evaluated (Me_2SO, PG and EG) in an experiment carried out by Wang et al. [51], and the highest post-thaw mussel larval survival was obtained with Me_2SO 0.5 M (55.3 ± 7.8%). Rusk [40] cryopreserved *Perna canaliculus* D-larvae with EG 10% + TRE 0.4 M. After cryopreservation, he reported abnormalities in shell as a consequence of cryopreservation, and a delay in development at cryopreserved D-larval stage, in contrast of control individuals. Nascimento et al. [24] found similar abnormalities in D-larvae after exposure of CPAs on gametes and embryos of *Crassostrea rhizophorae*, thus CPAs may have a specific effect on cells of shell gland but there is no current research on this topic. According with our toxicity results, abnormalities showed by Rusk might be only due to cryopreservation itself and not caused by toxicity damage of CPAs, given harmless effect CPAs showed for the D-larvae.

Previous research on the cryopreservation of D-larvae hadn't produced the general successful results once would expect if indeed those larvae could withstand exposure and equilibration to such high concentration of cryoprotecting agents. Is it the cryopreservation of cells with hard structures, like the D-larvae shell an obstacle? Further studies are needed in order to gather more information regarding this.

On the other side, another possibility to consider is that the CPAs might not be entering the cells completely, hence the lower toxicity presented and the lower survivals to cryopreservation. This might be explained considering its capacity to perceive harmful substances and to isolate their mantle from the external environment: several authors have described that D-larvae presents an apical sense organ, which is able to detect chemicals, light and orientation [10,26,40]. If D-larvae could detect the CPA during first seconds of exposure and close the shell to reduce the CPA interaction with tissues during our 15 min equilibration time, then it would be impossible for us to appreciate cell injury, abnormality or any delay in development due to toxicity. It would be very useful to check how much of the CPAs could be found in the larvae internal tissues by non invasive methods like x-ray computed tomography [7] or similar methods. If this were the case, the capacity of D-larvae to keep closed during exposure to CPAs period should be studied further and procedures about the exposure time for CPA equilibration would need to be reconsidered. Indeed D-larvae are quite evolved cells, the shell gland appears at trochophore larval stage, by organogenesis formed from ectodermic cells of early embryo stage, which starts producing periostracum [52]. In the case of Bivalve species with a planktotrophic larval stage, they secrete the prodisoconch shell layer first [29]. Then, the shell is compressed laterally and takes the shape of a D. Consequently, the developed larvae are called D-veliger larvae at this point [53].

Recent preliminary cryopreservation trials of 48 and 72 h old D-larvae of *M. galloprovincialis* [39] were carried out by using different combinations of EG and TRE, preliminary results registered on Table 3. The 48 h old D-larvae showed lower survival post-cryopreservation, in contrast, cryopreservation of D-larvae of 72 h post-fertilization yielded better normal D-larval percentages for at least 48 h post-thaw. Long-term survival data still lacking.

Toxicity tests showed minimal injurious effects for EG concentrations up to 3 M, so if equilibration between larvae and CPA, then the abnormalities found in 48 h D-larvae's after cryopreservation could be due to freezing protocol. These low percentages of normal D-larvae found after cryopreservation might evidence the unsuccessful protective effect of the CPA to D-larvae and could be considered as an additional demonstration to refute the incapacity of permeant CPAs to go across larval shell to reach tissues and post-thaw survival percentages of 72 h. old D-larvae could be due only to intrinsic resistance of older D-larvae to low temperatures.

This work was carried out to select the best CPA that adjusts to each development stage evaluated of *M. galloprovincialis*, on basis of their toxicity values under 15 min of exposure. Several factors have been

Table 3
Percentage of normal D-larvae resulting from *M. galloprovincialis* D-larvae cryopreserved with different combinations of CPAs.

CPA	% Normal Larvae	
	$D\text{-larvae}_{48}$	$D\text{-larvae}_{72}$
EG 10% (1.80 M)	6.66 ± 3.21	86.15 ± 3.51
EG 10% (1.80 M) + 0.4 M TRE	2.49 ± 2.16	92.41 ± 3.00
EG 15% (2.69 M)	0.85 ± 0.36	97.64 ± 3.56
EG 15% (2.69 M) + 0.4 M TRE	0.37 ± 0.31	91.22 ± 6.33

found that would need to be further investigated to produce successful protocols for this species: permeability parameters for all development stages, improvement of the actual protocols by researching the balance between toxicity and cryoprotection and finally, long-term development of the larvae post-thaw.

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